Safety Assessment of Hyaluronates as Used in Cosmetics

Status:	Draft Report for Panel Review
Release Date:	February 10, 2023
Panel Meeting Date:	March 6 – 7, 2023

The Expert Panel for Cosmetic Ingredient Safety members are: Chair, Wilma F. Bergfeld, M.D., F.A.C.P.; Donald V. Belsito, M.D.; David E. Cohen, M.D.; Curtis D. Klaassen, Ph.D.; Allan E. Rettie, Ph.D.; David Ross, Ph.D.; Thomas J. Slaga, Ph.D.; Paul W. Snyder, D.V.M., Ph.D.; and Susan C. Tilton, Ph.D. The Cosmetic Ingredient Review (CIR) Executive Director is Bart Heldreth, Ph.D. This safety assessment was prepared by Priya Cherian, M.S., Senior Scientific Analyst/Writer, CIR.

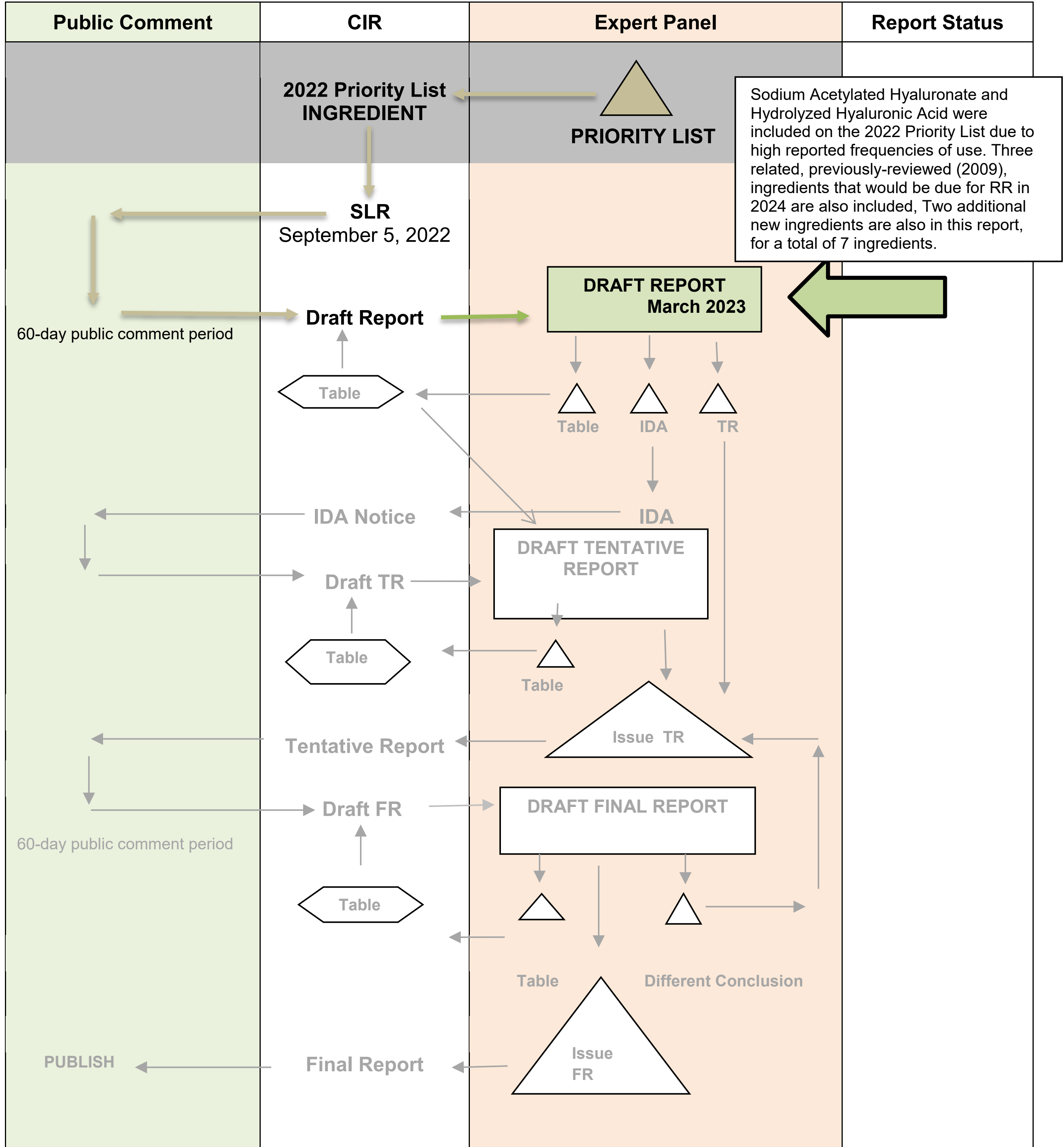
© Cosmetic Ingredient Review

1620 L Street, NW, Suite 1200 ◊ Washington, DC 20036-4702 ◊ ph 202.331.0651
cirinfo@cir-safety.org

SAFETY ASSESSMENT FLOW CHART

INGREDIENT/FAMILY __ Hyaluronates

MEETING March 2023



Cosmetic Ingredient Review
Commitment & Credibility since 1976

Memorandum

To: Expert Panel for Cosmetic Ingredient Safety Members and Liaisons
From: Priya Cherian, M.S., Senior Scientific Analyst/Writer, CIR
Date: February 10, 2023
Subject: Safety Assessment of Hyaluronates as Used in Cosmetics

Enclosed is the Draft Report of the Safety Assessment of Hyaluronates as Used in Cosmetics (identified in the pdf as *report_Hyaluronates_032023*). The 7 hyaluronates reviewed in this report include the following:

Hyaluronic Acid
Hydrolyzed Calcium Hyaluronate
Hydrolyzed Hyaluronic Acid
Hydrolyzed Sodium Hyaluronate
Potassium Hyaluronate
Sodium Acetylated Hyaluronate
Sodium Hyaluronate

Sodium Acetylated Hyaluronate and Hydrolyzed Hyaluronic Acid were included on the 2022 Priority List due to reported frequencies of use. It was noted that 3 related ingredients previously reviewed by the Panel in a report published in 2009, i.e., Hyaluronic Acid, Potassium Hyaluronate, and Sodium Hyaluronate, would soon be considered for re-review. Accordingly, the Panel deemed it appropriate to include the 3 previously-reviewed ingredients in this new safety assessment. (The Panel had concluded that these 3 ingredients are safe in cosmetics in the present practices of use and concentration, as described in that 2009 safety assessment.) The 2009 report (*originalreport_Hyaluronates_032023*) along with corresponding minutes of the deliberations (*originalminutes_Hyaluronates_032023*) have been included herein.

The Scientific Literature Review (SLR) on this group of 7 hyaluronate ingredients was announced on October 5, 2022. Since the issuing of the SLR, ample unpublished data have been received including manufacturing, composition and impurities, genotoxicity, developmental and reproductive toxicity, dermal irritation, dermal sensitization, phototoxicity, and ocular irritation data. These unpublished data files may be found in the packet as *data2_Hyaluronates_032023*, *data3_Hyalurates_032023*, *data4_Hyaluronates_032023*, *data5_Hyaluronates_032023*).

As per the Panel's request at the December 2022 meeting, an updated use table format has been implemented. The frequency and concentration of use is presented both cumulatively by likely duration and exposure and individually by product category.

Comments on the SLR provided by Council (*PCPCcomments_Hyaluronates_032023*) were addressed, as indicated in the responses to these comments (*response-PCPCcomments_Hyaluronates_032023*).

The following documents are also included in this packet:

- 2021 concentration of use data (*data1_Hyaluronates_032023*)
- report history (*history_Hyaluronates_032023*)
- data profile (*dataprofile_Hyaluronates_032023*)
- search strategy (*search_Hyaluronates_032023*)
- flow chart (*flow_Hyaluronates_032023*)

After reviewing these documents, if the available data are deemed sufficient to make a determination of safety, the Panel should issue a Tentative Report with a safe as used, safe with qualifications, unsafe, or split conclusion, and Discussion items should be identified. If the available data are insufficient, the Panel should issue an Insufficient Data Announcement (IDA), specifying the data needs therein.

1620 L Street, NW Suite 1200, Washington, DC 20036
(Main) 202-331-0651
(email) cirinfo@cir-safety.org (website) www.cir-safety.org

Memorandum

TO: Bart Heldreth, Ph.D.
Executive Director - Cosmetic Ingredient Review

FROM: Alexandra Kowcz, MS, MBA
Industry Liaison to the CIR Expert Panel

DATE: October 17, 2022

SUBJECT: Scientific Literature Review: Safety Assessment of Hyaluronates as Used in Cosmetics (release date: October 5, 2022)

The Personal Care Products Council has no suppliers listed for Potassium Hyaluronate.

The Personal Care Products Council respectfully submits the following comments on the scientific literature review, Safety Assessment of Hyaluronates as Used in Cosmetics.

Key Issues
Information about cross-linked hyaluronic acid dermal fillers should be removed from the report as similar materials have been given INCI names other than Hyaluronic Acid. For example, Sodium Hyaluronate Crosspolymer-4 is defined as "the sodium salt of a polymer of Hyaluronic Acid (q.v.) crosslinked with 1,4-butanediol diglycidyl ether." The CIR report should clearly state that it does not cover crosslinked Hyaluronic Acid.

The case reports summarized in Table 10 are not relevant and should be deleted from the CIR report. The route of exposure is not relevant, and it is not clear that the reactions are due to Hyaluronic Acid. Without providing details, it would be helpful to cite a review where the reader can find more information about reactions observed following injection of crosslinked Hyaluronic dermal fillers. One example of a recent review article is:

Owczarczyk-Saczonek A, Zdanowska N, Wygonoska E, et al. 2021. The Immunogenicity of Hyaluronic Acid Fillers and Its Consequences. *Clinical, Cosmetic and Investigational Dermatology* 14: 921-934.

This paper indicates that the reactions range from immediate (anaphylactic reactions or histamine release edema) to delayed (foreign body granulomas, delayed inflammatory reactions, or possibly autoimmune/autoinflammatory syndrome induced by adjuvants). This paper also suggests that additives or bacterial components introduced during injection may be responsible for some of the reactions.

Additional Considerations

Introduction – In the Introduction, it would be helpful to state why this report is not a standard re-review with added ingredients. Which new ingredient is driving this review?

Cosmetic Use; Summary – Please include the maximum use concentration for the new ingredient with the most uses.

Dermal Penetration, old report summary – Please indicate the radiolabel that was used. Since Hyaluronic Acid is metabolized, if only radioactivity was measured, it should state the results in terms of radioactivity, not in terms of the test compound or Hyaluronic Acid. How is it known that Hyaluronic Acid, rather than a metabolite, penetrated the skin?

DART, old report summary – Please state when in relation to gestation the rats and rabbits were treated subcutaneously. Were the effects in the adrenal glands observed in parental animals or their offspring?

Genotoxicity – Please indicate that the maximum dose tested was 5 mg/plate (rather than just 5 mg). Please correct “strands” to “strains”.

Other Relevant Studies, old report summary – Please state the type of injection used in the studies in rabbits and rats.

Immediate and Delayed Hypersensitivity to Intracutaneous Hyaluronic Acid; Summary – As this section is about fillers which are generally crosslinked, it should be removed from the report. If it is left in the report, please indicate the differences among the 6 dermal fillers.

Summary; Table 9 – For the genotoxicity studies, the doses should be stated as mg/plate.

Table 3 – The title of Table 3 should indicate that MW can also be found in this table.

Table 4 – Please delete the word “Current” from the title of this table as it will not be current when it is published. The dates in the headings are sufficient to indicate when the information was collected.

Table 6, third study – In the Dose/Protocol column, please correct “Animals were killed at the end of the study to measure the residual rate in the body.” It is not clear how a “rate” can be measured in a carcass. It is more likely that they measured “residual radioactivity”. In the Results column, please change “cadaver” to “carcass”. Cadaver is a term used for a human body, and this is a rat study.

Table 6, reference 20 – It would be helpful to include the conclusion from the abstract of this study: Hyaluronic Acid is “degraded by intestinal bacteria and oligosaccharide HA absorbed in the large intestines is widely distributed”. Please also indicate how the Hyaluronic Acid was measured in the excretion study, e.g., measured as described in the metabolism/distribution study above (if correct).

Table 6, reference 21 – If only radioactivity was measured, the results should be reported as "radioactivity" rather than "test substance" (unless they confirmed that the radioactivity was still associated with the unmetabolized test substance).

Hyaluronates - March 2023 Meeting – Priya Cherian	
Comment Submitter: Personal Care Products Council **Date of Submission: October 17, 2022**	
Comment	**Response/Action**
Information about cross-linked hyaluronic acid dermal fillers should be removed from the report as similar materials have been given INCI names other than Hyaluronic Acid. For example, Sodium Hyaluronate Crosspolymer-4 is defined as "the sodium salt of a polymer of Hyaluronic Acid (q.v.) crosslinked with 1,4-butanediol diglycidyl ether." The CIR report should clearly state that it does not cover crosslinked Hyaluronic Acid	Addressed
The case reports summarized in Table 10 are not relevant and should be deleted from the CIR report. The route of exposure is not relevant, and it is not clear that the reactions are due to Hyaluronic Acid. Without providing details, it would be helpful to cite a review where the reader can find more information about reactions observed following injection of crosslinked Hyaluronic dermal fillers. One example of a recent review article is: Owczarczyk-Saczonek A, Zdanowska N, Wygonoska E, et al. 2021. The Immunogenicity of Hyaluronic Acid Fillers and Its Consequences. Clinical, Cosmetic and Investigational Dermatology 14: 921-934. This paper indicates that the reactions range from immediate (anaphylactic reactions or histamine release edema) to delayed (foreign body granulomas, delayed inflammatory reactions, or possibly autoimmune/autoinflammatory syndrome induced by adjuvants). This paper also suggests that additives or bacterial components introduced during injection may be responsible for some of the reactions.	Hypersensitivity studies following intradermal exposure to Hyaluronic Acid facial fillers were summarized in this report to address potential allergenicity concerns regarding these ingredients. The Panel should indicate whether or not this table should be removed and replaced with a summary of a review article in text.
Introduction – In the Introduction, it would be helpful to state why this report is not a standard re-review with added ingredients. Which new ingredient is driving this review?	Addressed
Cosmetic Use; Summary – Please include the maximum use concentration for the new ingredient with the most uses	Only the ingredient with the highest concentration of use is typically noted in the cosmetic use section text. However, for the Panel's purposes, the highest concentration of use among the 4 ingredients not previously reviewed was reported to be 0.2% in Hydrolyzed Hyaluronic Acid.
Dermal Penetration, old report summary – Please indicate the radiolabel that was used. Since Hyaluronic Acid is metabolized, if only radioactivity was measured, it should state the results in terms of radioactivity, not in terms of the test compound or Hyaluronic Acid. How is it known that Hyaluronic Acid, rather than a metabolite, penetrated the skin?	When the radiolabel was stated, it was noted in the report. The summary included in this report is as written in the original report.
DART, old report summary – Please state when in relation to gestation the rats and rabbits were treated subcutaneously. Were the effects in the adrenal glands observed in parental animals or their offspring?	As this a summary from the previous report, and numerous DART studies are being referred to in this statement, all with differing treatment periods, specific treatment periods were not stated in this summary.
Genotoxicity – Please indicate that the maximum dose tested was 5 mg/plate (rather than just 5 mg). Please correct "strands" to "strains".	Addressed
Other Relevant Studies, old report summary – Please state the type of injection used in the studies in rabbits and rats.	Addressed

Immediate and Delayed Hypersensitivity to Intracutaneous Hyaluronic Acid; Summary – As this section is about fillers which are generally crosslinked, it should be removed from the report. If it is left in the report, please indicate the differences among the 6 dermal fillers	Kept in report as is; the only distinguishing factors between the dermal fillers mentioned in the report were the product trade names, which are generally not mentioned in CIR reports
Summary; Table 9 – For the genotoxicity studies, the doses should be stated as mg/plate	Addressed
Table 3 – The title of Table 3 should indicate that MW can also be found in this table	Addressed
Table 4 – Please delete the word “Current” from the title of this table as it will not be current when it is published. The dates in the headings are sufficient to indicate when the information was collected	Addressed
Table 6, third study – In the Dose/Protocol column, please correct “Animals were killed at the end of the study to measure the residual rate in the body.” It is not clear how a “rate” can be measured in a carcass. It is more likely that they measured “residual radioactivity”. In the Results column, please change “cadaver” to “carcass”. Cadaver is a term used for a human body, and this is a rat study	Addressed
Table 6, reference 20 – It would be helpful to include the conclusion from the abstract of this study: Hyaluronic Acid is “degraded by intestinal bacteria and oligosaccharide HA absorbed in the large intestines is widely distributed”. Please also indicate how the Hyaluronic Acid was measured in the excretion study, e.g., measured as described in the metabolism/distribution study above (if correct).	Addressed
Table 6, reference 21 – If only radioactivity was measured, the results should be reported as “radioactivity” rather than “test substance” (unless they confirmed that the radioactivity was still associated with the unmetabolized test substance)	Addressed

Hyaluronates – History

July 2021

- Concentration of use received for Hydrolyzed Calcium Hyaluronate, Hydrolyzed Hyaluronic Acid, Hydrolyzed Sodium Hyaluronic Acid, and Sodium Acetylated Hyaluronate

January 2022

- Concentration of use received for Hyaluronic Acid, Sodium Hyaluronate, and Potassium Hyaluronate

October 2022

- SLR posted on CIR website
- Comments received on SLR from PCPC

November 2022

- Unpublished data received:
    - In vitro dermal and ocular irritation assays on several trade name mixtures containing 1-3% Hyaluronic Acid
    - In vitro dermal and ocular irritation assays on trade name mixture containing 0.5% Sodium Hyaluronate
    - HRIPT on formula containing 0.2% Sodium Acetylated Hyaluronate
    - HRIPT on formula containing 0.2% Hyaluronic Cid
    - HRIPT on formula containing 1.5% Sodium Hyaluronate

December 2022

- Unpublished data received:
    - Composition, impurities, manufacturing, and summarized toxicity data on Sodium Hyaluronate
    - Composition, impurities, manufacturing, and summarized toxicity data on Hydrolyzed Sodium Hyaluronate
    - Composition and Manufacturing Data on Hydrolyzed Hyaluronic Acid

January 2023

- Composition, manufacturing, instruction, and summarized safety data on Sodium Hyaluronate

March 2023

- Panel reviews Draft Report on 7 hyaluronate ingredients

Hyaluronates Data Profile – March 2023 – Priya Cherian

				Toxicokinetics			Acute Tox			Repeated Dose Tox			DART		Genotox		Carci		Dermal Irritation			Dermal Sensitization				Ocular Irritation		Clinical Studies	
	Reported Use	Method of Mfg	Impurities	log P/log K_{ow}	Dermal Penetration	ADME	Dermal	Oral	Inhalation	Dermal	Oral	Inhalation	Dermal	Oral	In Vitro	In Vivo	Dermal	Oral	In Vitro	Animal	Human	In Vitro	Animal	Human	Phototoxicity	In Vitro	Animal	Retrospective/ Multicenter	Case Reports
Hyaluronic Acid	**XO**	**XO**	XO		XO	X		O			O								X	O	O	X		X		X	X		X
Hydrolyzed Calcium Hyaluronate	**X**																												
Hydrolyzed Hyaluronic Acid	**X**	**X**	X																										
Hydrolyzed Sodium Hyaluronate	**X**	**X**	X												X				X		X			X	X	X			
Potassium Hyaluronate	**XO**																												
Sodium Acetylated Hyaluronate	**X**																							X					
Sodium Hyaluronate	**XO**	**X**	X					X			X			XO	X	X			X		O	X		X	X	X			X

* "X" indicates that data were available in a category for the ingredient

* "O' indicates that data were available in a category for the ingredient (data is in italics in report as it is summarized from the previous report on Hyaluronic Acid, Potassium Hyaluronate, and Sodium Hyaluronate published in 2009

Hyaluronates

Ingredient	CAS #	InfoB	PubMed	TOXNET	FDA	EU	ECHA	IUCLID	SIDS	ECETOC	HPVIS	NICNAS	NTIS	NTP	WHO	FAO	NIOSH	FEMA	Web
Sodium Acetylated Hyaluronate		x	x			x													x
Hydrolyzed Hyaluronic Acid		x				x													
Hydrolyzed Calcium Hyaluronate		x				x													
Hyaluronic Acid	9004-61-9	x	x			x	x												x
Sodium Hyaluronate	9067-32-7	x	x		x	x	x												x
Potassium Hyaluronate	31799-91-4	x				x													
Hydrolyzed Sodium Hyaluronate		x				x													

An "x" indicates that relevant data were found in the database/website

Search Strategy

Ingredient names and CAS numbers were searched in combination with the search terms listed below.
*these terms were searched from 2003 onwards for previously reviewed ingredients (Hyaluronic Acid, Potassium Hyaluronate, and Sodium Hyaluronate)

Typical Search Terms

- INCI names
- CAS numbers
- chemical/technical names
- metabolism
- impurities
- composition
- dermal
- inhalation
- skin
- toxicity
-
- drugs
- medicine
- clinical
- case report
- irritation
- ocular
- eye
- sensitization
- allergy
- manufacture
- pharmacokinetics
- cancer
- carcinogenicity
- mutagenicity
- Ames
- Reproductive
- Teratogenicity
- Synthesis

LINKS

Search Engines

- Pubmed (- http://www.ncbi.nlm.nih.gov/pubmed)
- Toxnet (https://toxnet.nlm.nih.gov/); (includes Toxline; HSDB; ChemIDPlus; DART; IRIS; CCRIS; CPDB; GENE-TOX)

appropriate qualifiers are used as necessary
search results are reviewed to identify relevant documents

Pertinent Websites

- wINCI - http://webdictionary.personalcarecouncil.org

- FDA databases http://www.ecfr.gov/cgi-bin/ECFR?page=browse
- FDA search databases: http://www.fda.gov/ForIndustry/FDABasicsforIndustry/ucm234631.htm;,
- EAFUS: http://www.accessdata.fda.gov/scripts/fcn/fcnnavigation.cfm?rpt=eafuslisting&displayall=true
- GRAS listing: http://www.fda.gov/food/ingredientspackaginglabeling/gras/default.htm
- SCOGS database: http://www.fda.gov/food/ingredientspackaginglabeling/gras/scogs/ucm2006852.htm
- Indirect Food Additives: http://www.accessdata.fda.gov/scripts/fdcc/?set=IndirectAdditives
- Drug Approvals and Database: http://www.fda.gov/Drugs/InformationOnDrugs/default.htm
- http://www.fda.gov/downloads/AboutFDA/CentersOffices/CDER/UCM135688.pdf
- FDA Orange Book: https://www.fda.gov/Drugs/InformationOnDrugs/ucm129662.htm
- OTC ingredient list: https://www.fda.gov/downloads/aboutfda/centersoffices/officeofmedicalproductsandtobacco/cder/ucm135688.pdf
- (inactive ingredients approved for drugs: http://www.accessdata.fda.gov/scripts/cder/iig/

- HPVIS (EPA High-Production Volume Info Systems) - https://ofmext.epa.gov/hpvis/HPVISlogon
- NIOSH (National Institute for Occupational Safety and Health) - http://www.cdc.gov/niosh/
- NTIS (National Technical Information Service) - http://www.ntis.gov/
- NTP (National Toxicology Program) - http://ntp.niehs.nih.gov/
- Office of Dietary Supplements https://ods.od.nih.gov/
- FEMA (Flavor & Extract Manufacturers Association) - http://www.femaflavor.org/search/apachesolr_search/

- EU CosIng database: http://ec.europa.eu/growth/tools-databases/cosing/
- ECHA (European Chemicals Agency – REACH dossiers) – http://echa.europa.eu/information-on-chemicals;jsessionid=A978100B4E4CC39C78C93A851EB3E3C7.live1
- ECETOC (European Centre for Ecotoxicology and Toxicology of Chemicals) - http://www.ecetoc.org
- European Medicines Agency (EMA) - http://www.ema.europa.eu/ema/
- IUCLID (International Uniform Chemical Information Database) - https://iuclid6.echa.europa.eu/search
- OECD SIDS (Organisation for Economic Co-operation and Development Screening Info Data Sets)- http://webnet.oecd.org/hpv/ui/Search.aspx
- SCCS (Scientific Committee for Consumer Safety) opinions: http://ec.europa.eu/health/scientific_committees/consumer_safety/opinions/index_en.htm
- NICNAS (Australian National Industrial Chemical Notification and Assessment Scheme)- https://www.nicnas.gov.au/

- International Programme on Chemical Safety http://www.inchem.org/
- FAO (Food and Agriculture Organization of the United Nations) - http://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/jecfa-additives/en/
- WHO (World Health Organization) technical reports - http://www.who.int/biologicals/technical_report_series/en/

- www.google.com - a general Google search should be performed for additional background information, to identify references that are available, and for other general information

APRIL 2006 PANEL MEETING – DRAFT REPORT

Belsito Team – April 3, 2006

Dr. Belsito stated that since Hyaluronic Acid comes from animals, the caveat needs to be employed as well as the boilerplate for bacterially engineered sources. The new data does not change anything and has little relevance. There is enhanced metastasis with the presence of Hyaluronic Acid. Is there any concern for people with squamous cell carcinoma? Would the carcinoma be enhanced? There is not enough data on antibody injections. There is little on delayed reactions if Hyaluronic Acid is rubbed onto the skin. There is not enough photosensitive data; ten guinea pigs is not sufficient for our needs. There is a lack of data on T-cells. We need more data on enhanced metastasis.

Dr. Eisenmann asked if Hyaluronic Acid causes cancer to metastasize or is its presence caused by the cancer. Dr. Belsito asked if Hyaluronic Acid caused increased metastasis. Dr. Snyder stated that he thought it was a downstream event, not caused by Hyaluronic Acid's presence. Dr. Belsito pointed out that the Antila study shows increased Hyaluronic Acid associated with increased malignacy. Dr. Snyder pointed out that this does not mean that the patient will develop the disease. Dr. Belsito pointed out that with tumors, the more Hyaluronic Acid, the more it will metastasize. Dr. Snyder stated that it could be either way with the current available data.

Dr. Belsito pointed out that there is nothing new on the subject in the additional data. Dr. Snyder pointed out that the Goomer study and the studies on hypersensitivity are useful. Dr. Belsito stated that nothing else was new. Dr. Snyder stated that some studies address the issue of hypersensitivity. Dr. Belsito pointed out that this is deep tissue, not topical. Dr. Klaassen stated that additional data should not be put in; the injection subcutaneously is safe and not a problem.

Dr. Belsito stated that dermal tumors have less exposure than epidermal and squamous cell tumors. The compound is fine for joints and eyes. The exposure is different and we need to address that. Maybe Dr. Slaga will have information to help. Dr. Snyder pointed out that the conclusion could go either way and that the Panel did not want to over-interpret the data.

Dr. Belsito asked Ms. Becker what the other group did. She replied that they decided safe as used and that they were concerned with avian flu and bovine sources.

Dr. Snyder pointed out that Hyaluronic Acid has a short half-life and it is degraded in the skin.

Dr. Belsito asked for any other comments or concerns.

It was decided that more sensitization and irritation data were needed and the question on metastasis addressed more completely.

Marks Team – April 3, 2006

Dr. Marks pointed out that this is the first time for the Panel to see the Hyaluronic Acid group. He asked if more data was necessary.

Dr. Shank stated that the report has all the data necessary to make an assessment. There is more data than necessary and that the report could be shortened. A summary table of the reproduction data could be inserted. The Panel has all it needs to say that Hyaluronic Acid is safe as used. What about bovine sources and the avian flu?

Dr. McEwen stated that the World Health Organization has a report that the H5N1 bird flu virus is sensitive to heat and will inactivate the virus.

Dr. Marks asked about prions. Dr. McEwen stated that there are no bovine sources of Hyaluronic Acid in cosmetics. Dr. Marks pointed out that needs to be in the discussion. Ms. Weintraub asked if the virus is always inactivated. Dr. McEwen replied yes, the manufacturing process always kills the virus.

Dr. Shank made an editorial comment.

Dr. Marks asked if Hyaluronic Acid penetrates the skin. Dr. Bronough (FDA) stated that it is not possible for molecule of this size to penetrate the skin but there may be tritium transfer.

Dr. Slaga stated that there is more than enough data to make an assessment. Dr. Marks asked if the report should go to tentative report, safe as used.

Dr. McEwen asked if the new guinea pig data is needed.

Dr. Bergfeld asked if there is a recommendation on the medical uses of Hyaluronic Acid. Do we need tables? Dr. Marks pointed out that all the adverse effects were in medical uses. He asked how should that be handled. Dr. Slaga stated that a few lines on non-cosmetic uses are needed. Dr. McEwen stated that a table of medical uses could be considered by the Panel. Dr. Shank pointed out that has not been done in the past and Dr. McEwen stated that the table must be detailed. Dr. Slaga agreed that the Panel must address the other uses. Dr. Bergfeld stated that the uses must be addressed to mention that reactions did occur.

Dr. Andersen stated that the Panel can describe the collection of information as representative. The reader will understand that it is not a total list of the literature. The same issue is coming up with corn oil. This approach will not provide the reader with all the studies but will be representative. This would be useful.

Dr. Shank pointed out that the data would be additional, not representative.

Dr. Bergfeld pointed out that all the adverse reactions were injections.

Dr. Marks agreed that a table with the adverse reactions and a short paragraph describing the table and data would work. Dr. Shank said that Table 4 is a good representation of what is needed and to expand on that.

There were editorial comments.

It was decided to go to tentative report, safe as used with changes to the text.

Full Panel – April 4, 2006

Dr. Belsito noted that the Panel is reviewing this group of ingredients for the first time, and that Hyaluronic Acid, Sodium Hyaluronate, and Potassium Hyaluronate are now being used as dermal fillers.

After reviewing the available data, Dr. Belsito noted that his Team was surprised at the relative lack of dermal sensitization, irritation, phototoxicity, and photosensitization data in the draft report. The available animal data involved 12 guinea pigs, and human data are absent from the report.

Dr. Belsito said that the notion of enhancement of metastases being related to elevated levels of Hyaluronic Acid is discussed in the draft report, and that his Team remains concerned about this finding.

Dr. Belsito's Team determined that the available data are insufficient for determining the safety of Hyaluronic Acid, Sodium Hyaluroante, and Potassium Hyaluraonte in cosmetics, and that the following data are needed: (1) additional dermal irritation and sensitization data, (2) UV absorption data; if UV absorption occurs, then photosensitization and photoirritation data will be needed, (3) an explanation of the association between Hyaluronic Acid and metastases in the studies, looking to see whether application of a cosmetic product containing Hyaluronic Acid to the skin might enhance cutaneous metastases of squamous cell carcinomas existing in the skin at that time.

Dr. Belsito said that because these ingredients are from bacterial and animal sources, the usual statement indicating that they must be pyrogen-free and free of infectious organisms should be added to the draft report.

Dr. McEwen said that the Panel is talking about the addition of approximately the same percentage of Hyaluronic Acid that is in the skin, in the interstitial fluid. With this in mind, he wanted to understand the Panel's concerns relating to topical application, particularly, the concerns regarding irritation or sensitization. He added that the Panel has reviewed guinea pig sensitization test data and a photo test as well.

In terms of irritation and sensitization potential, Dr. Belsito noted that the chemical is sitting down in the dermal layer of the skin, and not on the stratum corneum.

Dr. McEwen said that Hyaluronic Acid is actually in the epidermis as well, in the interstitial fluid and in the matrix around the cells, at a concentration of 10 mg/g.

Dr. Belsito was referred to the following text on page 22 of the draft report: The largest amount of Hyaluronic Acid (7 to 8 g per average adult human, ~50% of the total in the body) resides in skin tissue, where it is present in both the dermis (~0.5 mg/g wet tissue) and the epidermis (~0.1 mg/g wet tissue). The actual concentrations of Hyaluronic Acid in the matrix around the cells in the epidermis (estimated to be 2 to 4 mg/ml) are an order of magnitude higher than in the dermis (estimated to be ~0.5 mg/ml).

After further consideration of the preceding text and current use concentration data, Dr. Belsito noted that items 1 and 2 from the informal request could be eliminated, leaving the following request: an explanation of the association between Hyaluronic Acid and metastases in the studies, looking to see whether application of a cosmetic product containing Hyaluronic Acid to the skin might enhance cutaneous metastases of squamous cell carcinomas existing in the skin at that time.

Dr. Slaga said that there are a number of substances that can be added to tumor cells in culture that increase metastatic potential, and that, to his knowledge, none has caused this effect in whole animal studies.

Still, Dr. Belsito said that the results of the two in vitro studies (Toole, 2002; Auninen et al., 2000) need to be clarified. He noted that Toole (2002) is a review article. According to the report text, elevated levels of Hyaluronic Acid are associated with tumor progression and cancer migration due to its influence on cell division and attachment as well as its stimulation of angiogenesis (Toole 2002; Auninen et al., 2000).

Dr. Slaga said that, to his knowledge, there are no data showing that if exogenous Hyaluronic Acid is increased, the metastatic potential of tumor cells is also increased.

Dr. Slaga also noted that, according to the draft report text (page 76, Zeng et al., 1998), Hyaluronic Acid (lower molecular weight) has been shown to inhibit tumor growth.

Dr. Belsito said that the draft report on Hyaluronic Acid needs to be tabled until the next Panel meeting, pending receipt of all of the primary sources relating to Hyaluronic Acid and metastatic potential.

The Panel agreed that the issue of Hyaluronic Acid and enhanced metastatic potential needs to be clarified.

Dr. Belsito said that the Panel expects to receive the original reports on all of the articles referencing enhanced metastases prior to the June 2006 Panel meeting.

Dr. Marks noted that his Team recommended the following revisions for the draft report: (1) The report needs to be shortened, i.e., text needs to be replaced with tables and summaries. (2) Figure 3 on page 12 needs to be corrected; i.e., the oxygen should be surrounded by 2 bonds, not 6 bonds.

Dr. Andersen recalled that Dr. Belsito had commented that the abstracts of all of the publications (i.e., medical data) that CIR had found were not particularly persuasive, and wanted to know if this means that these studies do not need to be included in the draft report. Dr. Andersen said that, from an educational standpoint, Dr. Marks' Team had discussed mentioning all of the medical data as being useful. In so doing, the reader would get the full picture regarding use.

Dr. Belsito disagreed, noting that the medical data relate to intraarticular, intradermal, and intraocular injections and the side effects and benefits of those injections; however, the Panel is concerned with products that are applied topically to the skin. Furthermore, Dr. Belsito said that the data are totally irrelevant and do not enhance any of the safety conclusions.

Dr. Snyder said that one of the publications relates to skin penetration enhancement and should be included.

Dr. Marks said that since the draft report will be shortened by adding data to tables, it may also be useful to include the medical data in tabular form. He noted that it may be helpful to know that there are immediate and delayed-type reactions to the medical uses of Hyaluronic Acid.

Dr. Belsito said that if the medical data will be included, the following references would be helpful and should be used: Hyaluronic Acid as a vehicle for drug delivery (Brown reference), native hyaluronan producing less hypersensitivity (Goomer reference), and delayed inflammatory reaction to restalyn (Jordan reference).

Dr. Bergfeld said that the references on the metastatic potential of Hyaluronic Acid as well as those on the inhibitory activity of this ingredient on tumor growth will be obtained for the Panel's review.

Dr. Andersen said that this will offer an opportunity for any member of the public to comment on this issue between now and the June Panel meeting.

JUNE 2006 PANEL MEETING – DRAFT TENTATIVE REPORT

Belsito Team – June 12, 2006

Dr. Belsito pointed out that the tabling of Hyaluronic Acid resulted from his concern about metastasis. After reading the literature, he is still unsure.

Dr. Snyder pointed out the quote from Toole (2004).

Dr. Belsito stated that the issue may be a breakdown by hyaluronidase in the dermal matrix and not a cause of metastasis. He continued that is appears that some people are hypersensitive to Hyaluronic Acid, including a paper received today on a patient reaction after a skin test. Hyaluronic Acid is in the skin at a greater concentration than in cosmetics. Is there a problem with no studies of cosmetic grade Hyaluronic Acid? What is the cause of the reactions by injectable Hyaluronic Acid. He stated that he was not concerned, but that the data on reports of reactions of injected Hyaluronic Acid needed to be addressed. Goomer (2005) pointed out the differences between native and cross-linked Hyaluronic Acid.

Dr. Snyder stated that there are no clear cut reports of problems. Many of the problems are in people who had problems with collagen also.

Dr. Belsito, Dr. Snyder and Dr. McEwen all agreed that all of the reactions relate to injectable Hyaluronic Acid.

Dr. McEwen clarified that Hyaluronic Acid is approved as a drug, not as a medical device.

Dr. Belsito pointed out that in the Short-Term studies, there was an inhalation study. The boilerplate regarding inhalation should be included.

Dr. Belsito asked what Dr. Mark's group decided. Ms. Becker relayed the information.

Dr. Belsito pointed out the all of the ill effects were from injections, many of the them included Freund's Adjuvant, and the guinea pig skin test was negative. Hopefully, metastasis will be clarified by the papers quoted by Toole.

Marks Team – June 12, 2006

Dr. Marks reviewed what happened at the April 2006 Panel Meeting. His team found that Hyaluronic Acid was safe and Dr. Belsito's team had a concern about the role of Hyaluronic Acid in enhancing metastasis. The Tentative Report was delayed until the Panel could review the literature on cancer and metastasis.

Dr. Slaga stated that there was much published information on Hyaluronic Acid and metastatic potential. Most of the studies were in vitro. There was a good correlation, but this is a common occurrence with many things. There is little in vivo data. The number of tumors is higher in the presence of Hyaluronic Acid and that is a reasonable relationship. There is also the same relationship with other things in the blood. Since high molecular weight Hyaluronic Acid does not penetrate the skin, the amount in cosmetics would not raise the amount in the blood. However, there is no real data available on the effects of Hyaluronic Acid in cosmetics. The majority of the cases are of a low level. The level of Hyaluronic Acid that would cause an effect is a large level, and, therefore, is probably not a concern.

Wounds with a cancerous cell in it may have an effect. Concentration-wise, there is probably not an effect but there are no data for squamous and melanoma cells.

Dr. Marks stated that a chronic wound may be a problem, but that it is not likely that makeup would be applied to it.

Dr. Shank expressed the same concerns as Dr. Slaga. The presence of Hyaluronic Acid in the blood is not the problem, but, down in the basal level. This is not a problem for rinse-off products, but would request animal study for UV light/melanoma.

The Panel discussed using in vivo and in vitro models as well as animal models. They decided that if a study were done, an in vitro study with squamous and melanoma cells (preferably human) should be performed. If exposure to Hyaluronic Acid resulted in metastasis, then it should be further tested in vivo.

They discussed what molecular weight range should be used. Dr. Eisenmann stated that cosmetics use 1 to 5 kd. They then discussed concentration. Dr. Eisenmann pointed out that there was 0.1 to 0.5 mg/g in the skin.

Ms. Becker pointed out that in a paper in their packet (Toole 2004) it is stated that "...hyaluronan levels did not correlate with progress in melanomas or in some epidermal carcinomas." The originals were requested. An attempt to make them available the next day will be made.

It was decided that no assays were necessary unless Dr. Belsito's team wants them. With confirmation of the statement in the Toole paper, the recommendation will be to issue a tentative final report.

Full Panel – June 13, 2006

Dr. Marks stated that the Draft Report on this group of ingredients was reviewed for the first time at the April 3-4, 2006 Panel meeting. He added that his Teams' main concern was that Hyaluronic Acid may facilitate tumor metastasis, and, therefore, wanted to obtain the primary references relating to this effect.

Dr. Marks said that, after reviewing the primary references and considering how much Hyaluronic Acid would be added following a cosmetic application (compared to that already present in the epidermis and dermis), his Team agreed that a Tentative Final Report with a safe as used conclusion could be issued.

Dr. Belsito said that, in his opinion, reviewing the original reports on possible enhanced metastatic carcinoma was not very helpful; however, he noted that there are two publications that are very pertinent. One is on melanoma (Karjalainen et al., 2000), where there was a reduced level of hyaluronan, which was associated with an unfavorable prognosis of clinical stage 1 cutaneous melanoma. Dr. Belsito said that these results suggest that, in melanoma, hyaluronan does not play a role in the metastatic process.

In the other study (Karvinen et al. 2003), hyaluronan was studied using the hyaluronan receptor, CD44 (a cell surface glycoprotein that is involved in cell/cell and cell/matrix interactions) on epidermal keratinocyte tumors, specifically, basal cell carcinomas and squamous cell carcinomas. In basal cell carcinomas, CD44 expression was quite low. In squamous cell carcinomas, CD44 expression was variable and associated with an interesting finding. As the malignancy became less differentiated (and, therefore, would be expected to have a higher risk for metastases), the expression of hyaluronan decreased.

Dr. Belsito said that the preceding results suggest that Hyaluronic Acid and hyaluronan do not play a role in metastases. He added that these results, together with the levels of Hyaluronic Acid that would be applied to the skin, would further insure the safety of this ingredient in cosmetic products.

Dr. Slaga emphasized that, in some cancers, an increase in CD44 expression is correlated with a potential increase in metastatic potential, whereas, in other cancers, a decrease in CD44 expression is correlated with a potential increase in metastatic potential.

The Panel voted unanimously in favor of issuing a Tentative Final Report with a conclusion stating that Hyaluronic Acid, Sodium Hyaluronate, and Potassium Hyaluronate are safe as cosmetic ingredients in the practices of use and concentration as described in the safety assessment.

DECEMBER 2006 PANEL MEETING – DRAFT FINAL REPORT

Belsito Team – December 4, 2006

Dr. Belsito stated we need newer concentration data than from 2004. We should add restriction of pyrogens to the discussion. The penetration enhancement needs to be added to the discussion. There are some editorial comments in the book.

It was suggested that we need to expand the impurities section; it is short on details such as the concentration of proteins in high and low molecular weight polymers; It was also suggested that we need to clarify that tumors secrete hyaluronic acid and hyaluronic acid does not increase tumors.

Marks Team – December 4, 2006

Dr. Marks noted that new data have been added and the conclusion is safe. Team members stated that the write up on the new data is acceptable and the Discussion is complete.

Full Panel – December 5, 2006

Dr. Belsito stated that a Tentative Final Report with the following conclusion was issued at the June 12-13, 2006 Panel meeting: The CIR Expert Panel concluded that Hyaluronic Acid, Sodium Hyaluronate, and Potassium Hyaluronate are safe as cosmetic ingredients in the practices of use and concentrations as described in this safety assessment.

Dr. Belsito noted that data (on carcinogenicity and the metastases of tumors) reviewed at the June meeting have been incorporated into the report, and that these data do not warrant a change in the Panel's conclusion. He also mentioned that the reference for use concentration data in Table 3 should be changed to (CTFA, 2005) and that the report discussion should be revised to include the following statement: One of the sources of Hyaluronic Acid is a bacterial source, and it would be expected that Hyaluronic Acid from bacterial sources would be pyrogen free.

Dr. Belsito also requested inclusion of the following statement in the report summary and discussion: Hyaluronic Acid is a skin penetration enhancer, and, therefore, care should be taken when formulated with other chemicals for which there is a concern relating to skin penetration.

The Panel voted unanimously in favor of issuing a Final Report with the following conclusion: The CIR Expert Panel concluded that Hyaluronic Acid, Sodium Hyaluronate, and Potassium Hyaluronate are safe as cosmetic ingredients in the practices of use and concentrations as described in this safety assessment.

Safety Assessment of Hyaluronates as Used in Cosmetics

Status: Draft Report for Panel Review
Release Date: February 10, 2023
Panel Meeting Date: March 6 – 7, 2023

The Expert Panel for Cosmetic Ingredient Safety members are: Chair, Wilma F. Bergfeld, M.D., F.A.C.P.; Donald V. Belsito, M.D.; David E. Cohen, M.D.; Curtis D. Klaassen, Ph.D.; Allan E. Rettie, Ph.D.; David Ross, Ph.D.; Thomas J. Slaga, Ph.D.; Paul W. Snyder, D.V.M., Ph.D.; and Susan C. Tilton, Ph.D. The Cosmetic Ingredient Review (CIR) Executive Director is Bart Heldreth, Ph.D. This safety assessment was prepared by Priya Cherian, M.S., Senior Scientific Analyst/Writer, CIR.

© Cosmetic Ingredient Review
1620 L Street, NW, Suite 1200 ◊ Washington, DC 20036-4702 ◊ ph 202.331.0651
cirinfo@cir-safety.org

ABBREVIATIONS

ARE	antioxidant response element
BCOP	bovine corneal opacity and permeability
BDDE	1,4-butanediol diglycidyl ether
CAMVA	chorioallantoic membrane vascular assay
CAS	Chemical Abstracts Service
CD44	cluster of differentiation 44
CFR	Code of Federal Regulations
cfu	colony forming units
CIR	Cosmetic Ingredient Review
Council	Personal Care Products Council
CPSC	Consumer Product Safety Commission
Da	dalton
DART	developmental and reproductive toxicity
DMSO	dimethyl sulfoxide
DPRA	direct peptide reactivity assay
ECHA	European Chemicals Agency
EPA	Environmental Protection Agency
EU	endotoxin units
FCC	*Food Chemicals Codex*
FDA	Food and Drug Administration
FIGO	International Federation of Gynecology and Obstetrics
FW	formula weight
HA	Hyaluronic Acid
h-CLAT	human cell line activation test
HRIPT	human repeated insult patch test
I-NOSE	Nasal Obstruction Symptom Evaluation Instrument
K_{ow}	n-octanol/water partition coefficient
kDa	kiloDaltons
LC_{50}	median lethal concentration
LD_{50}	median lethal dose
Log K_{ow}	n-octanol/water partition coefficient
MBq	megabecquerels
MDa	megadaltons
MW	molecular weight
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
ND	not detected
NIBUT	non-invasive break-up time
NICNAS	National Industrial Chemicals Notification and Assessment Scheme
NOEL	no-observed-effect-level
NOAEL	no-observed-adverse-effect-level
NR	not reported
Nrf2	nuclear factor erythroid 2-related factor 2
OECD	Organisation for Economic Cooperation and Development
Panel	Expert Panel for Cosmetic Ingredient Safety
PBS	phosphate-buffered saline
PSL	photo-stimulated luminescence
SDS	sodium dodecyl sulfate
SPECT	single photon emission computed tomography
^{99m}Tc	technetium-99m (a radionuclide nuclear agent)
TG	test guidelines
US	United States
UVB	ultraviolet light B (mid-wavelength)
VCRP	Voluntary Cosmetic Registration Program
wINCI; *Dictionary*	web-based *International Cosmetic Ingredient Dictionary and Handbook*

INTRODUCTION

This assessment reviews the safety of the following 7 ingredients as used in cosmetic formulations:

Hyaluronic Acid*
Hydrolyzed Calcium Hyaluronate
Hydrolyzed Hyaluronic Acid
Hydrolyzed Sodium Hyaluronate
Potassium Hyaluronate*
Sodium Acetylated Hyaluronate
Sodium Hyaluronate*

* previously reviewed by the Expert Panel for Cosmetic Ingredient Safety (Panel)

Sodium Acetylated Hyaluronate and Hydrolyzed Hyaluronic Acid were included on the 2022 Priority List due to high reported frequencies of use in the US Food and Drug Administration (FDA) Voluntary Cosmetic Registration Program (VCRP). Three structurally-similar ingredients (i.e., Hyaluronic Acid, Potassium Hyaluronate, and Sodium Hyaluronate) have previously been reviewed by the Panel in a safety assessment that was published in 2009.[1] Accordingly, in that these ingredients would soon be considered for re-review, the Panel deemed it appropriate to include the 3 previously-reviewed ingredients in this safety assessment. Additionally, two hydrolyzed salts of Hyaluronic Acid are included in this grouping. Hence, all ingredients reviewed in this report are structurally similar as they are salts or acetylated esters derived from Hyaluronic Acid.

According to the web-based *International Cosmetic Ingredient Dictionary and Handbook* (wINCI; *Dictionary*), the majority of ingredients included in this assessment are reported to function in cosmetics as skin and/or hair conditioning agents (Table 1).[2] Sodium Acetylated Hyaluronate is reported to function in cosmetics only as a humectant.

This safety assessment includes relevant published and unpublished data that are available for each endpoint that is evaluated. Published data are identified by conducting an exhaustive search of the world's literature. A listing of the search engines and websites that are used and the sources that are typically explored, as well as the endpoints that the Panel typically evaluates, is provided on the Cosmetic Ingredient Review (CIR) website (https://www.cir-safety.org/supplementaldoc/preliminary-search-engines-and-websites; https://www.cir-safety.org/supplementaldoc/cir-report-format-outline). Unpublished data are provided by the cosmetics industry, as well as by other interested parties.

In its original 2009 review of Hyaluronic Acid, Potassium Hyaluronate, and Sodium Hyaluronate, the Panel concluded that these 3 ingredients are safe in the present practices of use and concentration, as described in the safety assessment.[1] Excerpts from the 2009 report are disseminated throughout the report, as appropriate, and are *identified by italicized text.* (This information is not included in the tables or the Summary section.) Accordingly, for these 3 ingredients, an exhaustive search of the world's literature was performed for studies dated 2004 forward, and relevant new data were included.

Information on cross-linked hyaluronic acid dermal fillers is available in the published literature. However, it should be noted that cross-linked hyaluronic acid ingredients are assigned separate INCI names, and these ingredients are not reviewed in this report. Accordingly, data on crosslinked hyaluronic acid ingredients are not included in this safety assessment. In addition, it should be noted that safety and efficacy data regarding Hyaluronic Acid (non-cross-linked and cross-linked) used as dermal fillers, as well in surgical procedures and arthritic therapy were found; however, with the exception of reference to studies regarding hypersensitivity reactions to injectable Hyaluronic Acid (which can be found in the Clinical Studies section), the other studies are not summarized in this report as no relevance to cosmetic use could be surmised, as exposure to Hyaluronic Acid and its derivatives would be topical when used in cosmetics.

CHEMISTRY

Definition and Structure

Hyaluronic Acid (CAS No. 9004-61-9; Figure 1) is a linear glycosaminoglycan composed of repeating disaccharides of β4-glucuronic acid-β3-*N*-acetylglucosamine.[3] The remaining ingredients in this report are derivatives of Hyaluronic Acid (e.g., Sodium Hyaluronate (CAS No. 9067-32-7) is a sodium salt of Hyaluronic Acid). The definitions of the ingredients included in this review are provided in Table 1.

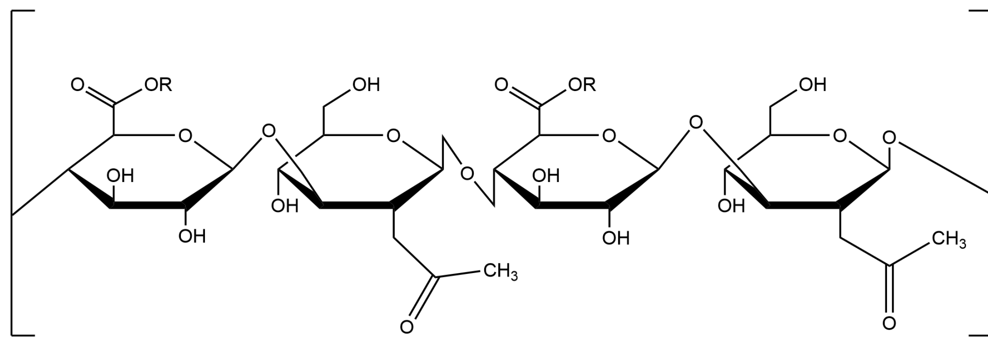


Figure 1. Hyaluronates (when R is hydrogen = Hyaluronic Acid; when R is sodium = Sodium Hyaluronate; etc.)

Chemical Properties

Hyaluronic Acid is a water-soluble substance that is available as a highly purified, freeze-dried powder or aqueous solution.[1] Hyaluronic Acid may also be presented as its potassium or sodium salt (i.e., Potassium Hyaluronate or Sodium Hyaluronate). The molecular weight (MW) of Hyaluronic Acid in cosmetics is highly variable and ranges from 5 – 1800 kiloDaltons (kDa), dependent upon manufacturing procedures. Hyaluronic Acid has a high capacity for water retention; 1 g of Hyaluronic Acid can hold up to 6 l of water.

These hyaluronates have a wide range of MW. For instance, according to the *Food Chemicals Codex* (*FCC*), the formula weight (FW) of Sodium Hyaluronate can vary from 80.2 to 4010 kDa. Other chemical properties of Hyaluronic Acid and Sodium Hyaluronate can be found in Table 2.

Method of Manufacture

Hyaluronic Acid is an ubiquitous substance that can be derived from several natural sources.[1] These sources can be found in the Natural Occurrence section of this report. According to unpublished data, Hyaluronic Acid obtained for cosmetic use is derived via either bacterial fermentation or extraction from rooster combs.[1]

Hyaluronic Acid

In order to manufacture Hyaluronic Acid from rooster combs, the frozen tissue is first thoroughly washed with water, acetone, ethanol, or a mixture of ethanol and chloroform.[4] The tissues are then grounded and extracted with a solvent. Examples of solvents include distilled water, salt solutions, and aqueous-organic mixtures. The substance then undergoes purification to remove potential impurities such as proteins, peptides, lipids, nucleic acids, mucopolysaccharides, and low MW precursors. Purification can be performed via extraction using ethanol, acetone, acetic acid, or a double volume of ethanol with sodium acetate. Proteins are typically removed using a water-chloroform or chloroform-iso-amyl alcohol extraction, followed by intensive stirring. In order to remove covalently bonded peptides and proteins, proteolytic enzymes such as pepsin, trypsin, papain, or pronase, may be used. A fractional precipitation with cetylpyridinium chloride followed by dissolution with sodium chloride may be performed to remove mucopolysaccharides from the final product. Polysaccharides can be removed with ion-exchange chromatography, cellulose, and gel-filtration. Other purification methods include ultrafiltration, sorption on the activated carbon, ion-exchange resin, electrodialysis, electrophoresis, and ultracentrifusion with caesium chloride.

Hyaluronic Acid derived from bacterial strains (e.g., *Streptococcus* sp.) involve the cultivation of these bacteria in conditions where the polysaccharide capsule containing Hyaluronic Acid is formed.[4] The cultural liquid containing accumulated Hyaluronic Acid is then ultrafiltrated, precipitated with an organic solvent, and purified using similar methods as described above for rooster comb-derived Hyaluronic Acid.

Hydrolyzed Hyaluronic Acid

Hydrolyzed Hyaluronic Acid (MW = 37 – 56 megaDaltons (MDa)) is manufactured via similar methods as stated below (see manufacturing process of Hydrolyzed Sodium Hyaluronate (FW = 5 - 10 kDa)).[5] However, when manufacturing Hydrolyzed Hyaluronic Acid, the Hyaluronic Acid product is mixed with ethanol and hydrochloric acid. When the Hyaluronic Acid is degraded to the set point, the pH is adjusted via a sodium hydroxide solution. Finally, the resulting solution is dehydrated and dried, yielding the final product.

Hydrolyzed Sodium Hyaluronate

Hydrolyzed Sodium Hyaluronate (formula weight (FW) = 5 - 10 kDa) manufactured for cosmetic use may be produced from the bacterial strain *Streptococcus equi* subsp. *Zooepidemicus*.[6] The process begins with the preparation of a seed broth prepared from seed culture, which is transferred from a fermenter containing sterilized fermentation medium. After fermentation, the seed broth is mixed with ethanol. The crude Sodium Hyaluronate precipitate is dissolved in water and filtered to remove impurities and inactivated fragments. The resulting filtrate is precipitated, dehydrated, and dried, yielding

the Hyaluronic Acid product. This product is dissolved in purified water and combined with an enzyme to create a solution that is then degraded, heated, filtered, precipitated, dehydrated, and dried.

Low-FW Hydrolyzed Sodium Hyaluronate (FW = 1 - 5 kDa) is produced via the hydrolysis by hyaluronidase from low MW Sodium Hyaluronate.[6] This process includes dissolution, enzymatic hydrolysis, inactivation, filtration, spray drying, sieving, and packaging. In order to produce Hydrolyzed Sodium Hyaluronate of a very low FW (FW < 1 kDa), Sodium Hyaluronate (FW > 1 MDa) undergoes enzymatic hydrolysis via purified water and hyaluronidase.[7] The resulting solution is ultrafiltrated and heated to denature and remove the remaining hyaluronidase. Activated carbon is then used to absorb the denatured hyaluronidase, and the residual hyaluronidase is removed via the removal of activated carbon through multistage filtration. The resulting filtrate is dried, yielding the final product.

<u>Sodium Hyaluronate</u>

According to a supplier, Sodium Hyaluronate, is manufactured via a similar process to that stated above for Hydrolyzed Sodium Hyaluronate (FW = 5 - 10 kDa; omitting enzymatic hydrolysis) using the bacterial strain *Streptococcus equi* subsp. *zooepidemicus*.[8,9] The manufacturing process for low MW Sodium Hyaluronate is the same; however, when manufacturing low MW Sodium Hyaluronate, the seed broth is degraded prior to mixing with ethanol.[10]

Impurities

When derived from animal sources, Hyaluronic Acid may contain several impurities.[1] These impurities include proteins, DNA, and chondroitin sulfate.

<u>Hyaluronic Acid</u>

The impurities (nucleic acid, protein, endotoxins) of Hyaluronic Acid obtained from several sources (e.g., *Streptococcus zooepidemicus*, rooster comb, bovine vitreous, human umbilical cord) were evaluated.[11] Nucleic acid and protein impurities were highest in human umbilical cord- and bovine vitreous-derived Hyaluronic Acid, and were lowest in bacterial- and rooster comb-derived Hyaluronic Acid. Human umbilical cord-, bovine vitreous-, and rooster comb-derived Hyaluronic Acid preparations contained high levels of endotoxin contaminants. Bacterially-derived Hyaluronic Acid was nearly endotoxin-free. The specific levels of impurities evaluated in these samples can be found in Table 3.

<u>Hydrolyzed Hyaluronic Acid</u>

A supplier reported that Hydrolyzed Hyaluronic Acid (MW = 37 - 56 kDa) contained < 0.5 endotoxin units (EU)/mg bacterial endotoxins, < 0.05% protein, < 0.5% chlorides, < 20 ppm total metals, and < 2 ppm arsenic.[12]

<u>Hydrolyzed Sodium Hyaluronate</u>

The same supplier as referenced above reported that several Hydrolyzed Sodium Hyaluronate ingredients of different FWs (<1 kDa, 1 - 5 kDa, and 5 - 10 kDa) contained < 0.5 EU/mg bacterial endotoxins, < 0.05% protein, < 0.5% chlorides, and ≤ 10 - 20 ppm total metals.[13]

<u>Sodium Hyaluronate</u>

According to a manufacturer, Sodium Hyaluronate contained < 5000 ppm residual solvents (ethanol), < 20 ppm heavy metals, < 2 ppm arsenic, and < 0.1% protein.[14] A different manufacturer reported that both Sodium Hyaluronate (FW ≥ 1 MDa) and low FW Sodium Hyaluronate (FW = 100 kDa -1 MDa) contained < 0.5 EU/mg bacterial endotoxins, < 0.05% protein, < 0.5% chlorides, and ≤ 20 ppm total metals.

The *FCC* states that Sodium Hyaluronate manufactured for use in foods may not contain more than 1 mg/kg lead, 2 mg/kg arsenic, or 0.5% chloride.[15] A manufacturer of food-use Sodium Hyaluronate states that potential contaminants of Sodium Hyaluronate include microbes and heavy metals.[16] This manufacturer requires a purity level of ≥ 93% Sodium Hyaluronate, and maximum lead and arsenic levels of 1 and 2 ppm, respectively. The same manufacturer also requires bacteria counts of ≤ 500 colony forming units (cfu)/g, yeast and mold counts of ≤ 100 cfu/g, and negative test readings for *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella* sp.

Natural Occurrence

Hyaluronic Acid and its derivatives can be found distributed throughout vertebrate tissues such as the brain, vitreous humor, umbilical cord, synovial fluid, skin, rooster combs, neural tissues, and epithelium.[3,17] Hyaluronic Acid is also a signaling molecule involved in biological processes such as embryonic development, wound healing, inflammation, and cancer. In addition, Hyaluronic Acid can be found in the extracellular capsule formed by gram-positive microorganisms such as *Streptococcus* sp. and *Pasteurella* sp..[4]

<u>USE</u>

Cosmetic

The safety of the cosmetic ingredients addressed in this assessment is evaluated based on data received from the US FDA and the cosmetics industry on the expected use of these ingredients in cosmetics, and does not cover their use in

airbrush delivery systems. Data are submitted by the cosmetic industry via the FDA's VCRP database (frequency of use) and in response to a survey conducted by the Personal Care Products Council (Council) (maximum use concentrations). The data are provided by cosmetic product categories, based on 21CFR Part 720. For most cosmetic product categories, 21CFR Part 720 does not indicate type of application and, therefore, airbrush application is not considered. Airbrush delivery systems are within the purview of the US Consumer Product Safety Commission (CPSC), while ingredients, as used in airbrush delivery systems, are within the jurisdiction of the FDA. Airbrush delivery system use for cosmetic application has not been evaluated by the CPSC, nor has the use of cosmetic ingredients in airbrush technology been evaluated by the FDA. Moreover, no consumer habits and practices data or particle size data are publicly available to evaluate the exposure associated with this use type, thereby preempting the ability to evaluate risk or safety.

According to 2022 FDA VCRP data, Sodium Hyaluronate has the highest frequency of use (4048 total formulations; 3680 leave-on formulations, 366 rinse-off formulations, and 2 formulations diluted for bath use; Table 4).[18] This use of this ingredient has increased significantly since it was last reviewed; it was reported to be used in 601 formulations in 2005.[1] All other ingredients are reported to be used in 568 formulations or less. The results of the 2021 concentration of use survey conducted by the Council indicate Sodium Hyaluronate also has the highest concentration of use; it is used at up to 7.5% in face and neck products (not spray).[19] In 2005, Sodium Hyaluronate was reported to be used at up to 2%. Current FDA VCRP data on the four hyaluronate ingredients included in this report that have not been previously reviewed (i.e., Hydrolyzed Calcium Hyaluronate, Hydrolyzed Hyaluronic Acid, Hydrolyzed Sodium Hyaluronate, and Sodium Acetylated Hyaluronate) can be found in Table 5.

Incidental ingestion of several of these ingredients may occur as they are reported to be used in lipstick formulations (e.g., Sodium Hyaluronate is used in lipsticks at up to 0.39%). In addition, these ingredients are also reported to be used in products that are applied near the eye; for example, Sodium Hyaluronate is used in eye shadows at up to 0.96%. Sodium Hyaluronate is also used in baby products at up to 0.005%.

Some of these hyaluronate ingredients are used in cosmetic sprays and powders, and could possibly be inhaled; for example, Sodium Hyaluronate is reported to be used at up to 0.01% in other skin care preparations (spray) and at up to 0.099% in face powders. In practice, as stated in the Panel's respiratory exposure resource document (https://www.cir-safety.org/cir-findings), most droplets/particles incidentally inhaled from cosmetic sprays would be deposited in the nasopharyngeal and tracheobronchial regions and would not be respirable (i.e., they would not enter the lungs) to any appreciable amount. Conservative estimates of inhalation exposures to respirable particles during the use of loose powder cosmetic products are 400-fold to 1000-fold less than protective regulatory and guidance limits for inert airborne respirable particles in the workplace.

Although products containing some of these ingredients may be marketed for use with airbrush delivery systems, this information is not available from the VCRP or the Council survey. Without information regarding the frequency and concentrations of use of these ingredients (and without consumer habits and practices data or particle size data related to this use technology), the data are insufficient to evaluate the exposure resulting from cosmetics applied via airbrush delivery systems.

All of the hyaluronate ingredients named in the report are not restricted from use in any way under the rules governing cosmetic products in the European Union.[20]

Non-Cosmetic

Hyaluronic Acid and Sodium Hyaluronate are reported to be used in FDA-approved medical devices as dermal fillers, surgical fluids, topical wound creams, osteoarthritis treatments, periodontitis treatments, and ophthalmic surgery aids.[21] In addition, Sodium Hyaluronate is used as in inactive ingredient in several FDA-approved medications.[22] These medications include injectable intra-articular, intramuscular, and intravitreal treatments containing Sodium Hyaluronate at up to 2.3% for various conditions such as arthritis and hypotony. Sodium Hyaluronate is also used in FDA-approved topical medications at up to 0.01% as a skin lubricant. In addition to human medicine, Sodium Hyaluronate has FDA-approved uses in veterinary medicine as an implantable or injectable treatment for joint ailments, as indicated in CFR 522.1145.[23]

Hyaluronic Acid is found as a natural component in foods as it is present in animal products.[16] Rooster combs, which are rich in Hyaluronic Acid, are eaten alone or in dishes such as chicken soups or stews in European countries. Sodium Hyaluronate is used as an ingredient in food (e.g., ready-to-eat cereal preparations and candies) and beverages including fruit drinks, soft drinks, milk, and milk products.[16] In addition, both Hyaluronic Acid and Sodium Hyaluronate are reported to be ingredients of dietary supplements on the market in the US.[24]

TOXICOKINETIC STUDIES

Dermal Penetration

Autoradiography was used to detect the dermal penetration of Hyaluronic Acid (in the form of [^{3}H]hyaluronan) in SKh/1 hairless mice (4 animals/group; sex not stated; one group treated with radioactive gel; one group treated without radioactive gel).[1] Mice were treated for either 3 or 12 total applications (12 h intervals). Twelve to 16 h after the last application, animals were examined. Radioactivity was found mainly in the dermis, from the outermost layer to the

lymphatic and blood vessels. In a second experiment using 10 mice (strain and sex not reported; performed according to similar procedures), radioactivity was found in the same distribution within the dermis. In both assays, grains were found in the keratinized layer of the skin and hair follicles. In a dermal penetration assay performed in 11 Sprague-Dawley rats, Hyaluronic Acid (1.35 – 4.5 kDa) was applied dermally, twice daily, for 5 d. The Hyaluronic Acid penetrated to a maximum depth of 136 µm beneath the epidermis. In a different assay, radiolabeled Hyaluronic Acid was placed on the back of one Sprague-Dawley rat (sex not stated; singular dose). After a 4-h absorption period, the test substance was found to penetrate the rat skin to a maximum depth of approximately 800 µm. Autoradiography was used to detect the dermal penetration of [^{3}H]Hyaluronic Acid gel (56.3 – 56.4 mg) in the forearm of one male subject (2 total applications 12 h apart; skin removed by biopsy 7 h after last treatment). The test substance was shown to disseminate through all layers of the skin.

Hyaluronic Acid

The dermal penetration of three Hyaluronic Acid solutions, with three different MW (20 - 50 kDa, 100 - 300 kDa, and 1000 - 1400 kDa), was evaluated via Raman microimaging.[25] Test solutions contained 1% Hyaluronic Acid in distilled water. The solution (300 µl) was placed on human dermatomed skin samples for 8 h. Control skin samples were treated with either water (negative control) or glycerin (positive control). After the diffusion period, the skin surface was cleaned, samples were frozen, 10 µm-thick transverse skin sections were obtained, and spectral images were recorded. Spectral images revealed that the Hyaluronic Acid solution with the lowest MW (20 – 50 kDa) was present in the skin section at around 100 µm (full epidermal depth). The Hyaluronic Acid solution with a MW range of 100 - 300 kDa was present at an epidermal depth of approximately 50 µm. Permeation did not exceed 25 µm for the 1 – 14 MDa Hyaluronic Acid solution. The majority of each of the Hyaluronic Acid solutions, regardless of MW, was found in the stratum corneum, around 25 µm from the skin surface.

Penetration Enhancement

According to a review article evaluating Hyaluronic Acid's influence as a drug delivery system for diclofenac, it was observed that dermal penetration was dependent on animal species.[1,26] The drug reservoir was formed in the deeper layers of the skin (dermis) in mice, while the drug reservoir was formed in more shallow layers of the skin (epidermis) in humans.

Absorption, Distribution, Metabolism, and Excretion (ADME)

Details on the oral absorption, distribution, and excretion studies summarized below can be found in Table 6.

In an absorption assay in which male Sprague-Dawley rats (n = 3) were dosed with 25 mg/kg [^{14}C]Hyaluronic Acid (MW = 920 kDa) via gavage, the peak plasma radioactivity level was 7.6 µg eq/ml 8 h post-administration.[27] The highest amount of radioactivity was observed in intestinal contents 8 h post-administration. When evaluating excretion, the total excretion of radioactivity in the urine, feces, and expired air was 91.3% by 168 h post-administration. In a different assay, male Sprague-Dawley rats (6/group) were orally administered Hyaluronic Acid (MW = 300 kDa; 200 mg/kg bw), and blood, cecal content, and ventral skin were evaluated at different time intervals.[28] The recovery rate of unsaturated hyaluronic disaccharides and unsaturated hyaluronic tetrasaccharides in the serum and skin was approximately 25 and 70%, respectively. Male Sprague-Dawley rats (8/group) were given Hyaluronic Acid (MW = 300,000 Da) in distilled water (5 ml/kg bw/d; 1 and 5% concentrations; 5 d administration) via gavage, and excretion parameters were evaluated. Hyaluronic Acid was below the detection limit in the feces for all treated groups. The distribution of [^{99m}Tc]Hyaluronic Acid (0.2 ml; single dose; MW = 1.1 – 1.5 MDa) was evaluated in Wistar rats (3/group).[29] A rapid uptake of radioactivity was observed in the bone, muscle, small intestine, and large intestine at the 5- and 15-min time points. Excretion assays were performed in Wistar rats (n = 5) and Beagle dogs (n = 2) using the same test substance ([^{99m}Tc]Hyaluronic Acid; 0.2 ml in rats and 1.5 ml in dogs; single dose administration). In rats, the total radioactivity excretion in urine and feces was 86.7 ± 8.0% of the ingested radiolabel-dose. In dogs, urinary radioactivity clearance appeared after 30 min, and returned to background after 12 h.

TOXICOLOGICAL STUDIES

Acute Toxicity Studies

No deaths were observed in an acute oral toxicity assay in which ICR mice were given > 1200 mg/kg Hyaluronic Acid.[1] No other details were provided.

Details on the acute oral toxicity studies summarized below can be found in Table 7.

Several acute oral toxicity assays were performed using Sodium Hyaluronate in mice (at up to 15,000 mg/kg bw) and rats (at up to 5280 mg/kg bw).[16] No signs of toxicity or deaths were reported in any of these assays.

Short-Term and Subchronic Toxicity Studies

No toxicity was observed in in a short-term inhalation toxicity assay performed in male Beagle dogs exposed to 10% Hyaluronic Acid formulations containing insulin. No other details were provided for this study.

Details on the short-term and subchronic oral toxicity assays summarized below can be found in Table 8.

No signs of toxicity were observed in short-term and subchronic oral toxicity assays of Sodium Hyaluronate.[16] These assays include a 30-d study in which Wistar rats (10/sex/group) were given up to 1500 mg/kg bw Sodium Hyaluronate via

feed, a 90-d assay in which Sprague-Dawley rats (5 - 10/sex/group) were given up to 48 mg/kg bw/d of a 1% Sodium Hyaluronate ophthalmic solution via gavage, a 90-d assay in which Wistar rats (10/sex/group) were given up to 1000 mg/kg bw/d Sodium Hyaluronate via feed, and a 90-d study using Wistar rats (12/sex/group) given up to 1333 mg/kg bw/d Sodium Hyaluronate in corn oil via gavage.

DEVELOPMENTAL AND REPRODUCTIVE TOXICITY STUDIES

Several reproductive and developmental studies on subcutaneously injected Hyaluronic Acid (up to 60 mg/kg/d) and Sodium Hyaluronate (up to 50 mg/kg/d) were performed in rats and rabbits.[1] In the majority of assays, treatment with the test substance had no effect on mortality, necropsy observations post-delivery, food or water consumption, or fertility in dams. However, in one assay in which rats were given up to 60 mg/kg bw of a 1% Hyaluronic Acid solution in physiological saline via subcutaneous injection, nodular hyperplasia of reticular zone cells were present in the adrenal glands of treated dams (treatment on day 17 of pregnancy to day 20 after parturition). No severe fetal abnormalities were observed in rats or rabbits.

Sodium Hyaluronate

A sperm malformation assay was performed in adult male mice (strain not stated; 10/group).[30] Sodium Hyaluronate (20 ml/kg bw), cyclophosphamide (40 mg/kg bw (positive control), and distilled water (negative control) was given to animals via gavage, once a day, for 5 d. Mice were killed 30 d after the last administration. No other details were provided. The test substance had no influence on sperm malformation rate.

A teratogenicity assay was performed in Wistar rats (15/group) given Sodium Hyaluronate (FW = 270 kDa) via gavage in doses of 0, 170, 330, or 670 mg/kg bw/d (administration during gestation days 7 - 16).[16] Dams were euthanized and evaluated on day 20 of gestation. No statistically significant differences ($P > 0.05$) were observed in the maternal, uterine, and ovary weights or in the number of corpus lutea and nidation between test and control groups. In addition, no statistically significant differences ($P > 0.05$) were observed between the control and treated groups regarding the weight, length, and number of living embryos. No evidence of maternal or embryo toxicity resulting from the test substance administration was observed.

In a different teratogenicity assay, Sprague Dawley rats (12/group) were given Sodium Hyaluronate, via gavage, in doses of 333, 667, or 1333 mg/kg bw/d, on gestation days 7 - 16.[16] A negative control group was given water and a positive control group was given aspirin on the same gestation days. Animals were euthanized and evaluated on gestation day 20. No statistical differences in maternal body weight, number of corpus lutea, implantations, uterine weight, placental weight, live fetus rate, fetal death rate, or absorbed fetus rate were observed between test and negative control treated groups. Fetal development and growth were similar between control and negative control treated groups. No evidence of maternal toxicity or teratogenicity resulting from test substance administration was observed.

GENOTOXICITY STUDIES

No genotoxicity was observed in an Ames assays evaluating Sodium Hyaluronate (up to 1%; up to 5000 µg/plate) in Staphylococcus aureus, Salmonella typhimurium and Escherichia coli or in an in vitro chromosomal aberration assay evaluating the genotoxic potential of Sodium Hyaluronate (up to 1000 µg/ml) in Chinese hamster lung fibroblasts. Negative results were also observed in an in vivo micronucleus assay using 1% Sodium Hyaluronate (up to 400 mg/kg) in CD-1 (ICR) mice and in a micronucleus assay evaluating ICR (Crj: CD-1) mice treated with 360 mg/kg Sodium Hyaluronate for up to 4 d.

Details on the genotoxicity assays summarized below can be found in Table 9.

Hydrolyzed Sodium Hyaluronate (FW = 5-10 kDa), Hydrolyzed Sodium Hyaluronate (FW < 1 kDa) and Sodium Hyaluronate were determined to be non-genotoxic in several Ames assays when tested at up to 5 mg/plate, with and without metabolic activation (assays performed in *S. typhimurium* strains TA97a, TA98, TA100, TA102, TA1535, TA537 and *E. coli* WP2 *uvrA*).[16,30-32] In addition, Sodium Hyaluronate (up to 5000 mg/kg bw) was non-mutagenic in mouse micronucleus assays.[30]

CARCINOGENICITY STUDIES

Mouse melanoma cell lines with high Hyaluronic Acid production had increased lung metastasis and lower survival than melanoma cell lines with lower Hyaluronic Acid production.[1] Aneuploid human breast adenocarcinoma cells modified with antisense inhibition of hyaluronan synthase 2 expression produced more high MW Hyaluronic Acid. Injection of these cells into mice did not result in primary tumors. In other studies, well-differentiated tumors (e.g., salivary gland, stomach, colon) had intense Hyaluronic Acid-staining in the tumor cells, intratumoral and associated surrounding stroma. Poorly differentiated tumor samples (e.g., astrocytomas, infiltrating breast, stomach, gallbladder) with carcinoma or sarcoma had almost no Hyaluronic Acid when stained. Enhanced motility of human pancreatic carcinoma cells was dependent on the cluster of differentiation 44 (CD44)-hyaluronic acid interaction where low MW Hyaluronic Acid induced angiogenesis,

enhanced CD44 cleavage, and promoted the migration of the tumor cells in a CD44-dependent manner. In a different study, stromal Hyaluronic Acid was not related to survival or recurrence-free survival from cutaneous melanoma. Compared with normal epidermis, in situ carcinomas and well-differentiated squamous cell carcinomas showed an enhanced Hyaluronic Acid signal on carcinoma cells, while CD44 expression resembled normal skin. Less-differentiated squamous cell carcinoma samples had reduced and irregular expression of Hyaluronic Acid and CD44 on carcinoma cells. In basal cell carcinoma samples, Hyaluronic Acid was frequently present on cell nuclei but not in the other types of samples. Hyaluronidase applied to tumors or tumor cells injected into the footpads of mice reduced growth rates in human breast carcinoma. Hyaluronic Acid levels have been found to be increased in tissues surrounding some breast cancer, gastric cancer, poorly differentiated, serous histological type, advanced stage, and large primary tumor epithelial ovarian cancer, endometrial cancer, ganglioma, thyroid cancer, and salivary gland cancer. Normal and low levels of stromal Hyaluronic Acid were associated with early International Federation of Gynecology and Obstetrics (FIGO) stage, mucinous histological-type epithelial ovarian cancer, and murine astrocytoma. Increased Hyaluronic Acid intensity in breast cancer patients was related to axillary lymph node positivity and poor survival.

OTHER RELEVANT STUDIES

Multiple injections of Hyaluronic Acid derived from human umbilical cords or streptococcal fermentation did not result in sensitization in immunogenicity studies performed in rabbits (administration of test substance via either subcutaneous or intramuscular route). In an antigenicity assay using injected streptococcal-derived Hyaluronic Acid in rabbits, precipitating antibodies were observed. A similar assay was performed using purified Hyaluronic Acid derived from rooster combs and human umbilical cords in rabbits. No formations of passive cutaneous anaphylaxis reactive antibodies were observed. Antibody response by rooster comb-derived Hyaluronic Acid caused an enhanced secondary antibody response to birch pollen, egg albumen, and dog albumen in rats. Neither commercial Sodium Hyaluronate preparations nor a crude rooster comb Sodium Hyaluronate preparation elicited a Hyaluronic Acid-specific antibody response in rabbits.

Use in Dissolving Microarray Patches

Hyaluronic Acid

The following study is included in this report as it may be helpful in addressing cosmetic safety concerns regarding irritation following dermal exposure to Hyaluronic Acid. Dissolving microarray patches containing 30% Hyaluronic Acid (in distilled water) were placed under and at the corner of the eyes of 30 female subjects aged 35 - 60.[33] Patches were applied 3x/wk for 4 wk. Safety was assessed by the degree of adverse effects, including facial itching, prickling, burning, erythema, edema, and swelling. These parameters were evaluated by participant questionnaires. No adverse effects on the skin or eyes were reported throughout the study.

DERMAL IRRITATION AND SENSITIZATION STUDIES

Hyaluronic Acid was non-irritating in a single-stimulus skin test using Japanese rabbits and Hartley guinea pigs.[1] In addition, no irritation was observed in a human closed skin patch test using Hyaluronic Acid produced via fermentation. No details were provided for either study. A skin prick test was performed in 9 subjects. The forearm of each subject was pricked with Sodium Hyaluronate (10 mg/ml), and evaluated 15 min, and 2, 6, and 24 h after pricking. No skin reactions were observed.

Details on the dermal irritation and sensitization data summarized below can be found in Table 10.

In vitro dermal irritation assays performed on two trade name mixtures containing 1% Hyaluronic Acid (tested neat), a trade name mixture containing 3% Hyaluronic Acid (tested neat), Hydrolyzed Sodium Hyaluronate (FW < 1 kDa; concentration not reported), a trade name mixture containing 0.5% Sodium Hyaluronate (tested neat), and 100% Sodium Hyaluronate yielded negative results.[30,32,34-37] No irritation was observed in human dermal irritation assays performed under occlusive conditions using Hydrolyzed Sodium Hyaluronate (several FW tested; 30 - 32 subjects; tested at up to 2%).[32] No sensitization was noted in a direct peptide reactivity assay (DPRA) performed on a trade name mixture containing 1% Hyaluronic Acid (tested at up to 25 mM), in a KeratinoSens™ assay performed on a trade name mixture containing 1% Hyaluronic Acid (up to 2 mM), and in a human cell line activation test (h-CLAT) performed on Sodium Hyaluronate (tested at 1 mg/ml).[30,38,39] Similarly, no sensitization was observed in human repeat insult patch tests (HRIPTs) performed using a formula containing 0.2% Hyaluronic Acid (114 subjects; tested neat), a formula containing 0.2% Sodium Acetylated Hyaluronate (104 subjects; tested neat), Hydrolyzed Sodium Hyaluronate (FW = 5 - 10 kDa; 55 subjects; tested at 0.5%); Sodium Hyaluronate (50 - 100 subjects; tested at 0.2%), and a formula containing 1.5% Sodium Hyaluronate (198 subjects; tested neat).[30-32,40-42]

Phototoxicity

In Vitro

Summary data were provided from a supplier on 3T3 neutral red uptake phototoxicity assays performed on Hydrolyzed Sodium Hyaluronate (FW < 1 kDa; up to 128 mg/ml) and Sodium Hyaluronate (125 µg/ml).[30,32] Neither test substance was predicted to induce phototoxicity.

OCULAR IRRITATION STUDIES

Details on the ocular irritation summarized below can be found in Table 11.

No ocular irritation was observed in EpiOcular^{TM} assays performed on 2 trade name mixtures containing 1% Hyaluronic Acid (tested neat), a trade name mixture containing 3% Hyaluronic Acid (tested neat), and a trade name mixture containing 0.5% Sodium Hyaluronate (tested neat).[34-37] Test substances were considered to be non-irritating/slightly irritating in chorioallantoic membrane vascular assays (CAMVA) performed using Hydrolyzed Sodium Hyaluronate (FW < 1 kDa; concentration tested not reported) and Sodium Hyaluronate (tested neat).[30,32] Similar results were observed in a bovine corneal opacity and permeability (BCOP) test performed using Sodium Hyaluronate (tested neat). In addition, a *Bacillus*-derived and *Streptococcus*-derived Hyaluronic Acid (up to 0.3%) was considered to be very well-tolerated when tested in the eyes of New Zealand white rabbits (3/group).[43]

CLINICAL STUDIES

Nebulized Nasal Hypertonic Solution in the Treatment of Chronic Rhinosinusitis

Hyaluronic Acid

Eighty patients with chronic rhinosinusitis were instructed to use a nasal spray containing high MW Hyaluronic Acid and sodium chloride, twice daily (2 puffs per nostril at each administration), for 20 d.[44] Patients were assessed at baseline, on day 10, and day 20. An endoscopic nasal examination and nasal obstruction symptom evaluation instrument (I-NOSE) questionnaire were performed during each visit, and safety parameters (adverse effects and local tolerability (burning, irritation, congestion) were assessed. Patients were instructed to keep diaries to log changes in symptoms. The improvement in chronic rhinosinusitis compared to baseline as measured by the I-NOSE score, was statistically significantly ($P < 0.001$). According to patient diaries, nasal blockage, congestion, drainage, and rhinorrhea was significantly improved between baseline and day 20 ($P < 0.01$). Fourteen patients experienced at least one adverse effect; however, these effects were not related to study treatment. No symptoms related to local tolerability at the site of administration were reported.

Immediate and Delayed Hypersensitivity to Intracutaneous Hyaluronic Acid

Hyaluronic Acid

Twelve patients with previously reported inflammatory responses to Hyaluronic Acid fillers were subjected to intracutaneous tests.[45] Approximately 0.1 ml of each filler was tested on the inner sides of the upper arms in a randomized manner. Tests were read after 15 min, and 2, 3, 4, and 7 d following application. Potential late reactions were monitored after 2 and 4 wk, and patients were instructed to contact study conductors in case at any later reaction. No positive reactions were observed for any of the tested Hyaluronic Acid fillers during the testing period, or during the 4 mo follow-up.

Treatment of Dry Eye

Sodium Hyaluronate

The effectiveness of Sodium Hyaluronate eye drops was evaluated in 13 patients with moderate dry eye.[46] Patients were treated with instillations of 40 µl of 0.1% Sodium Hyaluronate, 0.3% Sodium Hyaluronate, or 0.9% saline, in a randomized, double-masked manner (vehicles for treatment not stated). Symptom intensity and non-invasive break-up time (NIBUT) were evaluated at 5, 15, 30, 45, 60 min, and hourly, until 6 h after drop instillation. This process was repeated twice following an interval of approximately 7 d, but with a different treatment, so that at the end of the final visit, each subject had trialed all products. Symptoms and NIBUT improved with all treatments; however, improvement (reduction in eye irritation) was of a greater magnitude and longer duration with Sodium Hyaluronate drops. Eye drops containing 0.3% Sodium Hyaluronate performed better than 0.1% Sodium Hyaluronate ($P = 0.04$).

Treatment of Rosacea

Sodium Hyaluronate

The effect of a cream containing 0.2% Sodium Hyaluronate (containing low MW Hyaluronic Acid) was evaluated in 14 patients with mild to moderate facial rosacea.[47] Patients were instructed to apply the cream, following cleansing, on the whole face, twice daily, for 4 wk. After 4 wk, patients continued the cleansing regimen for an additional 4 wk, but discontinued the use of the cream containing Sodium Hyaluronate. Patients were evaluated for papules, pustules, erythema, edema, telangiectasia, burning, stinging, and/or dryness at baseline and at 2-wk intervals following administration. No patients experienced adverse effects throughout the study. The largest reduction in erythema was observed at the 2-wk visit (48.3% reduction). At the 4-wk visit, it was reported that treatment with 0.2% Sodium Hyaluronate cream resulted in a reduction of papules, erythema, burning/stinging, and dryness in all patients.

Case Reports

Numerous case reports were found in the literature regarding hypersensitivity/adverse reactions to Hyaluronic Acid used as an injectable dermal filler. A summary of these studies has been provided and can be found in Table 12. In addition, it should be noted that case reports were also found on the adverse effects of Hyaluronic Acid following other methods of administration (e.g., intra-articular injections for osteoarthritis, injection during surgical procedures). These studies were not

summarized in the table, as their relevance to the cosmetic use of Hyaluronic Acid is not likely; however, two case report regarding photodermatitis following the intra-articular administration of Hyaluronic Acid in the knee has been summarized below, as it may have relevance in evaluating the photosensitivity-inducing potential of Hyaluronic Acid. In addition, a case report regarding an anaphylactic response in an elderly patient following oral exposure to Hyaluronic Acid has also been included.

A 71-yr-old man with a history of osteoarthritis reported previous treatment with three-series 2 ml Hyaluronic Acid injections in the knee for 5 yr with no adverse reactions.[48] The patient switched to a single 6 ml Hyaluronic Acid injection and immediately developed pain and swelling at injection site. These effects were also seen 5 mo later following a second injection of 6 ml Hyaluronic Acid. Six mo after the second injection, the patient received another 6 ml Hyaluronic Acid injection in the knee and developed a similar localized inflammatory reaction with chills. Eight days later, the patient developed erythematous, pruritic, scaly papules and plaques near the injection site. Several weeks later, he presented with photo-distributed scale and scattered excoriations on the bilateral cheeks and all four extremities. A similar reaction was observed in a 65-yr-old woman who also switched from three-series 2 ml Hyaluronic Acid injections in the knee, with no adverse effects for 13 yr, to a singular 6 ml Hyaluronic Acid injection. Following the singular injection, the patient displayed a localized inflammatory reaction at the injection site, followed by the development of photo-distributed erythematous macules and papules with pustules and scale on the face and all four extremities. Both patients recovered following treatment with triamcinolone and prednisone.

Upper airway angioedema was observed in a 100-yr-old woman following application of a spray containing xylitol and Hyaluronic Acid (0.01%) to the inner lower lip and gums to treat gingival sores for the third time in 2 d.[49] The previous two applications were much smaller in quantity. Following admission to the emergency department, the patient became dyspneic and hypoxemic, with edema of the lip, lower face, and epiglottis. The patient recovered following treatment with oxygen, epinephrine, methylprednisolone, diphenhydramine, ranitidine, and icatibant.

SUMMARY

The safety of Hyaluronic Acid and 6 hyaluronate ingredients are reviewed in this safety assessment. The majority of these ingredients are reported to function in cosmetics as skin and/or hair conditioning agents. Sodium Acetylated Hyaluronate is reported to function in cosmetics as a humectant. In cosmetics, these hyaluronates are derived from either bacterial fermentation or rooster combs. Hyaluronic Acid, Potassium Hyaluronate, and Sodium Hyaluronate have previously been reviewed by the Panel and were considered safe in the present practices of use and concentration as described in the safety assessment published in 2009.

According to 2022 VCRP survey data, Sodium Hyaluronate is reported to be used in 4048 formulations (3680 leave-on formulations, 366 rinse-off formulations, and 2 formulations diluted for bath use), and Hyaluronic Acid is reported to be used in 568 formulations (493 leave-on formulations, 72 rinse-off formulations, and 3 formulations diluted for bath use). All other ingredients are reported to be used in 378 formulations or less. The results of the 2021 concentration of use survey conducted by the Council indicate Sodium Hyaluronate also has the highest concentration of use in a leave-on formulation; it is used at up 7.5% in face and neck products (not spray).

A dermal penetration assay was performed in human dermatomed skin samples using Hyaluronic Acid solutions of three different MW (20 - 50 kDa, 100 - 300 kDa, and 1 – 1.4 MDa). Hyaluronic Acid solutions, from lowest to highest MW, were present at epidermal depths of 100 µm, 50 µm, and 25 µm, respectively. Regardless of the MW of the Hyaluronic Acid solution, the majority quantity of Hyaluronic Acid was found in the stratum corneum, approximately 25 µm from the skin surface.

In an absorption assay in which male Sprague-Dawley rats (n = 3) were dosed with 25 mg/kg [^{14}C]Hyaluronic Acid (MW = 920 kDa) via gavage, the peak plasma radioactivity level was 7.6 µg eq/ml 8 h post-administration. When evaluating excretion, the total excreted radioactivity in the urine, feces, and expired air was 91.3% by 168 h post-administration. In a different assay, male Sprague-Dawley rats were orally administered Hyaluronic Acid (MW = 300 kDa; 200 mg/kg bw), and blood, cecal content, and ventral skin were evaluated at different time intervals. The recovery rate of unsaturated hyaluronic disaccharides and unsaturated hyaluronic tetrasaccharides in the serum and skin was approximately 25 and 70%, respectively. Male Sprague-Dawley rats were given Hyaluronic Acid (MW = 300 kDa) in distilled water (5 ml/kg bw/d; 1 and 5% concentrations; 5 d administration) via gavage, and excretion parameters were evaluated. Hyaluronic Acid was below the detection limit in the feces for all treated groups. The distribution and excretion of [^{99m}Tc]Hyaluronic Acid (MW = 1.1 – 1.5 MDa) was evaluated in Wistar rats and Beagle dogs. In rats, a rapid uptake of radioactivity was observed in the bone, muscle, small intestine, and large intestine at the 5- and 15-min time points. The total urine and feces excretion of the ingested dose was 86.7 ± 8.0%. In dogs, urinary clearance of the test substance appeared after 30 min and returned to background after 12 h.

No signs of toxicity were observed in several acute oral toxicity assays performed using Sodium Hyaluronate in mice (at up to 15,000 mg/kg bw) and rats (at up to 5280 mg/kg bw). Similarly, no signs of toxicity were observed in 30- and 90-d oral toxicity assays performed in rats given Sodium Hyaluronate (up to 1333 mg/kg bw/d).

Sodium Hyaluronate (20 ml/kg) did not have an influence on sperm malformation in adult male mice in a sperm malformation assay. No maternal toxicity or teratogenicity resulting from Sodium Hyaluronate (up to 1333 mg/kg bw/d) were observed in two reproductive toxicity assays using rats. In both assays, animals were treated via gavage, on gestation days 7 - 16. All measured parameters (e.g., ovary weights, number of living embryos, implantations) were similar among control and treated groups.

Hydrolyzed Sodium Hyaluronate and Sodium Hyaluronate were determined to be non-genotoxic in several Ames assays performed using strains of *S. typhimurium*, at concentrations up to 5 mg/plate, with and without metabolic activation. Similarly, no mutagenicity was observed in micronucleus assays using mice given up to 5000 mg/kg bw Sodium Hyaluronate.

The safety of dissolving microarray patches containing Hyaluronic Acid (30% in distilled water) placed under the eyes was evaluated in 30 subjects (patches applied 3x/wk for 4 wk). No adverse dermal or ocular effects were reported throughout the study.

In vitro dermal irritation assays performed on two trade name mixtures containing 1% Hyaluronic Acid (tested neat), a trade name mixture containing 3% Hyaluronic Acid (tested neat), Hydrolyzed Sodium Hyaluronate (FW < 1 kDa; concentration not reported), a trade name mixture containing 0.5% Sodium Hyaluronate (tested neat), and 100% Sodium Hyaluronate yielded negative results. No irritation was observed in human dermal irritation assays performed under occlusive conditions using Hydrolyzed Sodium Hyaluronate (tested at up to 2%). No sensitization was noted in a DPRA performed on a trade name mixture containing 1% Hyaluronic Acid (tested at up to 25 mM), in a KeratinoSens^{TM} assay performed on a trade name mixture containing 1% Hyaluronic Acid (up to 2 mM), or in an h-CLAT performed on Sodium Hyaluronate (tested at 1 mg/ml). Similarly, no sensitization was observed in HRIPTs performed using a formula containing 0.2% Hyaluronic Acid (tested neat), a formula containing 0.2% Sodium Acetylated Hyaluronate (tested neat), Hydrolyzed Sodium Hyaluronate (FW = 5 - 10 kDa; tested at 0.5%); Sodium Hyaluronate (tested at 0.2%), and a formula containing 1.5% Sodium Hyaluronate (tested neat). No potential for phototoxicity was observed in in vitro phototoxicity assays performed on Hydrolyzed Sodium Hyaluronate (FW < 1 kDa) and Sodium Hyaluronate.

In vitro ocular irritation assays performed on several test substances (trade name mixtures containing 1% Hyaluronic Acid, trade mixture containing 3% Hyaluronic Acid, Hydrolyzed Sodium Hyaluronate, Sodium Hyaluronate (100%), and a trade name mixture containing 0.5% Sodium Hyaluronate) yielded slightly irritating/non-irritating or non-irritating results. In addition, a *Bacillus*-derived and *Streptococcus*-derived Hyaluronic Acid (up to 3%) was considered to be very well-tolerated when tested in the eyes of rabbits.

Eighty patients with chronic rhinosinusitis were treated with a nasal spray containing high MW Hyaluronic Acid and sodium chloride (2 puffs/nostril/d for 20 d). A statistically significant improvement in rhinosinusitis symptoms was observed at the end of treatment compared to baseline ($P < 0.01$). No study-related adverse effects were observed. The treatment was considered to be well-tolerated.

Twelve patients with previously-reported inflammatory responses to Hyaluronic Acid fillers were subjected to intracutaneous tests using 6 different types of Hyaluronic Acid fillers. No positive reactions were observed for any of the tested Hyaluronic Acid fillers during the testing period, or during the 4 mo follow-up

The effect of Sodium Hyaluronate eye drops was evaluated in 13 patients with dry eye. Patients were treated with instillations of 40 µl of 0.1% Sodium Hyaluronate, 0.3% Sodium Hyaluronate, or 0.9% saline, in a randomized, double-masked manner (vehicles for treatment not stated). Symptoms of dry eye improved with the use of all treatments; however, improvement was greatest with the use of 0.3% Sodium Hyaluronate drops.

The effect of a cream containing 0.2% Sodium Hyaluronate was evaluated in 14 patients with facial rosacea. Use of the cream (2x/d for 4 wk) resulted in a reduction in rosacea symptoms. No adverse effects were reported throughout the study.

Case reports were found in the literature regarding hypersensitivity/adverse reactions to Hyaluronic Acid used as an injectable dermal filler. Two case reports stated that treatment of osteoarthritis with three-series 2 ml Hyaluronic Acid injections was performed without adverse effects; however, switching to a single 6 ml Hyaluronic Acid injection did result in adverse effects. In another case report, upper airway angioedema was observed in a 100-yr-old woman after use of a spray containing xylitol and Hyaluronic Acid (0.01%) to the inner lower lips and gums; the patient recovered following treatment.

DISCUSSION FROM 2009 REPORT ON HYALURONIC ACID, SODIUM HYALURONATE, AND POTASSIUM HYALURONATE

While Hyaluronic Acid has multiple sources, including rooster combs, bovine sources, and bacterial fermentation, in cosmetics, the only sources of Hyaluronic Acid used are bacterial fermentation and rooster combs.[1] Because there is an avian source for these cosmetic ingredients, the matter of avian flu was considered. Because the heat from the manufacturing process reliably kills the avian flu virus, no safety concern exists in this regard. While there are no specific infectious agent concerns, the Panel is mindful of the need to derive these ingredients only from disease-free animals. Bacterial sources should be free of pyrogens.

The Expert Panel recognized that these ingredients can enhance the penetration of other ingredients through the skin (e.g., HC Yellow No. 4, Disperse Yellow 3). The Panel cautioned that care should be taken in formulating cosmetic products that may contain these ingredients in combination with any ingredients whose safety was based on their lack of dermal absorption data or when dermal absorption was a concern.

After reviewing inhalation toxicity data on dogs and sheep, the Expert Panel determined that Hyaluronic Acid, Sodium Hyaluronate, and Potassium Hyaluronate can be used safely in sprays because the ingredient particle size is not respirable. The Panel reasoned that, for example, the particle size of anhydrous hair sprays (60-80 mm) and pump hair sprays (>80 mm) is large compared with the median aerodynamic diameter of 4.25 + 1.5 mm for a respirable particulate mass.

The Expert Panel considered that the amount of Hyaluronic Acid present naturally in human skin was relevant to considering the effect of exogenous Hyaluronic Acid. The amount of Hyaluronic Acid in the skin is approximately 0.6 mg/g skin. The average woman has a total surface area of 16,900 cm^2; approximately 15% of a 60-kg woman is skin which is approximately 9000 g. Dividing the weight of skin by the area of skin on a woman (9000 g/16,900 cm^2), the figure of 0.53 g skin/cm^2 is reached. The Expert Panel estimated the amount of Hyaluronic Acid in skin by area to be 0.318 mg Hyaluronic Acid/cm^2 skin.

The Expert Panel compared the amount of Hyaluronic Acid found in the skin to the maximum amount of Hyaluronic Acid applied to the skin by cosmetic products, as noted in this report, of 0.02 mg/cm^2 by a product with the maximum concentration of 2%, and found the contribution via application of such a cosmetic product to be negligible. Acute, short-term, and chronic toxicological studies indicated low toxicity.

The Expert Panel recognized that Hyaluronic Acid has been linked to metastatic cancer and sought to resolve whether the relationship was causal. In that regard, one seminal study reported a reduced level of Hyaluronic Acid associated with an unfavorable prognosis of clinical stage 1 cutaneous melanoma. These results suggest that, in melanoma, Hyaluronic Acid does not play a role in the metastatic process. In another pivotal study, Hyaluronic Acid was studied using the Hyaluronic Acid receptor, CD44 (a cell surface glycoprotein that is involved in cell/cell and cell/ matrix interactions) on epidermal keratinocyte tumors, specifically, basal cell carcinomas and squamous cell carcinomas. In basal cell carcinomas, CD44 expression was quite low. In squamous cell carcinomas, CD44 expression was variable. As the malignancy became less differentiated and, therefore, would be expected to have a higher risk for metastasis, the expression of Hyaluronic Acid decreased.

These key findings suggest that Hyaluronic Acid likely does not play a causal role in metastasis and that increased expression of Hyaluronic Acid genes may be a consequence of metastatic growth, not the converse. These results, together with the levels of Hyaluronic Acid that would be applied to the skin, would further ensure the safety of this ingredient in cosmetic products.

The Expert Panel discussed the possible need for additional dermal irritation and sensitization, UV absorption, and/or photosensitization and photoirritation data. Taking into consideration the above-mentioned calculation on the amount of Hyaluronic Acid in the skin compared to the amount that might be contributed by the application of cosmetics, the Expert Panel decided that the amount of Hyaluronic Acid in cosmetics would be negligible and not a concern in these areas. Even though adverse reactions to injected Hyaluronic Acid used in the treatment of osteoarthritis and tissue augmentation are reported, these data do not raise safety concerns regarding the use of Hyaluronic Acid in cosmetics. There were no reported reactions to topically applied Hyaluronic Acid, further supporting that hyaluronic acid at levels currently used in cosmetics applied to the skin should not be of concern.

The Expert Panel recognizes that there are data gaps regarding use and concentration of these ingredients. However, the overall information available on the types of products in which these ingredients are used and at what concentrations indicate a pattern of use, which was considered by the Expert Panel in assessing safety.

DISCUSSION

To be developed

CONCLUSION

To be determined.

TABLES

Table 1. Definitions and reported functions of the hyaluronate ingredients[2]

Ingredient	Definition	Function
Hyaluronic Acid (9004-61-9)	Hyaluronic Acid is the natural mucopolysaccharide formed by bonding *N*-acetyl-D-glucosamine with glucuronic acid. *See Figure 1, wherein "R" is hydrogen.*	Skin-Conditioning Agents - Miscellaneous; Viscosity Increasing Agents - Aqueous
Hydrolyzed Calcium Hyaluronate	Hydrolyzed Calcium Hyaluronate is the hydrolysate of the calcium salt of Hyaluronic Acid derived by acid, enzyme, or other method of hydrolysis. *See Figure 1, wherein 2 "R" are replaced by 1 calcium cation.*	Skin-Conditioning Agents - Miscellaneous
Hydrolyzed Hyaluronic Acid	Hydrolyzed Hyaluronic Acid is the hydrolysate of Hyaluronic Acid derived by acid, enzyme or other method of hydrolysis.	Hair Conditioning Agents; Skin-Conditioning Agents - Humectant
Hydrolyzed Sodium Hyaluronate	Hydrolyzed Sodium Hyaluronate is the hydrolysate of Sodium Hyaluronate derived by acid, enzyme, or other method of hydrolysis.	Skin-Conditioning Agents - Miscellaneous
Potassium Hyaluronate (31799-91-4)	Potassium Hyaluronate is the potassium salt of Hyaluronic Acid. *See Figure 1, wherein R is potassium.*	Skin-Conditioning Agents - Miscellaneous
Sodium Acetylated Hyaluronate	Sodium Acetylated Hyaluronate is the acetyl ester of Sodium Hyaluronate. *See Figure 1, wherein "R" is sodium, and one or more hydroxyl groups are acetylated.*	Humectants
Sodium Hyaluronate (9067-32-7)	Sodium Hyaluronate is the sodium salt of Hyaluronic Acid. *See Figure 1, wherein "R" is sodium.*	Skin-Conditioning Agents - Miscellaneous

Table 2. Chemical properties of Hyaluronic Acid and Sodium Hyaluronate

Property	Value	Reference
Hyaluronic Acid		
Physical Form	powder	1
MW (kDa)	5 - 1800	1
Sodium Hyaluronate		
Physical Form	powder	1
Color	white	1
Odor	faint odor	1
FW (kDa)	80.2 - 4010	15

FW = formula weight; MW = molecular weight

Table 3. Molecular weight (MW) and impurities measurements of Hyaluronic Acid derived from different sources (per 1 mg Hyaluronic Acid)[11]

	Human umbilical cord	Bacterially-derived	Bacterially-derived*	Rooster comb	Bovine vitreous
MW (x 10^6 Da)	1.3 ± 0.1	1.6	1.4	1.4	0.4
Endotoxin (EU/mg HA)	> 100	< 0.02	0.022	23	> 100
Total Protein (µg/ml HA)	47.7 ± 3	1.1	ND	1.0	36.2
RNA (µg/mg HA)	6.7 ± 0.1	ND	ND	ND	1.9
DNA (µg/mg HA)	16.8 ± 4.5	ND	ND	ND	1.1

*two different bacterially-derived (*Streptococcus zooepidemicus*) samples were tested; EU = endotoxin units; HA = Hyaluronic Acid; ND = not detected

Table 4. 2022/2021 and historical frequency and concentration of use according to duration and exposure

	# of Uses		Max Conc of Use (%)		# of Uses		Max Conc of Use (%)		# of Uses		Max Conc of Use (%)	
	2022[18]	2005[1]	2021[19]	2005[1]	2022[18]	2005[1]	2022[18]	2005[1]	2022[18]	2005[1]	2022[18]	2005[1]
	Hyaluronic Acid				Potassium Hyaluronate				Sodium Hyaluronate			
Totals	**568**	**223**	**0.000002 – 0.83**	**0.00005 – 1**	**44**	**11**	**NR**	**NR**	**4048**	**601**	**0.00001 – 7.5**	**0.000001 – 2**
summarized by likely duration and exposure*												
Duration of Use												
Leave-On	*493*	*194*	*0.000002 – 0.3*	*0.00005 – 1*	*43*	*10*	*NR*	*NR*	*3680*	*552*	*0.00001 – 7.5*	*0.000001 – 2*
Rinse-Off	*72*	*29*	*0.002 – 0.83*	*0.001 – 0.3*	*1*	*1*	*NR*	*NR*	*366*	*49*	*0.0001 – 0.12*	*0.000001 – 0.5*
Diluted for (Bath) Use	*3*	*NR*	*0.0089*	*NR*	*NR*	*NR*	*NR*	*NR*	*2*	*NR*	*NR*	*0.001 – 0.5*
Exposure Type*												
Eye Area	45	33	0.001	0.001 – 0.07	NR	NR	NR	NR	259	49	0.0001 – 0.96	0.0001 – 0.7
Incidental Ingestion	3	NR	0.003 – 0.05	0.01	NR	NR	NR	NR	219	96	0.24 – 0.39	0.0002 – 0.5
Incidental Inhalation-Spray	3; 213[a]; 158[b]	57[a]; 21[b]	NR	0.001[a]; 0.001 – 1[b]	22[a]; 14[b]	4[a]; 6[b]	NR	NR	10; 1376[a]; 1250[b]	1; 180[a]; 73[b]	0.01; 2[a]	0.000001 – 1[a]; 0.0001 – 2[b]
Incidental Inhalation-Powder	4; 158[b]; 5[c]	6; 21[b]	0.003 – 0.3[c]	0.00005; 0.001 – 1[b]	14[b]	6[b]	NR	NR	34; 1250[b]; 9[c]	16; 73[b]	0.001 – 0.099; 0.00002 – 7.5[c]	0.0005 – 0.5; 0.0001 – 2[b]; 0.5[c]
Dermal Contact	542	216	0.000002 – 0.83	0.00005 – 1	44	11	NR	NR	3746	482	0.00001 – 7.5	0.000001 – 2
Deodorant (underarm)	NR	NR	NR	NR	NR	NR	NR	NR	4[a]	NR	0.013	0.5[a]
Hair - Non-Coloring	22	4	0.0036	NR	NR	NR	NR	NR	62	12	0.005 – 2	0.001 – 0.5
Hair-Coloring	NR	1	0.002	NR	NR	NR	NR	NR	1	2	NR	0.5
Nail	NR	2	NR	0.001 – 0.01	NR	NR	NR	NR	1	NR	0.025	0.01 – 0.5
Mucous Membrane	27	1	0.003 – 0.05	0.01	NR	NR	NR	NR	251	97	0.01 – 0.39	0.0002 – 0.5
Baby Products	6	NR	NR	0.001	NR	NR	NR	NR	14	NR	0.005	0.5
as reported by product category												
Baby Products												
Baby Shampoos	1	NR	NR	NR					NR	NR	NR	0.5
Baby Lotions/Oils/Powders/Creams	5	NR	NR	NR					9	NR	NR	0.5
Other Baby Products	NR	NR	NR	0.001					5	NR	0.005	0.5
Bath Preparations (diluted for use)												
Bath Oils, Tablets, and Salts	1	NR	NR	NR					1	NR	NR	0.5
Bubble Baths	1	NR	NR	NR					NR	NR	NR	0.001 – 0.5
Bath Capsules									NR	NR	NR	0.5
Other Bath Preparations	1	NR	0.0089	NR					1	NR	NR	0.001 – 0.5
Eye Makeup Preparations												
Eyebrow Pencil	3	NR	NR	NR					1	3	NR	0.5
Eyeliner	1	NR	NR	NR					9	4	NR	0.001 – 0.5
Eye Shadow	1	15	NR	0.02					21	11	0.097 – 0.96	0.0001 – 0.5
Eye Lotion	16	5	0.001	NR					95	6	0.1	0.001 – 0.7
Eye Makeup Remover	1	2	NR	0.001					7	NR	0.12	NR
Mascara									19	9	0.0001 – 0.1	0.0001 – 0.5
Other Eye Makeup Preparations	23	11	NR	0.07					107	16	0.001 – 0.1	0.0001 – 0.5
Fragrance Preparations												
Cologne and Toilet Water									1	NR	NR	NR
Perfumes									NR	1	NR	0.5
Powders (dusting/talcum, excl aftershave talc)	NR	1	NR	NR					NR	1	NR	0.5
Sachets									ǂ	NR	ǂ	0.5
Other Fragrance Preparation	2	NR	NR	NR					8	NR	NR	0.0002
Hair Preparations (non-coloring)												
Hair Conditioner	9	2	0.0036	NR					15	5	NR	0.001 – 0.5
Hair Spray (aerosol fixatives)	1	NR	NR	NR					1	NR	NR	0.5

Table 4. 2022/2021 and historical frequency and concentration of use according to duration and exposure

	# of Uses		Max Conc of Use (%)		# of Uses		Max Conc of Use (%)		# of Uses		Max Conc of Use (%)	
	2022[18]	2005[1]	2021[19]	2005[1]	2022[18]	2005[1]	2022[18]	2005[1]	2022[18]	2005[1]	2022[18]	2005[1]
Hair Straighteners									2	1	NR	0.5
Permanent Waves	NR	1	NR	NR								0.5
Rinses (non-coloring)									1	NR	NR	0.001 – 0.5
Shampoos (non-coloring)	5	NR	0.0036	NR					21	6	0.01	0.001 – 0.5
Tonics, Dressings, and Other Hair Grooming Aids	3	1	NR	NR					8	NR	2	0.02 – 0.5
Wave Sets									NR	NR	NR	0.5
Other Hair Preparations	3	NR	NR	NR					14	NR	0.005	0.5
Hair Coloring Preparations												
Hair Dyes and Colors (all types requiring caution statements and patch tests)									NR	NR	NR	0.5
Hair Tints	NR	NR	0.002	NR					NR	NR	NR	0.5
Hair Rinses (coloring)	NR	1	NR	NR					NR	NR	NR	0.5
Hair Shampoos (coloring)									1	NR	NR	NR
Hair Color Sprays (aerosol)									NR	NR	NR	0.5
Hair Lighteners with Color									NR	NR	NR	0.5
Hair Bleaches									NR	NR	NR	0.5
Other Hair Coloring Preparation									NR	2	NR	0.5
Makeup Preparations												
Blushers (all types)	NR	7	NR	0.02					11	20	0.05	0.001 – 0.5
Face Powders	4	5	NR	0.00005					34	15	0.001 – 0.099	0.0005 – 0.5
Foundations	4	24	0.000002 – 0.1	0.002					67	27	0.015 – 0.2	0.001 – 0.5
Leg and Body Paints									1	1	NR	0.001 – 0.5
Lipstick	3	NR	0.003 – 0.05	0.01					213	96	0.24 – 0.39	0.0002 – 0.5
Makeup Bases	4	22	0.1	NR	3	NR	NR	NR	29	15	NR	0.002 – 0.5
Rouges									1	10	0.001	0.0001 – 0.5
Makeup Fixatives									6	3	NR	0.05 – 0.5
Other Makeup Preparations	4	NR	NR	0.001					61	17	0.025 – 0.1	0.0001 – 0.5
Manicuring Preparations (Nail)												
Basecoats and Undercoats									NR	NR	NR	0.5
Cuticle Softeners	NR	1	NR	0.001					NR	NR	NR	0.01 – 0.5
Nail Creams and Lotions									NR	NR	NR	0.5
Nail Extenders									NR	NR	NR	0.5
Nail Polish and Enamel									NR	NR	NR	0.5
Nail Polish and Enamel Removers									NR	NR	NR	0.5
Other Manicuring Preparations	NR	1	NR	0.01					1	NR	0.025	0.5
Oral Hygiene Products												
Dentifrices									3	NR	NR	NR
Mouthwashes and Breath Fresheners									3	NR	NR	NR
Personal Cleanliness Products												
Bath Soaps and Detergents	15	NR	NR	NR					11	1	0.01	0.001 – 0.5
Deodorants (underarm)									4	NR	0.013 (not spray)	0.5
Douches	1	NR	NR	NR								
Feminine Deodorants									1	NR	NR	0.001
Other Personal Cleanliness Products	5	1	NR	NR					18	NR	NR	0.5
Shaving Preparations												
Aftershave Lotion	1	1	NR	NR					10	6	0.1	0.001 – 0.5

Table 4. 2022/2021 and historical frequency and concentration of use according to duration and exposure

	# of Uses		Max Conc of Use (%)		# of Uses		Max Conc of Use (%)		# of Uses		Max Conc of Use (%)	
	2022[18]	**2005[1]**	**2021[19]**	**2005[1]**	**2022[18]**	**2005[1]**	**2022[18]**	**2005[1]**	**2022[18]**	**2005[1]**	**2022[18]**	**2005[1]**
Beard Softeners									NR	NR	NR	0.5
Mens Talcum									NR	NR	NR	0.5
Preshave Lotions (all types)									NR	NR	NR	0.5
Shaving Cream	NR	3	NR	0.3					7	NR	NR	0.001 – 0.5
Shaving Soap									NR	NR	NR	0.5
Other Shaving Preparations	3	NR	0.008	NR					5	2	0.01	0.5
Skin Care Preparations												
Cleansing	24	6	NR	0.001					146	20	0.0001 – 0.1	0.000001 – 0.5
Depilatories									1	NR	NR	0.5
Face and Neck (exc shave)	147	8	0.003 – 0.3 (not spray)	0.1	12	6	NR	NR	1104	48	0.005 – 7.5 (not spray)	0.005 – 1
Body and Hand (exc shave)	10	12	0.05 (not spray)	0.001 – 1	2	NR	NR	NR	145	25	0.00002 – 0.86 (not spray)	0.0001 – 2
Foot Powders and Sprays	1	1	NR	NR					NR	NR	NR	1
Moisturizing	179	37	0.08 – 0.2 (not spray)	0.001 – 0.1	22	4	NR	NR	1170	151	0.001 – 0.4 (not spray)	0.000001 - 1
Night	14	17	0.15 (not spray)	0.02					122	11	0.00001 – 0.3 (not spray)	0.0001 - 1
Paste Masks (mud packs)	8	13	0.83	0.001	1	1	NR	NR	125	12	0.024	0.005 – 0.5
Skin Fresheners	13	NR	NR						59	10	0.01	0.05 – 0.5
Other Skin Care Preparations	47	23	NR	0.001	4	NR	NR	NR	329	38	0.02 – 0.1	0.001 - 1
Suntan Preparations												
Suntan Gels, Creams, and Liquids	1	1	NR	NR					2	4	NR	0.000001 – 1
Indoor Tanning Preparations	3	1	NR	0.001[a]					5	1	NR	0.001 – 0.5
Other Suntan Preparations									7	3	NR	0.001 – 0.5

NR – not reported

*likely duration and exposure is derived based on product category (see Use Categorization https://www.cir-safety.org/cir-findings)

**Because each ingredient may be used in cosmetics with multiple exposure types, the sum of all exposure types may not equal the sum of total uses.

[a] It is possible these products are sprays, but it is not specified whether the reported uses are sprays.

[b] Not specified whether a spray or a powder, but it is possible the use can be as a spray or a powder, therefore the information is captured in both categories

[c] It is possible these products are powders, but it is not specified whether the reported uses are powders.

ǂ Sachets are no longer listed as a product category in the VCRP.

Table 5. Frequency (2022) and concentration (2021) of use of ingredients not previously reviewed[18,50]

	# of Uses	Max Conc of Use (%)	# of Uses	Max Conc of Use (%)	# of Uses	Max Conc of Use (%)	# of Uses	Max Conc of Use (%)
	Hydrolyzed Calcium Hyaluronate		Hydrolyzed Hyaluronic Acid		Hydrolyzed Sodium Hyaluronate		Sodium Acetylated Hyaluronate	
Totals	**2**	**NR**	**362**	**0.002 – 0.2**	**108**	**0.0015 – 0.15**	**378**	**0.002 – 0.1**
summarized by likely duration and exposure*								
Duration of Use								
Leave-On	*2*	*NR*	*320*	*0.01 – 0.2*	*105*	*0.0015 – 0.15*	*353*	*0.002 – 0.1*
Rinse-Off	*NR*	*NR*	*42*	*0.002 – 0.01*	*3*	*NR*	*25*	*NR*
Diluted for (Bath) Use	*NR*	*NR*	*NR*	*NR*	*NR*	*NR*	*NR*	*NR*
Exposure Type*								
Eye Area	NR	NR	9	0.01	8	NR	43	NR
Incidental Ingestion	NR	NR	NR	NR	NR	NR	68	NR
Incidental Inhalation-Spray	2[a]	NR	166[a]; 94[b]	0.02	49[a]; 29[b]	NR	107[a]; 50[b]	0.0085 – 0.1[a]
Incidental Inhalation-Powder	NR	NR	4; 94[b]; 4[c]	0.01[c]	29[b]	0.15[c]	11; 50[b]; 3[c]	0.1[c]
Dermal Contact	2	NR	352	0.01 – 0.2	108	NR	307	0.002 – 0.1
Deodorant (underarm)	NR	NR	NR	NR	NR	NR	NR	NR
Hair - Non-Coloring	NR	NR	10	0.002 – 0.02	NR	NR	1	NR
Hair-Coloring	NR	NR	NR	0.002	NR	NR	NR	NR
Nail	NR	NR	NR	NR	NR	NR	NR	NR
Mucous Membrane	NR	NR	7	NR	NR	NR	71	NR
Baby Products	NR	NR	7	NR	NR	NR	6	NR
as reported by product category								
Baby Products								
Baby Shampoos								
Baby Lotions/Oils/Powders/Creams			4	NR			3	NR
Other Baby Products			3	NR			3	NR
Bath Preparations (diluted for use)								
Bath Oils, Tablets, and Salts								
Bubble Baths								
Bath Capsules								
Other Bath Preparations								
Eye Makeup Preparations								
Eyebrow Pencil							1	NR
Eyeliner							8	NR
Eye Shadow							13	NR
Eye Lotion			2	NR	5	NR	12	NR
Eye Makeup Remover								
Mascara							2	NR
Other Eye Makeup Preparations			7	0.1	3	NR	7	NR
Fragrance Preparations								
Cologne and Toilet Water								
Perfumes								
Powders (dusting/talcum, excl aftershave talc)								
Other Fragrance Preparation								
Hair Preparations (non-coloring)								
Hair Conditioner			4	NR			1	NR
Hair Spray (aerosol fixatives)								
Hair Straighteners								
Permanent Waves								
Rinses (non-coloring)								

Table 5. Frequency (2022) and concentration (2021) of use of ingredients not previously reviewed[18,50]

	# of Uses	Max Conc of Use (%)	# of Uses	Max Conc of Use (%)	# of Uses	Max Conc of Use (%)	# of Uses	Max Conc of Use (%)
Shampoos (non-coloring)			3	0.002				
Tonics, Dressings, and Other Hair Grooming Aids			1	0.02				
Wave Sets								
Other Hair Preparations			2	NR				
Hair Coloring Preparations								
Hair Dyes and Colors (all types requiring caution statements and patch tests)			NR	0.002				
Hair Tints								
Hair Rinses (coloring)								
Hair Shampoos (coloring)								
Hair Color Sprays (aerosol)								
Hair Lighteners with Color								
Hair Bleaches								
Other Hair Coloring Preparation								
Makeup Preparations								
Blushers (all types)							4	NR
Face Powders			4	NR			11	NR
Foundations			1	NR			42	0.002
Leg/Body Paints								
Lipstick							68	NR
Makeup Bases			3	NR	3	NR	4	NR
Rouges								
Makeup Fixatives			1	NR	1	NR	2	NR
Other Makeup Preparations			3	NR	1	NR	5	NR
Manicuring Preparations (Nail)								
Basecoats and Undercoats								
Cuticle Softeners								
Nail Creams and Lotions								
Nail Extenders								
Nail Polish and Enamel								
Nail Polish and Enamel Removers								
Other Manicuring Preparations								
Oral Hygiene Products								
Dentifrices								
Mouthwashes and Breath Fresheners								
Personal Cleanliness Products								
Bath Soaps and Detergents			4	NR			1	NR
Deodorants (underarm)								
Douches								
Feminine Deodorants								
Other Personal Cleanliness Products			3	NR			2	NR
Shaving Preparations								
Aftershave Lotion								
Beard Softeners								
Mens Talcum								
Preshave Lotions (all types)								
Shaving Cream								
Shaving Soap								

Table 5. Frequency (2022) and concentration (2021) of use of ingredients not previously reviewed[18,50]

	# of Uses	Max Conc of Use (%)	# of Uses	Max Conc of Use (%)	# of Uses	Max Conc of Use (%)	# of Uses	Max Conc of Use (%)
Other Shaving Preparations								
Skin Care Preparations								
Cleansing			24	0.01	2	NR	18	NR
Depilatories								
Face and Neck (exc shave)			89	0.01 (not spray)	28	0.15 (not spray)	43	0.1 (not spray)
Body and Hand (exc shave)					1	NR	7	NR
Foot Powders and Sprays			5	NR				
Moisturizing	2	NR	145	0.1 – 0.2 (not spray)	4	0.0015 (not spray)	96	0.0085 – 0.1
Night			12	0.15 (not spray)	2	NR	7	NR
Paste Masks (mud packs)			4	NR	1	NR	3	NR
Skin Fresheners			8	NR	14	NR	4	NR
Other Skin Care Preparations			30	0.2			11	0.1
Suntan Preparations								
Suntan Gels, Creams, and Liquids								
Indoor Tanning Preparations								
Other Suntan Preparations								

NR – not reported
*likely duration and exposure is derived based on product category (see Use Categorization https://www.cir-safety.org/cir-findings)
**Because each ingredient may be used in cosmetics with multiple exposure types, the sum of all exposure types may not equal the sum of total uses.
[a] It is possible these products are sprays, but it is not specified whether the reported uses are sprays.
[b] Not specified whether a spray or a powder, but it is possible the use can be as a spray or a powder, therefore the information is captured in both categories
[c] It is possible these products are powders, but it is not specified whether the reported uses are powders.

Table 6. Oral ADME studies

Parameter Measured	Test Substance	Animals	No./Group	Vehicle/Dose	Dose/Protocol	Results	References
Absorption	[^{14}C]Hyaluronic Acid (MW = 920,000 (units not specified)	Male Sprague-Dawley rats	3	Distilled water; 25 mg/kg	Animals were given a single oral dose of the test substance via gavage. Administered radioactivity was 2.04 megabecquerel (MBq)/kg bw. The transition of plasma ^{14}C radioactivity was evaluated by collecting blood from the tail vein of treated animals at 5, 15, and 30 min, and 1, 2, 4, 8, 24, 48, 72, 96, 120, 144, and 168 h post-administration.	The peak plasma radioactivity level was 7.6 µg eq/ml, at 8 h. The half-life was approximately 1.9 d. The area under the concentration-time curves of plasma was determined to be 309 µg of eq/ml/h.	27
Distribution	[^{14}C]Hyaluronic Acid (MW = 920,000 (units not specified)	Male Sprague-Dawley rats	1/group	Distilled water; 25 mg/kg	Animals were given a single dose of the test substance via gavage, and killed under anesthesia at 8, 24, or 96 h after administration. Whole-body autoradiographs were prepared from radioactivity images recorded on imagine plates.	^{14}C was detected in the skin as follows: 2.36 PSL/mm^2 at 8 h, 3.81 PSL/mm^2 at 24 h, and 1.98 PSL/mm^2 at 96 h after administration. ^{14}C was detected in the blood as follows: 2.12 PSL/mm^2 at 8 h, 1.68 PSL/mm^2 at 24 h, and 0.84 PSL/mm^2 at 96 h after administration. Radioactivity was higher in the skin than in the blood at 24 and 96 h post-administration. In other tissues, the highest levels of radioactivity were observed in the intestinal contents 8 h post-administration (710 PSL/mm^2). Readings in the pancreas (17.45 PSL/mm^2), harderian gland (12.27 PSL/mm^2), liver (9.22 PSL/mm^2), and mandibular gland (7.49 PSL/mm^2) were also high 8 h post-administration. At 96 h post-administration, all radioactivity dropped.	27
Distribution	[^{14}C]Hyaluronic Acid (MW = 920,000 (units not specified)	Male Sprague-Dawley rats	3	Distilled water; 25 mg/kg	Animals were given a single oral dose of the test substance via gavage, and housed in metabolic cages. ^{14}C-excretion rates in the urine, feces, and expired air were evaluated at predetermined times (0-168 h post-administration). Animals were killed at the end of the study to measure the residual radioactivity in the body.	Radioactivity was excreted in the urine as follows: 2.5% of the dose by 24 h, 2.9% by 96 h, and 3% by 168 h. In feces, radioactivity was excreted as follows: 7.8% by 24 h, 11.6% by 96 h, and 11.9% by 168 h. In expired air, radioactivity was excreted as follows: 70.7% of the dose by 24 h, 75.4% by 96 h, and 76.5% by 168 h. The total excretion rate in the urine, feces, and expired air was 91.3% of the administered dose by 168 h post-administration. Approximately 8.8% of the dose remained in the carcass 168 post-administration.	27
Distribution	[^{99m}Tc]Hyaluronic Acid (MW = 1.1 – 1.5 MDa)	Wistar rats (sex not stated)	3/group	0.2 ml (vehicle not stated)	A single dose of the test substance was given to each animal via gavage. Animals were killed at each of the following times post-administration: 5, 15, 30 min, 1, 2, 4, 6, 12, and 24 h. Radioactivity was examined in organs, tissues, and carcasses. Tissues measured include blood, bone, brain, heart, kidney, knee joints, large intestine, liver, lung, muscle, small intestine, skin, spleen, thyroid, and bladder. In addition, whole-body scintigraphs SPECT scans were performed in order to further evaluate biodistribution of radioactivity.	Radioactivity appeared to remain in the GI tract, keeping with normal gut transition times. A very rapid uptake of radioactivity in bone, muscle, small intestine, and large intestine was seen at the 5- and 15-min time points. Tissue uptake of radioactivity into blood and peripheral tissue coincided with the presence of the test substance in absorptive sections of the GI tract. Whole-body scintigraphs revealed non-alimentary radioactivity concentrated in the joints, vertebrae, and salivary glands 4 h post-administration. Approximately 10% of the ingested radioactivity remained in the tissues 72 h post-administration.	29

Table 6. Oral ADME studies

Parameter Measured	Test Substance	Animals	No./Group	Vehicle/Dose	Dose/Protocol	Results	References
Metabolism/ Distribution	Hyaluronic Acid (MW = 300 kDa)	Male Sprague-Dawley rats	6/group	Vehicle not stated; 200 mg/kg bw	After overnight fasting, rats were given a single dose of the test substance (method of oral administration not stated). Samples of cecal content, blood, and shaved ventral skin were collected 0, 2, 4, 6, and 8 h after administration. Unsaturated Hyaluronic Acid disaccharides (u-HA2) and tetrasaccharides (u-HA4) in the serum and the supernatant of homogenized skin were analyzed via liquid chromatography-tandem mass spectrometry (LC/MS/MS).	Hyaluronic Acid is degraded by intestinal bacteria and oligosaccharide Hyaluronic Acid is absorbed in the small intestines and widely distributed. Oligosaccharide (di-, tetra-, hexa-, octa-, and decasaccharides) Hyaluronic Acid was observed in cecal content 2 h after test substance administration. The recovery rate of u-HA2 and u-HA4 in the serum and skin was approximately 25 and 70%, respectively. U-HA2 was observed in the serum 2 h after test substance administration, and peaked in concentration after approximately 6-8 h post-administration. U-HA4 was observed in the serum 8 h after test substance administration. Both u-HA2 and u-HA4 were observed in the skin 6 h after test substance administration, and peaked after 8 h.	28
Excretion	Hyaluronic Acid (MW = 300 kDa)	Male Sprague-Dawley rats	8/group	Distilled water; 5 ml/kg bw/d; 1 and 5%	Animals were orally administered the test substance (method of oral administration not stated) for 5 d. Control animals received distilled water only. On the last 3 d of treatment, feces were collected, freeze-dried, and ground for analysis. Hyaluronic Acid concentration was measured using a hyaluronan assay kit with a Hyaluronic Acid binding protein.	Hyaluronic Acid was below the detection limit (10 µg/3 d) in all groups.	28
Excretion	[^{99m}Tc]Hyaluronic Acid (MW = 1.1 – 1.5 MDa)	Wistar rats (sex not stated)	5 rats total	0.2 ml (vehicle not stated)	A single dose of the test substance was administered to the animals via gavage. Urine and feces were collected 12, 24, 48, and 72 h post-administration.	The average total excretions of radioactivity over the 72-h period in feces and urine were 84.6 ± 7.8% and 2.0 ± 0.63% of the ingested radiolabel-dose, respectively. Almost all radioactivity was excreted within 24 h.	29
Excretion	[^{99m}Tc]Hyaluronic Acid (MW = 1.1 – 1.5 MDa)	Beagle dogs (sex not stated)	2 dogs total	1.5 ml (vehicle not stated)	A single dose of the test substance was administered to the animals via gavage. Blood and urine samples were collected 2, 5, 15, 30 min, 1, 2, 4, 6, 12, 24, 48, and 72 h post-administration. Results were reported as the percent of administered dose/gram of whole blood or urine.	After administration, blood clearance rose immediately, and returned to background by 6 h. Urinary radioactivity clearance appeared after 30 min, and returned to background after 12 h.	29

kDa = kilodalton; MW = molecular weight; PSL = photo-stimulated luminescence; SPECT = single photon emission computed tomography

Table 7. Acute oral toxicity studies on Sodium Hyaluronate[16]

Test Article	Vehicle	Animals/Group	Concentration/Dose	Protocol	LD_{50}/Results
Sodium Hyaluronate (FW = 1800-2100 kDa)	NR	ICR mice (number of animals not stated)	500 mg/kg bw	single dose; method of oral administration not stated	LD_{50} > 500 mg/kg bw
Sodium Hyaluronate	peanut oil	Kunming mice (10/sex)	2000 mg/kg bw	single dose; gavage; 14-d evaluation	LD_{50} > 2000 mg/kg bw; no signs of toxicity or deaths
Sodium Hyaluronate	peanut oil	Kunming mice (20/sex)	1000, 2150, 4640, or 10,000 mg/kg bw	single dose; gavage; 14-d evaluation	LD_{50} > 10,000 mg/kg bw; no signs of toxicity or deaths
Sodium Hyaluronate	corn germ oil	Kunming mice (10/sex)	15,000 mg/kg bw	single dose; gavage; 14-d evaluation	LD_{50} > 15,000 mg/kg bw; no signs of toxicity or deaths
Sodium Hyaluronate (FW = 1800 – 2100 kDa)	NR	Rats (strain and number of animals not stated)	200 mg/kg bw	single dose; method of oral administration not stated	LD_{50} > 200 mg/kg bw; no signs of toxicity or deaths
Sodium Hyaluronate (FW = 270 kDa)	distilled water	Wistar rats (10/sex)	5280 mg/kg bw	single dose; gavage	MTD > 5280 mg/kg bw; no signs of toxicity or deaths

LD_{50} = median lethal dose; MTD = maximum tolerable dose; MW = molecular weight; FW = formula weight; NR = not reported

Table 8. Oral repeated dose toxicity studies on Sodium Hyaluronate[16]

Test Article	Vehicle	Animals/Group	Study Duration	Dose/Concentration	Protocol	Results
Sodium Hyaluronate	feed	Wistar rats (10/sex/group)	30 d	0, 167, 500, or 1500 mg/kg bw	rats given test substance via feed; body weight changes, hematological, biochemistry, and macroscopic parameters were evaluated	no deaths or changes in body weight, food consumption, or weight gain; all blood chemistry parameters were within normal ranges; no changes in organ weight/histopathological parameters; NOAEL = 1500 mg/kg bw/d
ophthalmic solution containing 1% Sodium Hyaluronate	NR	Sprague-Dawley rats (5-10/sex/group)	90 d	0, 3, 12, or 48 mg/kg bw/d	rats given test substance via gavage; body weight, food efficiency, urinalysis, and gross pathological and histopathological parameters evaluated	no dose-dependent changes in body weight, histopathological parameters, or hematological parameters were observed; NOAEL = 48 mg/kg bw/d
Sodium Hyaluronate (FW = 2270 kDa)	feed	Wistar rats (10/sex/group)	90 d	0, 330, 670, or 1000 mg/kg bw	rats given test substance via feed; evaluated for 28 d following treatment period; body weight changes, hematological, biochemistry, and macroscopic, and gross pathological parameters were evaluated	no deaths or changes in body weight, food consumption, weight gain, hematological parameters, or histopathological parameters; NOAEL = 1000 mg/kg bw/d
Sodium Hyaluronate	corn germ oil	Sprague-Dawley rats (12/sex/group)	90 d	0, 667, 100, or 1333 mg/kg bw/d	rats given test substance via gavage; body weight changes, hematological, biochemistry, and macroscopic, and gross pathological parameters were evaluated	no changes in behavior, feeding, body weight, food consumption, hematological parameters, organ weights, or macroscopic/histological parameters were observed; NOAEL = 1333 mg/kg bw/d

FW = formula weight; NOAEL = no-observed-adverse-effect-level; NR = not reported

Table 9. Genotoxicity studies[16,30-32]

Test Substance	Test Concentration/Dose	Vehicle	Test System	Procedure	Results
			IN VITRO		
Hydrolyzed Sodium Hyaluronate (FW = 5-10 kDa)	up to 5 mg/plate (specific concentrations not stated)	NR	*S. typhimurium* strains TA97a, TA98, TA100, and TA102	Ames assay performed with and without metabolic activation	Non-genotoxic
Hydrolyzed Sodium Hyaluronate (FW < 1kDa)	up to 5 mg/plate (specific concentrations not stated)	NR	*S. typhimurium* strains TA97a, TA98, TA100, and TA102	Ames assay performed with and without metabolic activation	Non-genotoxic
Sodium Hyaluronate	1 mg/plate	NR	*S. typhimurium* strains TA97a, TA98, TA100, TA102, and TA1535	Ames assay performed with and without metabolic activation	Non-genotoxic
Sodium Hyaluronate	up to 1 mg/plate (specific concentrations not stated)	NR	*S. typhimurium strains TA97a, TA98, TA100, TA102, and TA1535*	Ames assay performed with and without metabolic activation	Non-genotoxic
Sodium Hyaluronate	0.008, 0.04, 0.2, 1, and 5 mg/plate	NR	*S. typhimurium* strains TA97a, TA98, TA100, and TA102	Ames assay performed with and without metabolic activation	Non-genotoxic
Sodium Hyaluronate	0.2, 0.5, 1, 2.5, and 5 mg/plate	NR	*S. typhimurium* strains TA97, TA98, TA100, and TA102	Ames assay performed with and without metabolic activation	Non-genotoxic
Sodium Hyaluronate	up to 5 mg/plate (specific concentrations not stated)	NR	*S. typhimurium* strains TA97a, TA98, TA100, and TA102	Ames assay performed with and without metabolic activation	Non-genotoxic
Sodium Hyaluronate	without metabolic activation/*S. typhimurium* and *E. coli*: 313, 625, 1250, 2500, 5000 µg/plate with metabolic activation/ *S. typhimurium*: 39.1, 78.1, 156, 313, 625, 1250 µg/plate with metabolic activation/*E. coli*: 313, 625, 1250, 2500, 5000 µg/plate	NR	*S. typhimurium strains TA98, TA100, TA1535, TA1537* and *E. coli* WP2 *uvrA*	Ames assay performed with and without metabolic activation	Non-genotoxic
			IN VIVO		
Sodium Hyaluronate	20 ml/kg bw	NR	mice (strain not reported; 5/sex/group)	Mouse micronucleus assay; cyclophosphamide used as positive control; distilled water used as negative control; administrations via gavage	non-mutagenic
Sodium Hyaluronate	440, 880, 1760 mg/kg bw	NR	KS mice (5/sex/group)	Mouse micronucleus assay; cyclophosphamide used as positive control; distilled water used as negative control; administrations via gavage	non-mutagenic
Sodium Hyaluronate	1250, 2500, and 5000 mg/kg bw	NR	KS mice (5/sex/group)	Mouse micronucleus assay; cyclophosphamide used as positive control; corn germ oil used as negative control; administrations via gavage	non-mutagenic

NR = not reported

Table 10. Dermal irritation and sensitization studies

Test Article	Vehicle	Test Concentration/ Dose	Test Population	Procedure	Results	Reference
IRRITATION						
IN VITRO						
Trade name mixture containing 1% Hyaluronic Acid	NR	100%: 30 µl	3	EpiDerm^{TM} assay; 60 min incubation period; sterile PBS and sterile deionized water used as negative controls; 5% SDS solution and methyl acetate used as positive control; cell viability evaluated via MTT assay	Non-irritating	34
Trade name mixture containing 1% Hyaluronic Acid	NR	100%: 30 µl	3	Same as above	Non-irritating	35
Trade name mixture containing 3% Hyaluronic Acid	NR	100%: 30 µl	3	Same as above	Non-irritating	36
Hydrolyzed Sodium Hyaluronate (FW < 1 kDa)	NR	NR	NR	OECD TG 439; reconstructed human epidermis assay; no details provided	Non-irritating	32
Trade name mixture containing 0.5% Sodium Hyaluronate	NR	100%; 30 µl	3	EpiDerm^{TM} assay; 60 min incubation period; sterile PBS and sterile deionized water used as negative controls; 5% SDS solution and methyl acetate used as positive control; cell viability evaluated via MTT assay	Non-irritating	37
Sodium Hyaluronate	NR	100%	NR	OECD TG 439; reconstructed human epidermis assay; no details provided	Non-irritating	30
HUMAN						
Hydrolyzed Sodium Hyaluronate (FW = 1-5 kDa)	NR	0.5%	32	Human skin closed patch test; test substance applied to skin of individuals with sensitive skin for 24 g	Non-irritating	32
Hydrolyzed Sodium Hyaluronate (FW = 1-5 kDa)	NR	0.5 and 2%	30	Human skin closed patch test; applications on healthy skin; no other details provided	Non-irritating	32
Hydrolyzed Sodium Hyaluronate (FW = 5-10 kDa)	NR	1%	NR	Human skin closed patch test; no details provided	Non-irritating	32
Hydrolyzed Sodium Hyaluronate (FW < 1 kDa)	NR	1%	30	Human skin closed patch test; applications on healthy skin; no other details provided	Non-irritating	32
SENSITIZATION						
IN CHEMICO/IN VITRO						
Trade name mixture containing 1% Hyaluronic Acid	NR	5 (0.05 ml) and 25 mM (250 µl)	3/concentration tested	OECD TG 442C; DPRA; 24 h incubation period; mean percent depletion of cysteine and lysine evaluated; positive control: cinnamic aldehyde in acetonitrile; negative control: peptide in buffer	Non-sensitizing	38
Trade name mixture containing 1% Hyaluronic Acid	NR	0.00098 – 2mM; 0.05 ml	3/concentration tested	OECD TG 442D: ARE-Nrf2 Luciferase Test Method; KeratinoSens^{TM} cell line; positive control: cinnamic aldehyde; negative control: DMSO	Non-sensitizing	39
Sodium Hyaluronate	NR	1 mg/ml	NR	OECD TG 442E;h-CLAT; no details provided	Non-sensitizing	30

Table 10. Dermal irritation and sensitization studies

Test Article	Vehicle	Test Concentration/ Dose	Test Population	Procedure	Results	Reference
			HUMAN			
Formula containing 0.2% Hyaluronic Acid	NR	100%; 20 mg	115	HRIPT; occlusive conditions; 9 applications over a 3-wk period for induction period; challenge phase (48-h patch application) after a 10-15 d rest period; challenge patches evaluated immediately after removal, and 24, 48 and 72 h after patch removal	Non-irritating and non-sensitizing	40
Formula containing 0.2% Sodium Acetylated Hyaluronate		100%; 0.02 ml	104	HRIPT; occlusive conditions; 9 applications over a 3-wk period for induction period; challenge phase (48-h patch application) after a 14-d rest period; challenge patches evaluated 15 min and around 48 h after patch removal	Non-irritating and non-sensitizing	41
Hydrolyzed Sodium Hyaluronate (FW = 5-10 kDa)	NR	0.5%	55	HRIPT; no details provided	Non-irritating and non-sensitizing	32
Sodium Hyaluronate	NR	0.2%	50	HRIPT; no details provided	Non-sensitizing	31
Sodium Hyaluronate	NR	0.2%	100	HRIPT; no details provided	Non-irritating and non-sensitizing	30
Formula containing 1.5% Sodium Hyaluronate	NR	100%; 0.2 ml	198	HRIPT; occlusive conditions; 9 applications over a 3-wk period for induction period; challenge phase (48-h patch application) after a 10-15 d rest period; challenge patches evaluated immediately after removal, and 24, 48 and 72 h after patch removal	Non-irritating and non-sensitizing	42

ARE = antioxidant response element; DMSO = dimethyl sulfoxide; DPRA - direct peptide reactivity assay; h-CLAT - human cell line activation test; HRIPT = human repeated insult patch test; MTT = 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; NR = not reported; Nfr2 = nuclear factor erythroid 2-related factor 2; OECD TG = Organisation for Economic Cooperation and Development Test Guidelines; PBS = phosphate-buffered saline; SDS = sodium dodecyl sulfate

Table 11. Ocular irritation studies

Test Article	Vehicle	Test Concentration/ Dose	Test Population	Procedure	Results	Reference
			IN VITRO			
Trade name mixture containing 1% Hyaluronic Acid	NR	100%; 50 µl	2	EpiOcular^{TM} assay; 90 min incubation period; sterile PBS and sterile deionized water used as negative controls; 5% SDS solution and methyl acetate used as positive control; cell viability evaluated via MTT assay	Non-irritating	34
Trade name mixture containing 1% Hyaluronic Acid	NR	100%; 50 µl	2	Same as above	Non-irritating	35
Trade name mixture containing 3% Hyaluronic Acid	NR	100%; 50 µl	2	Same as above	Non-irritating	36
Hydrolyzed Sodium Hyaluronate (FW < 1kDa)	NR	NR	NR	CAMVA (no details provided)	Non-irritating/slightly irritating	32
Trade name mixture containing 0.5% Sodium Hyaluronate	NR	100%; 50 µl	2	EpiOcular^{TM} assay; 90 min incubation period; sterile PBS and sterile deionized water used as negative controls; 5% SDS solution and methyl acetate used as positive control; cell viability evaluated via MTT assay	Non-irritating	37
Sodium Hyaluronate	NR	100%	NR	CAMVA (no details provided)	Non-irritating/slightly irritating	30
Sodium Hyaluronate	NR	100%	NR	BCOP test (no details provided)	Non-irritating/slightly irritating	30
			ANIMAL			
Bacillus-derived and *Streptococcus*-derived Hyaluronic Acid	NR	0.1 and 0.3%; 25 µl	New Zealand white rabbits (3/group)	Test substances were placed on the right eye, 4x/d, for 3 d. After the last instillation, rabbits were sedated, and eyes were evaluated via fluorescent imaging	The test substance was considered to be very well-tolerated	43

BCOP - bovine corneal opacity and permeability; CAMVA - chorioallantoic membrane vascular assay; MTT = 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; NR = not reported; PBS = phosphate-buffered saline; SDS = sodium dodecyl sulfate

Table 12. Case reports of hypersensitivity following injectable Hyaluronic Acid dermal fillers

Patient	Case report summary	Reference
50-yr-old female	-patient had previous Hyaluronic Acid injections in glabellar region and nasolabial fold with no adverse effects -1 yr later, patient received same treatment, and presented with erythematous, livedoid rash 3 d after injections -rash cleared within 10 d of treatment with antibiotics and steroids -patient repeated injections 3 yr later with no adverse effects	51
47-yr-old female	-facial Hyaluronic Acid injections at different locations of the face -12 mo later, patient complained of abdominal pain, asymmetric erythematous swelling of the lips, and pain and tingling of lips -angioedema resolved with injection of hyaluronidase	52
54-yr-old female	-facial injections of Hyaluronic Acid gel at multiple areas of face, once in April 1998 and again in November 1988, with no adverse effects -patient received Hyaluronic Acid injections in melolabial folds in June 1999, and developed indurated and erythematous papulocystic nodules 2 wk after injections -patient treated with corticosteroids and warm compresses, and nodules improved -2 wk later, patient returned with recurrent inflammation of the right mesolabial fold with tender nodules -patient again treated with corticosteroids and warm compresses, and experienced rapid resolution of symptoms	53
59-yr-old female	-injections of Hyaluronic Acid in melolabial folds, glabella, lips, and perioral rhytids -2 d after injections, patient noted significant swelling and pain at injection sites -5 d after injections, patient admitted to hospital for significant facial swelling -treated with corticosteroids and an immunosuppressant	54
52-yr-old female	-left and right upper lip injections of Hyaluronic Acid -5 min after injections, patient experienced worsening edema and erythema -patient treated with dexamethasone sodium phosphate, prednisone, and valacyclovir -the following day, the patient still had severe edema and fissures on lip mucosa -patient instructed to apply an emollient ointment, and reported improved symptoms	55
56-yr-old female	-melolabial fold injections of Hyaluronic Acid -27 d after injections, patient developed erythematous indurated papules at injection sites -treatment with steroids resolved symptoms	56
65-yr-old female	-prior to injections, skin prick tests of Hyaluronic Acid performed and yielded negative results -lip, nasolabial fold, and perioral rhytide injections of Hyaluronic Acid with no adverse effects -a second treatment was performed 3 mo later with no adverse effects -a third treatment was performed 6 mo later with no adverse effects -1 mo after the third treatment, patient re-treated, and presented with erythema, edema, and induration of injection regions 6 wk after 4th series of injections -symptoms improved with steroid treatment	57
54-yr-old female	-patient reported previous facial injections of Hyaluronic Acid (every 4 mo) with no adverse effects -10 d after an injection of Hyaluronic Acid to melolabial folds, patient reported granulomatous reaction -symptoms improved with betamethasone treatment	58
28-yr-old female	-swelling, pain, and tenderness at injection sites 3 mo after chin injections of Hyaluronic Acid -antibiotics did not resolve symptoms -treatment with corticosteroid, antihistamine, and clindamycin resolved symptoms	59
29-yr-old female	-asymmetry, edema, and inflammatory nodules seen at injection sites 112 d after facial injections of Hyaluronic Acid -treatment with steroids, antihistamines, and antibiotics resolved symptoms	60
49-yr-old female	-facial edema observed 28 d after glabella and eye area injections of Hyaluronic Acid -treatment with corticosteroids resolved symptoms	60
52-yr-old female	-inflammatory nodules, pustules, and fever observed 2 d after glabella injections of Hyaluronic Acid -treatment with steroids, antibiotics, and coloplast cream resolved symptoms	60
56-yr-old female	-pruritis and blisters 14 d after facial Hyaluronic Acid injections -treatment with steroids, antihistamines, saline dressings, and betamethasone resolved symptoms	60
42-yr-old- female	-inflammatory nodules 1 yr after facial Hyaluronic Acid injections -treatment with moxypen cefamezin resolved symptoms	60
60-yr-old female	-patient reported 2 previous series of Hyaluronic Acid injections with no adverse effects -erythema, pain, and edema observed 14 d after 3rd round of Hyaluronic Acid injections in the cheeks -patient treated with antibiotics, pulsed light therapy, and physical therapy	61
30-yr-old female	-patient reported previous facial Hyaluronic Acid injections with no adverse effects -5 yr after previous injections, patient was treated with Hyaluronic Acid injections in the cheeks, mandible, and chin -the following day, patient reported sore throat and treated with antibiotics -by day 10, patient presented with erythema and edema of lip and chin, treated with corticosteroids -by day 18, patient presented with painful, palpable, subcutaneous collections at the chin, cheekbone, and mandible -patient treated with antibiotics and incision/drainage of collections -patch and intradermal testing to evaluate the potential of a hypersensitivity reaction to Hyaluronic Acid was performed 3 mo later, and resulted in negative results	61
56-yr-old female	-swelling 4 mo after injections of Hyaluronic Acid to cheeks -1 yr later, patients re-treated with injections in the cheeks, and developed facial swelling 4 mo after treatment -patient treated with antibiotics and hyaluronidase	62

Table 12. Case reports of hypersensitivity following injectable Hyaluronic Acid dermal fillers

Patient	Case report summary	Reference
57-yr-old female	-patient reported previous treatment with Hyaluronic Acid to the perioral area, on 2 occasions, with no adverse effects -patient experienced erythema, warmth, and rigidity at injection sites 3 wk after 3rd series of facial Hyaluronic Acid injections -3 mo later, a nontender deep, firm, palpable thickening over both zygomatic arches was apparent -patient treated with antibiotics and hyaluronidase -a recurrent episode occurred 3 mo later, and was again treated with hyaluronidase	62
32-yr-old female	-patient reported previous Hyaluronic Acid injections lips with no adverse effects, and acute swelling after injection to hands -erythema and swelling at injection sites 6 mo after treatment with Hyaluronic Acid injections in cheeks -patient treated with hyaluronidase -recurrent redness and swelling occurred 2 mo later, and was again treated with hyaluronidase	62
48-yr-old female	-patient reported previous Hyaluronic Acid injection treatment in marionette lines with no adverse effects -swelling of cheeks 1 wk after Hyaluronic Acid injections to cheeks -treatment with corticosteroids -flare-ups occurred 3 and 4 mo post-injection, treated with corticosteroids	62
54-yr-old female	-redness and swelling of the nasolabial folds after Hyaluronic Acid injections -severe palpable and painful erythematous nodular papulocystic lesions 3 mo after injections -patient surgically treated	63
48-yr-old female	-blue/gray coloring of lips, cheek, and nose 8 h after Hyaluronic Acid injections -treatment with nitroglycerin and hyperbaric chamber	64
41-yr-old female	-erythematous nodules at injection sites 5 wk after melolabial, glabellar, and periorbital area injections of Hyaluronic Acid -treatment with antibiotics and steroids	65
49-yr-old female	-asymptomatic hard lesions along melomental folds 4 mo after lower facial injections of Hyaluronic Acid -treatment with corticosteroids and hyaluronidase -patch tests performed were negative at 48 and 96 h -intradermal injection into forearm was negative at 20 min and 96 h, but turned positive 2 mo later	66
72-yr-old female	-well-defined, millimetric, firm nodules on lips and oral mucosa 5 mo after Hyaluronic Acid injections -treatment with corticosteroids and hyaluronidase	67
45-yr-old female	-glabellar, neck, eyelid injections of Hyaluronic Acid and *Botulinum* toxin -1 mo after injection, patient developed facial pain, erythema, and edema -patient's symptoms improved following treatment with pain medication, antibiotics, and steroids	68
53-yr-old female	-patient reported previous Hyaluronic Acid injection to nasolabial folds and lips -asymmetry of nasolabial fold, palpable pea sized-lesions 1 yr after Hyaluronic Acid injections -patient treated with antibiotics and ibuprofen	69
40-yr-old female	-dusky, red, firm, linear rash 4 mo after injection of a mixture of Hyaluronic Acid gel and acrylic hydrogel to the nasolabial folds -treatment with betamethasone	70
66-yr-old female	-patient reported 12 treatments of facial Hyaluronic Acid injection over the course of 5 yr -patient reported an increasing number of hard lumps in areas that were repeatedly treated with Hyaluronic Acid gel and acrylic hydrogel -eventually developed into symmetrical linear purple plaques, nodules, induration of lips -treatment with steroids	71
65-yr-old female	-patient reported 3 treatments with a mixture of Hyaluronic Acid gel and acrylic hydrogel -hard subcutaneous nodules in nasolabial folds, upper lip, and glabella 2 yr after last treatment -treatment with steroids	71

*It should be noted that dermal fillers are derived from one of two methods: a non-animal method (bacterial fermentation using *Streptococcus*) or via extraction of chicken/rooster combs.[72]

REFERENCES

1. Becker LC, Bergfeld WF, Belsito DV, et al. Final report of the safety assessment of hyaluronic acid, potassium hyaluronate, and sodium hyaluronate. *Int J Toxicol.* 2009;28(4 Suppl):5-67.

2. Nikitakis J (ed), Kowcz A (ed). wINCI: *International Cosmetic Ingredient Dictionary and Handbook*. http://webdictionary.personalcarecouncil.org/jsp/Home.jsp. Washington, DC: Personal Care Products Council. Last Updated: 2022. Accessed: May 20, 2022.

3. Sze JH, Brownlie JC, Love CA. Biotechnological production of hyaluronic acid: a mini review. *3 Biotech.* 2016;6(1):67.

4. Boykov PY, Khabarov VN, Polyak F, Selyanin MA. Methods of Hyaluronic Acid Production. In: *Hyaluronic Acid.* Chichester, UK: John Wiley & Sons, Ltd; 2015:77-95.

5. Anonymous. 2022. Manufacturing process of Hydrolyzed Hyaluronic Acid. (Unpublished data submitted by Personal Care Products Council on December 1, 2022.)

6. Anonymous. 2022. Hydrolyzed Sodium Hyaluronate flow chart (1-5 kDa). (Unpublished data submitted by Personal Care Products Council on December 1, 2022.)

7. Anonymous. 2022. Manufacturing technique flow chart Hydrolyzed Sodium Hyaluronate (<1 kDa). (Unpublished data submitted by Personal Care Products Council on December 1, 2022.)

8. Anonymous. 2022. Manufacturing process of Sodium Hyaluronate. (Unpublished data submitted by Personal Care Products Council on December 1, 2022.)

9. Spera Nexus Inc. 2022. Flow chart hyaluronate IW series (Sodium Hyaluronate). (Unpublished data submitted by Personal Care Products Council on January 3, 2023.)

10. Anonymous. 2022 Manufacturing process of low molecular weight Sodium Hyaluronate. (Unpublished data submitted by Personal Care Products Council on December 1, 2022.)

11. Shiedlin A, Bigelow R, Christopher W, et al. Evaluation of hyaluronan from different sources: Streptococcus zooepidemicus, rooster comb, bovine vitreous, and human umbilical cord. *Biomacromolecules.* 2004;5(6):2122-2127.

12. Anonymous. 2022. Composition and impurities data of Hydrolyzed Hyaluronic Acid. (Unpublished data submitted by Personal Care Products Council on December 1, 2022.)

13. Anonymous. 2022. Composition and impurities data of Hydrolyzed Sodium Hyaluronate. (Unpublished data submitted by Personal Care Products Council on December 1, 2022.)

14. Spera Nexus Inc. 2022. Statement composition Sodium Hyaluronate. (Unpublished data submitted by Personal Care Products Council on January 3, 2023.)

15. United States Pharmacopeial Convention. Food Chemicals Codex *9th ed*. In: (USP) USP, ed. Rockville, MD 2014. Accessed June 6, 2022.

16. Bloomage Biotechnology Corp Ltd. Conclusion of GRAS Status of Sodium Hyaluronate. GRAS Associates, LLC;2020. https://www.fda.gov/media/152869/download. Accessed June 6, 2022.

17. Xin Y, Jianhao L, Tiansheng S, et al. The efficacy and safety of sodium hyaluronate injection (Adant®) in treating degenerative osteoarthritis: a multi-center, randomized, double-blind, positive-drug parallel-controlled and non-inferiority clinical study. *Int J Rheum Dis.* 2016;19(3):271-278.

18. US Food and Drug Administration (FDA) Center for Food Safety & Applied Nutrition (CFSAN). 2022. Voluntary Cosmetic Registration Program - Frequency of Use of Cosmetic Ingredients. (Obtained under the Freedom of Information Act from CFSAN; requested as "Frequency of Use Data" January 4, 2022; received January 11, 2022). College Park, MD.

19. Personal Care Products Council. 2021. Concentration of Use by FDA Product Category: Hyaluronic acid and its salts. (Unpublished data submitted by Personal Care Products Council on January 10, 2022.)

20. European Commission. CosIng database; following Cosmetic Regulation No. 1223/2009. http://ec.europa.eu/growth/tools-databases/cosing/. Last Updated: 2020. Accessed: May 23, 2022.

21. U.S. Food and Drug Administration. Devices @ FDA. https://www.accessdata.fda.gov/scripts/cdrh/devicesatfda/index.cfm. Last Updated: 2022. Accessed: June 8, 2022.

22. U.S. Food and Drug Administration. Inactive Ingredient Search for Approved Drug Products. https://www.accessdata.fda.gov/scripts/cder/iig/index.cfm. Last Updated: 2022. Accessed: June 8, 2022.

23. U.S. Food and Drug Administration. FDA Approved Animal Drug Products. https://animaldrugsatfda.fda.gov/adafda/views/#/search. Last Updated: 2022. Accessed: June 8, 2022.

24. National Institutes of Health Office of Dietary Supplements. Dietary Supplement Label Database. https://dsld.od.nih.gov/. Last Updated: 2022. Accessed: June 9, 2022.

25. Essendoubi M, Gobinet C, Reynaud R, Angiboust JF, Manfait M, Piot O. Human skin penetration of hyaluronic acid of different molecular weights as probed by Raman spectroscopy. *Skin Res Technol.* 2016;22(1):55-62.

26. Brown MB, Jones SA. Hyaluronic acid: a unique topical vehicle for the localized delivery of drugs to the skin. *J Eur Acad Dermatol Venereol.* 2005;19(3):308-318.

27. Oe M, Mitsugi K, Odanaka W, et al. Dietary hyaluronic acid migrates into the skin of rats. *Scientific World Journal.* 2014;2014:378024.

28. Kimura M, Maeshima T, Kubota T, Kurihara H, Masuda Y, Nomura Y. Absorption of Orally Administered Hyaluronan. *J Med Food.* 2016;19(12):1172-1179.

29. Balogh L, Polyak A, Mathe D, et al. Absorption, uptake and tissue affinity of high-molecular-weight hyaluronan after oral administration in rats and dogs. *J Agric Food Chem.* 2008;56(22):10582-10593.

30. Anonymous. 2022. Sodium Hyaluronate toxicity summary. (Unpublished data submitted by Personal Care Products Council on December 1, 2022.)

31. Spera Nexus Inc. 2022. Safety test of hyaluronate IW series (Sodium Hyaluronate). (Unpublished data submitted by Personal Care Products Council on January 3, 2023.)

32. Anonymous. 2022. Hydrolyzed Sodium Hyaluronate toxicity summary. (Unpublished data submitted by Personal Care Products Council on December 1, 2022.)

33. Ma Y, Li C, Mai Z, Yang J, Tai M, Leng G. Efficacy and safety testing of dissolving microarray patches in Chinese subjects. *J Cosmet Dermatol.* 2021.

34. Active Concepts. 2020. Dermal and ocular irritation tests AC HYA Solution 1% (contains 1% Hyaluronic Acid). (Unpublished data submitted by Personal Care Products Council on November 9, 2022.)

35. Active Concepts. 2018. Dermal and ocular irritation tests AC Hyalurosome (contains 1% Hyaluronic Acid). (Unpublished data submitted by Personal Care Products Council on Novemeber 9, 2022.)

36. Active Concepts. 2022. Dermal and ocular irritation tests AC NanoVesicular Systems P3 (contains 3% Hyaluronic Acid). (Unpublished data submitted by Personal Care Products Council on November 9, 2022.)

37. Active Concepts. 2022. Dermal and ocular irritation tests AC Moisture-Plex Advance (contains 0.5% Sodium Hyaluronate). (Unpublished data submitted by Personal Care Products Council on November 9, 2022.)

38. Active Concepts. 2018. *In chemico* skin sensitization AC Hyalurosome (contains 1% Hyaluronic Acid). (Unpublished data submitted by Personal Care Products Council on November 9, 2022.)

39. Active Concepts. 2018. OECD TG 422D: In vitro skin sensitization AC Hyalurosome (contains 1% Hyaluronic Acid). (Unpublished data submitted by Personal Care Products Council on November 9, 2022.)

40. Anonymous. 2020. Human repeat insult patch tests (formula contains 0.2% Hyaluronic Acid). (Unpublished data submitted by Personal Care Products Council on November 21, 2022.)

41. Anonymous. 2020. Human repeated insult patch test with challenge (formulat contains 0.2% Sodium Acetylated Hyaluronate). (Unpublished data submitted by Personal Care Products Council on November 21, 2022.)

42. Anonymous. 2019. Repeated insult patch test (formula contains 1.5% Sodium Hyaluronate). (Unpublished data submitted by Personal Care Products Council on November 21, 2022.)

43. Guillaumie F, Furrer P, Felt-Baeyens O, et al. Comparative studies of various hyaluronic acids produced by microbial fermentation for potential topical ophthalmic applications. *J Biomed Mater Res A.* 2010;92(4):1421-1430.

44. Monzani D, Molinari G, Gherpelli C, Michellini L, Alicandri-Ciufelli M. Evaluation of Performance and Tolerability of Nebulized Hyaluronic Acid Nasal Hypertonic Solution in the Treatment of Chronic Rhinosinusitis. *Am J Rhinol Allergy.* 2020;34(6):725-733.

45. Decates T, Kadouch J, Velthuis P, Rustemeyer T. Immediate nor Delayed Type Hypersensitivity Plays a Role in Late Inflammatory Reactions After Hyaluronic Acid Filler Injections. *Clin Cosmet Investig Dermatol.* 2021;14:581-589.

46. Johnson ME, Murphy PJ, Boulton M. Effectiveness of sodium hyaluronate eyedrops in the treatment of dry eye. *Graefe's Arch Clin Exp Ophthalmol.* 2006;244(1):109-112.

47. Schlesinger TE, Powell CR. Efficacy and tolerability of low molecular weight hyaluronic acid sodium salt 0.2% cream in rosacea. *J Drugs Dermatol.* 2013;12(6):664-667.

48. Sullivan DY, Hawkes JE. Photodermatitis following knee intra-articular hylan G-F 20 injection for osteoarthritis: two cases. *Dermatol Online J.* 2021;27(12).

49. Schattner A, Haj-Yahya A. Upper airway angioedema after topical hyaluronic acid in a patient treated with bisoprolol. *J Postgrad Med.* 2018;64(2):128.

50. Personal Care Products Council. 2021. Concentration of Use by FDA Product Category: Concentration of use by FDA Product Category – Hyaluronates Not Previously Reviewed by CIR. (Unpublished data submitted to Personal Care Products Council on July 6, 2021.)

51. Vidič M, Bartenjev I. An adverse reaction after hyaluronic acid filler application: a case report. *Acta Dermatovenerol Alp Pannonica Adriat.* 2018;27(3):165-167.

52. Alawami AZ, Tannous Z. Late onset hypersensitivity reaction to hyaluronic acid dermal fillers manifesting as cutaneous and visceral angioedema. *J Cosmet Dermatol.* 2021;20(5):1483-1485.

53. Lupton JR, Alster TS. Cutaneous hypersensitivity reaction to injectable hyaluronic acid gel. *Dermatol Surg.* 2000;26(2):135-137.

54. Arron ST, Neuhaus IM. Persistent delayed-type hypersensitivity reaction to injectable non-animal-stabilized hyaluronic acid. *J Cosmet Dermatol.* 2007;6(3):167-171.

55. Leonhardt JM, Lawrence N, Narins RS. Angioedema acute hypersensitivity reaction to injectable hyaluronic acid. *Dermatol Surg.* 2005;31(5):577-579.

56. Bisaccia E, Lugo A, Torres O, Johnson B, Scarborough D. Persistent inflammatory reaction to hyaluronic acid gel: a case report. *Cutis.* 2007;79(5):388-389.

57. Patel VJ, Bruck MC, Katz BE. Hypersensitivity reaction to hyaluronic acid with negative skin testing. *Plast Reconstr Surg.* 2006;117(6):92e-94e.

58. Bardazzi F, Ruffato A, Antonucci A, Balestri R, Tabanelli M. Cutaneous granulomatous reaction to injectable hyaluronic acid gel: another case. *J Dermatolog Treat.* 2007;18(1):59-62.

59. Wang C, Sun T, Li H, Li Z, Wang X. Hypersensitivity Caused by Cosmetic Injection: Systematic Review and Case Report. *Aesthetic Plast Surg.* 2021;45(1):263-272.

60. Bitterman-Deutsch O, Kogan L, Nasser F. Delayed immune mediated adverse effects to hyaluronic Acid fillers: report of five cases and review of the literature. *Dermatol Reports.* 2015;7(1):5851.

61. Homsy A, Rüegg EM, Jandus P, Pittet-Cuénod B, Modarressi A. Immunological reaction after facial hyaluronic acid injection. *Case Reports Plast Surg Hand Surg.* 2017;4(1):68-72.

62. O'Reilly P, Malhotra R. Delayed hypersensitivity reaction to Restylane ® SubQ. *Orbit.* 2011;30(1):54-57.

63. Hönig JF, Brink U, Korabiowska M. Severe granulomatous allergic tissue reaction after hyaluronic acid injection in the treatment of facial lines and its surgical correction. *J Craniofac Surg.* 2003;14(2):197-200.

64. Banh K. Facial ischemia after hyaluronic acid injection. *J Emerg Med.* 2013;44(1):169-170.

65. Ghislanzoni M, Bianchi F, Barbareschi M, Alessi E. Cutaneous granulomatous reaction to injectable hyaluronic acid gel. *Br J Dermatol.* 2006;154(4):755-758.

66. Pérez-Pérez L, García-Gavín J, Wortsman X, Santos-Briz Á. Delayed Adverse Subcutaneous Reaction to a New Family of Hyaluronic Acid Dermal Fillers With Clinical, Ultrasound, and Histologic Correlation. *Dermatol Surg.* 2017;43(4):605-608.

67. Caldas Pozuelo C, Domínguez De Dios J, Mota Rojas X. Multiple oral granulomatous nodules to hyaluronic acid filler. *J Cosmet Dermatol.* 2020;19(12):3453-3455.

68. Ross CL, Heitmiller K, Saedi N. Hypersensitivity reaction to RHA filler. *J Cosmet Dermatol.* 2022;21(1):412-413.

69. Pezier T, Morand G, Tischler V, Kleinjung T. Delayed granulomatous reaction to hyaluronic acid gel injection. *Eur J Plast Surg.* 2014;37(1):45-48.

70. Sidwell RU, Dhillon AP, Butler PE, Rustin MH. Localized granulomatous reaction to a semi-permanent hyaluronic acid and acrylic hydrogel cosmetic filler. *Clin Exp Dermatol.* 2004;29(6):630-632.

71. Angus JE, Affleck AG, Leach IH, Millard LG. Two cases of delayed granulomatous reactions to the cosmetic filler Dermalive, a hyaluronic acid and acrylic hydrogel. *Br J Dermatol.* 2006;155(5):1077-1078.

72. Matarasso SL, Herwick R. Hypersensitivity reaction to nonanimal stabilized hyaluronic acid. *J Am Acad Dermatol.* 2006;55(1):128-131.

Distributed for Comment Only -- Do Not Cite or Quote

International
Journal of Toxicology
Volume 28 Number 4S
July/August 2009 5-67
© 2009 The Author(s)
10.1177/1091581809337738
http://ijt.sagepub.com
hosted at
http://online.sagepub.com

Final Report of the Safety Assessment of Hyaluronic Acid, Potassium Hyaluronate, and Sodium Hyaluronate

Lillian C. Becker, MS, Wilma F. Bergfeld, MD, Donald V. Belsito, MD, Curtis D. Klaassen, PhD, James G. Marks Jr, MD, Ronald C. Shank, PhD, Thomas J. Slaga, PhD, Paul W. Snyder, DVM, PhD, Cosmetic Ingredient Review Expert Panel, and F. Alan Andersen, PhD

Hyaluronic acid, sodium hyaluronate, and potassium hyaluronate function in cosmetics as skin conditioning agents at concentrations up to 2%. Hyaluronic acid, primarily obtained from bacterial fermentation and rooster combs, does penetrate to the dermis. Hyaluronic acid was not toxic in a wide range of acute animal toxicity studies, over several species and with different exposure routes. Hyaluronic acid was not immunogenic, nor was it a sensitizer in animal studies. Hyaluronic acid was not a reproductive or developmental toxicant. Hyaluronic acid was not genotoxic. Hyaluronic acid likely does not play a causal role in cancer metastasis; rather, increased expression of hyaluronic acid genes may be a consequence of metastatic growth. Widespread clinical use of hyaluronic acid, primarily by injection, has been free of significant adverse reactions. Hyaluronic acid and its sodium and potassium salts are considered safe for use in cosmetics as described in the safety assessment.

Keywords: cosmetics; hyaluronic acid; safety

The safety of ingredients used in cosmetic formulations is reviewed by the Cosmetic Ingredient Review (CIR) program. Published studies relevant to assessing the safety of hyaluronic acid, sodium hyaluronate, and potassium hyaluronate as used in cosmetic products have been combined with unpublished data provided by interested parties. In a series of public meetings, with formal notice and comment opportunities for any interested party to provide additional data or comment, the CIR Expert Panel reviewed these data and reached a tentative and then final conclusion regarding safety of these ingredients as used in cosmetics.

Technical writer, Cosmetic Ingredient Review (LCB); Member, Cosmetic Ingredient Review Expert Panel (WFB, DVB, CDK, JGM, RCS, TJS, PWS); and Director, Cosmetic Ingredient Review (FAA), Washington, DC.

Please address correspondence to Lillian C. Becker, MS, Cosmetic Ingredient Review, 1101 17th Street, NW, Suite 412, Washington, DC 20036; e-mail: info@cir-safety.org.

Chemistry

Definition and Structure

Hyaluronic acid (CAS No. 9004-61-9) is the natural glycosaminoglycan formed by bonding N-acetyl-D-glucosamine with glucuronic acid.[1] Disaccharide units are formed at the plasma membrane in vertebrates and some bacteria.[2-4] These units are linked together with 2 to 4 glycosidic bonds to a long, linear (unbranched) molecule, which grows into a random coil as it becomes longer. A completed hyaluronic acid molecule can reach 10 000 or more disaccharide pairs, a molecular mass of approximately 4 million Da. The molecule is considerably rigid, and as it grows longer, the overall shape is spherical. It also entangles with adjacent coils to create a continuous network. At concentrations higher than 0.1%, the chains of hyaluronic acid form a continuous network. Only about 0.1% of the volume of the molecule

Distributed for Comment Only -- Do Not Cite or Quote

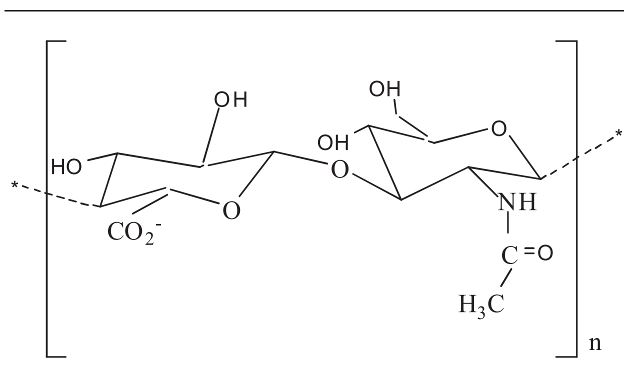


Figure 1. Structure of hyaluronic acid. *The repeating units, identical to the structure in brackets.

is hyaluronic acid, the rest being water which is mechanically immobilized within the coil. The rate of diffusion through the network is inversely related to the size of the polysaccharide molecules. The structure of the disaccharide is energetically very stable. The domain structure of hyaluronic acid allows small molecules such as water, electrolytes, and nutrients to freely diffuse through the solvent within the domain while large molecules such as proteins will be partially excluded from the domain because of their hydrodynamic sizes in solution.

In the presence of K^+, NH_4^+, Rb^+, and Cs^+, an antiparallel double-helical structure forms with hydrophobic and hydrophilic pockets between adjacent double helices.[5] Under physiological conditions (pH 7), this water soluble molecule exists in its salt form as sodium hyaluronate.[6] The structure of hyaluronic acid is shown in Figure 1.[4]

According to the *International Cosmetic Ingredient Dictionary and Handbook*, hyaluronic acid is also known as hyaluronan.[7] Trade names for hyaluronic acid and trade name mixtures containing hyaluronic acid are listed in Table 1.

Sodium hyaluronate (CAS no. 9067-32-7), also known as hyaluronic acid sodium and hyaluronate sodium, is the sodium salt of hyaluronic acid.[7] Other technical names are: hyaluronic acid, sodium salt; sodium hyaluronate (1); sodium hyaluronate (2); and sodium hyaluronate (3); and sodium hyaluronate solution. Its trade names and trade name mixtures are listed in Table 1. Potassium hyaluronate (CAS no. 31799-91-4) is the potassium salt of hyaluronic acid, also known as hyaluronic acid, potassium salt.[7]

Physical and Chemical Properties

Various authors have reported the water retention properties of hyaluronic acid. Hyaluronic acid has a greater capacity to hold water than any other natural or synthetic polymer.[8] One gram of hyaluronic acid can hold up to 6 L of water.[9]

Polymer Network

The structural factors underlying hyaluronic acid's unique properties are its high molecular weight and large molecular volume.[10] The large molecular volume forces the overlap of individual hyaluronic acid molecular domains, resulting in extensive chain entanglement and chain–chain interaction.[10-13]

Concentration (expressed in g/cc) and intrinsic viscosity (limiting viscosity number, expressed in cc/g), which are measures of molecular volume, are related to the ability of hyaluronic acid to form viscoelastic polymeric networks. The product of concentration and intrinsic viscosity has been called the coil overlap parameter, which expresses the degree of network formation.[14] Concentration and molecular volume (size) are totally interdependent in determining the physical properties of a hyaluronic acid solution.[13] They stated that the smaller the size of the individual hyaluronic acid molecules, the higher the concentration necessary for a viscoelastic network to form.

The carboxyl groups fully dissociate at physiological pH[15]; the structure is sensitive to ionic strength and pH. The molecule expands at lower ionic strength due to repulsions between the charges. This increases viscosity.[16,17]

The chains, when entangling, also interact with each other and form stretches of double helices so that the network becomes mechanically more firm.[18,19] Each glucuronate unit carries an anionic charge at physiological pH associated with its carboxylate group. There are often hundreds of negative charges fixed to each chain. These charges are balanced by mobile cations such as Na^+, K^+, Ca^{++}, and Mg^{++}. The charges are important in determining solubility in water because hyaluronic acid, converted into an uncharged polymer by fully esterifying with methyl groups, is insoluble. The molecule has the properties of a highly hydrophilic (polyhydroxylic) material simultaneously with hydrophobic domains characteristic of lipids.

Distributed for Comment Only -- Do Not Cite or Quote

Table 1. Trade Names for Hyaluronic Acid and Sodium Hyaluronate and Trade Name Mixtures Containing These Ingredients[7]

Trade Names of Hyaluronic Acid		
AEC Hyaluronic Acid	Biomatrix	Hyaluronic Acid SOL
Trade Name Mixtures of Hyaluronic Acid		
Amisil-HA	BioCare BHA-10	BioCare HA-24 Bio
BioCare Polymer BHA-10	BioCare Polymer HA-24	BioCare SA
Biosil Basics HMV - Hair Moisture Complex	Biosil Basics HMW - Hair Moisture Complex	Cromoist HYA
Cromoist WHYA	Glycoderm P	Lipocare HA/EC
Molecularsource LPC	PHYTO/CER.HA	
Trade Names of Sodium Hyaluronate		
Actimoist	AEC Sodium Hyaluronate	Avian Sodium Hyaluronate Powder
Avian Sodium Hyaluronate Solution	Bio-HE	Dekluron
HTL MYP Hyaluronic Acid	Hyaluronate Na F93	Hyaluronate Na F100
Hyaluronate Na 1.0% Gel	Hyaluronate Na P85	Hyaluronate Na P90
Hyaluronate Na P93	Hyaluronate Na P100	Hyaluronic Acid FCH-150
Hyaluronic Acid FCH-200	Hyaluronic Acid (Na)	Hyaluronic Acid, Sodium Salt
Hyaluronsan HA-L510	Hyaluronsan HA-M5070	Hyaluronsan HA-Q
Hyaluronsan HA-QSS	Hyaluronsan Solution HA-Q1P	Hyasol
Hyasol-BT	LMW Hyaluronic Acid Na Salt	Nikkol Sodium Hyaluronate
OriStart SH	RITA HA C-1-C	RITA HA C-1-P
Saccaluronate CW	Sodium Hyalronate HA-Q	Restylane
Hylaform		
Trade Name Mixtures of Sodium Hyaluronate		
Actiglide	Advanced Moisture Complex	Aragoline
Atecoron	Bellsilk HA	Biocrystal
Brookosome H	Chronosphere FHC/HA Blend	Chronosphere Hyaluronic
Collagen-Hyaluronic	Collagen-Hyaluronic Acid-Jelly	Desaron
EASHAVE	Essential Vital Elements - S	Gelhyperm (Avocado Oil)
Gelhyperm (Jojoba Oil)	Gelhyperm (Macadamia Nut Oil)	Gelhyperm (Seabukthorn Oil)
Gelhyperm (Wheat Germ Oil)	HA-Sol 2%	Hyaluronic Acid 1%
Hydralphatine 3P	Hydroxan	Hydroxan BG
Hydroxan CH	Iricalmin	Polyson HQ
Quiditat NwH	Ritacomplex DF 15	Ritacomplex DF 26
Rovisome H A	Saccaluronate CC	Saccaluronate LC
Spherica HA	Thioglycans	Toshiki BINS-3

Physical Form

Hyaluronic acid is available to the cosmetic formulator as a highly purified, freeze-dried powder or as an aqueous solution, and as its potassium or sodium salt.[20] In this form, hyaluronic acid slowly but fully dissolves in water to give viscous, clear to slightly opalescent and colorless solutions, which must be preserved in cosmetic usage. The viscosity can vary with the method of its preparation, decreasing sharply in the presence of electrolytes. Sodium hyaluronate is supplied as a white fiber-like or creamy white powder with a very faint odor. According to this author, it usually contains not less than 98% of the salt, although there are products with lower levels. It does not usually lose more than 10% of its weight on drying and normally gives not more than 20% of residue on incineration. A 0.2% water solution of sodium hyaluronate can have a pH range of 5.5 to 7.5. During the purification of sodium hyaluronate, which involves the removal of lipids, proteins, and nucleic acids, its molecular weight quickly drops. Sodium hyaluronate dissolves slowly but completely in water to give a clear to faintly opalescent colorless and highly viscous solution. The salt is soluble in sodium chloride solution but almost insoluble in organic solvents. Up to 90% of the salt can be insoluble in ethanol. This author also stated that aqueous solutions of sodium hyaluronate must be preserved, using 0.4% to 0.75% phenoxyethanol, for example, as the material is a rich source of nutrients for microbes.

Distributed for Comment Only -- Do Not Cite or Quote

Method of Manufacture/Sources

Hyaluronic acid and sodium hyaluronate historically are derived from rooster combs but can be prepared from human umbilical cords.[21] Hyaluronic acid is present in the perivascular connective tissue of the umbilical cord and is known as Wharton's jelly.[13] Hyaluronic acid also has been derived from bovine tracheas and bovine vitreous.[22] Hyaluronic acid and sodium hyaluronate of high molecular weight and purity are difficult to prepare because long chains of these molecules are easily broken by shear forces and are easily degraded by free radicals produced by ultraviolet radiation and oxidative agents.[1,13,23]

Billek and Billek described a process to produce a completely "protein-free" solution of hyaluronic acid.[8] A patent issued in Germany and several other European countries is for the process of purifying the vitreous bodies of pig eyes by lowering the pH to 4.2. The proteins form an insoluble complex with the hyaluronic acid that can be separated by centrifuging. The remaining clear, highly viscous solution is then adjusted to a physiological pH of 7.0, put into ampules, and sterilized.

Two of the hyaluronic acid gels manufactured specifically for dermal augmentation are from different sources.[24] Hylaform is produced by extraction from rooster combs, has a high molecular weight but a lower concentration (6 mg/mL), and its viscoelastic properties have a more elastic tendency. Restylane is produced by bacterial fermentation, has a lower molecular weight but a higher concentration (20 mg/mL), and its viscoelastic properties have a more viscous tendency.

In cosmetics, the only sources of hyaluronic acid used are from bacterial fermentation and rooster combs, with molecular weights between 5 and 1800 kD.[25] These 2 processes are described below.

From Bacteria

Hyaluronic acid of low molecular weight has been found in the capsules of bacteria such as Group A and C hemolytic *Streptococci* and *Pneumococcus* type II stain D39R.[26-32] Bacterial hyaluronic acid is industrially produced from *Streptococcus zooepidemicus* and *Streptococcus equi* where the microorganism produces hyaluronic acid and lactic acid from carbon and nitrogen sources.[9,22,31] Other bacteria that produce hyaluronic acid include *Streptococcus dysgalactiae*, *Staphylococcus aureus*, and *Streptococcus pyogenes*.[31,33-37]

To produce hyaluronic acid, bacteria was collected from a surface swab of guinea pig conjunctiva. *S zooedidemicus* was isolated and transferred to a Sakaguchi flask containing culture medium.[31] At the maximum cell titer, the culturing process was terminated, and benzyl alcohol was added for sterilization. After activated carbon and alumina were added to the growth media and agitated, the liquid was then filtered until transparent. Hyaluronic acid was precipitated by the addition of sodium and methanol. After thoroughly removing the methanol, the mixture was dried to a white powder.

From Cockscombs

Balazs was issued a patent in 1981 for sodium hyaluronate extracted from cockscombs to be used in cosmetics.[8,38] This product contained protein (50% to 400% relative to the extracted hyaluronic acid). By heating the extract to 100°C, some of the polysaccharide is broken down, leaving part of the product as high molecular weight (HMW) sodium hyaluronate and the rest as low molecular weight (LMW) sodium hyaluronate.

Crosslinking

Hyaluronic acid chains are crosslinked to stabilize the polysaccharide in such a way as to not affect the 2 specific groups of the molecule, the carboxylic and N-acetyl groups.[39] This crosslinking process produces a less dense structure than that of the native hyaluronic acid, resulting in a partial specific volume of 0.63 cc/g compared with hyaluronic acid's 0.57 cc/g (both in 0.15 N NaCl). Because only a limited number of the polysaccharide chains of hyaluronic acid are permanently associated through a methylene–bridge-protein–methylene bond, the hydrated molecules form an elastoviscous solution, which is called hylan fluid. The other hyaluronic acid crosslinking process utilizes vinyl sulfone, which reacts with the hydroxyl groups of the polysaccharide chain to form an infinite network through sulfonyl-bis-ethyl crosslinks.

Hyaluronic acid used for soft tissue augmentation is stabilized with carbon bridges every 2 to 500 U of disaccharide.[40] It is an epoxy, also used in many household glues, which hydrolyzes irreversibly into harmless carbon chains in hours.

Hylan B gel, sold as Hylaform (Biomatrix, Inc., Ridgefield, NJ) for tissue augmentation is stabilized

Distributed for Comment Only -- Do Not Cite or Quote

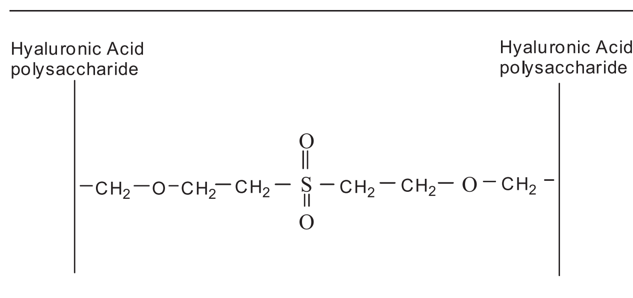


Figure 2. Chemical structure of the crosslink in insoluble Hylan B gel.[41]

hyaluronic acid.[41] The biomaterial retains the biocompatibility and biological properties of hyaluronic acid, but its residence time in dermal tissue is increased by introduction of sulfonyl-bis-ethyl crosslinks between the polysaccharide chains as shown in Figure 2.

Restylane (Q-Med Scandinavia Inc, Uppsala, Sweden) is produced in cultures of *Equine streptococci* by fermentation in the presence of sugar.[24] The fermentation material is then alcohol precipitated, filtered, and dried. The hyaluronic acid chains are then chemically stabilized through permanent epoxidic crosslinks that the manufacturer reports to alter only 1% of the hyaluronan molecular network. The material is heat sterilized in its final container.

Manna et al compared hyaluronic acid derivatives from rooster combs (Hylaform, subjected to crosslinking) and streptococcus (Restylane, stabilized as declared by the manufacturer), which were developed for soft tissue augmentation.[42] The researchers found that Hylaform functions as a strong hydrogel, has a minor quantity of crosslinked hyaluronic acid (about one fourth that of Restylane), and contains a low amount of protein (about one fourth that of Restylane). Restylane functions as a weak hydrogel and contains protein resulting from bacterial fermentation or added to enable crosslinking reaction. Hyaluronic acid and sodium hyaluronate may be crosslinked.[43]

Analytical Methods

Originally it was necessary to purify hyaluronic acid from tissue in order to measure the amount of hexosamine or hexuronic acid, which results in an accuracy within 100 μg.[2] Hyaluronate lyase from *Streptomyces hyalurolyticus* was also employed to degrade hyaluronic acid in the presence of other polysaccharides, and the resulting unsaturated disaccharides can be analyzed within 1 μg.

Hyaluronic acid can be detected and measured to within 1 ng in biological samples by specific radioassay making use of proteins with affinity for hyaluronic acid that are present in cartilage.[44] Proteins with affinity for hyaluronic acid also can be used for detection of the polymer using techniques similar to immunohistochemical methods.[45]

An absolute, weight average determination of molecular weight is obtained by using light scattering.[46] Milas et al suggested using this method to determine the range of molecular weights in a sample in combination with steric exclusion chromatography and refractometric detectors.[22]

Impurities

When hyaluronic acid is derived from animal sources, proteins may be present, which can affect the nonimmunogenic and immunogenic properties of the hyaluronic acid preparation.[6] To achieve high purity, a certain degree of depolymerization occurs, resulting in a lower grade product. For example, bovine hyaluronic acid (prepared by Etapharam, Vienna) contained approximately 10% of its total macromolecular content in protein.[47]

Proteins that bind to hyaluronic acid include hyaluronidases, antibodies, proteoglycans, and link proteins, cell-surface receptors for hyaluronic acid, and hyaluronic acid–binding proteins isolated from tissues and fluids.[2]

Balazs was granted a patent for "ultrapure" sodium hyaluronic acid.[8] The essential step that was different from other processes was to treat the hyaluronic acid solution, which had already had the proteins largely removed, with chloroform for up to 5 days. The purpose is to eliminate "a relatively undefined fraction ('inflammatory [H]yaluronic [A]cid') that is supposed to cause inflammation after injection into the eye."

The purity of hyaluronic acid may vary from one commercial batch to another.[48] The batch of human umbilical cord hyaluronic acid used in this study had <2% protein and <3% chondroitin sulfate. Using chondroitinase and protease digestion followed by thermic denaturation and dialysis against dissociating buffer to get rid of smaller fragments obtains an electrophoretically homogeneous hyaluronic acid preparation.

Distributed for Comment Only -- Do Not Cite or Quote

Clinically evident inflammatory reactions to viscous solutions of sodium hyaluronate are uncommon but may be related to impurities or deficiencies in formulation.[49] Reports of severe intraocular inflammation after cataract extraction with Viscoat (sodium hyaluronate; Alcon Laboratories, Fort Worth, TX) led to a recall of that product in 1987. The authors stated that the inflammation was probably due to the presence of endotoxins or other protein impurities in the visoelastic material, although the lots involved did meet standards for purity. The authors stated that this episode highlights the difficulty that manufacturers may have in detecting and eliminating impurities in long-chain HMW polymers from biological sources.

Three of the 7 hyaluronic acid preparations tested (source not given) contained significant levels of DNA in the range of 0.03 mg to 0.15 mg of DNA per milligram of hyaluronic acid with molecular weights ranging from 500 to >20 000 base pairs.[50]

Hyaluronic acid is easier to produce, extract, and purify as a polymer free of proteins from bacterial sources.[31] In contrast to animal sources, hyaluronic acid from a bacterial source exhibits superior reproducibility, high yields, and high degree of purity.[6] Hyaluronic acid for tissue augmentation from both rooster comb and bacterial sources is very pure and contains only low levels of impurities.[24]

Micheels concluded that hyaluronic acid was impure based on cited studies that showed that the hyaluronic acid extracted from sources other than rooster combs can be immunologically reactive.[40]

Based on the observation that avian influenza H5N1 virus is sensitive to heat, the 70°C temperature reached in the extraction of hyaluronic acid from cockscombs will also kill the virus.[51]

Use

Cosmetic Use

As given in the *International Cosmetic Ingredient Dictionary and Handbook*, hyaluronic acid functions as a skin-conditioning agent and/or as a viscosity increasing agent in cosmetic formulations.[7] Voluntary industry reports to the US Food and Drug Administration (FDA) include use in 223 products.[52] Hyaluronic acid is used in cosmetics in concentrations up to 1%.[53] The available data on frequency of use and use concentration as a function of product category are reported in Table 2.

Hyaluronic acid is used in "anti-aging" skin preparations.[6] LMW hyaluronic acid has been shown to increase the moisture level of damaged skin and to accelerate damage repair.[54] HMW hyaluronic acid solutions, when applied to the surface of the skin, form a hydrated viscoelastic film.[13] The film is permeable to air and cutaneous respiration is not obstructed while it "fixes" moisture to the skin surface.

Sodium hyaluronate is a skin conditioning agent–miscellaneous and is reportedly used in 601 products.[7,52] The maximum concentration of use for sodium hyaluronate is 2% in a body lotion.[53] At an application rate of 1 mg/cm^2 of a product, 0.02 mg hyaluronic acid/cm^2 of skin is contributed. The frequency of use as a function of cosmetic product category is reported in Table 2.

Potassium hyaluronate is a skin conditioning agent–miscellaneous and is reportedly used in 11 products.[7,52] No use concentrations were reported by industry.[53] The frequency of use as a function of cosmetic product category is reported in Table 2.

There are no restrictions of the use of hyaluronic acid, sodium hyaluronate, or potassium hyaluronate in cosmetics in Japan.[55] Hyaluronic acid, sodium hyaluronate, and potassium hyaluronate are not included among the substances listed as prohibited from use in cosmetic products marketed in the European Union.[56]

Cosmetics Aerosols

Hyaluronic acid, sodium hyaluronate, and potassium hyaluronate are used in products for which the application may be by aerosol spray.[52,53] Jensen and O'Brien reviewed the potential adverse effects of inhaled aerosols, which depend on the specific chemical species, the concentration, the duration of the exposure, and the site of deposition within the respiratory system.[57] Particle size is the most important factor affecting the location of deposition. The determination of the health consequences of exposure to an aerosol requires an analysis of the inhalation and deposition of the aerosol within the human respiratory system. The toxic action of an aerosol may be related to the number of particles, their surface area, or the mass deposited. Many occupational diseases are associated with the deposition of particles within a certain region of the respiratory tract. The aerosol properties associated with the location of deposition in the respiratory system are particle

Distributed for Comment Only -- Do Not Cite or Quote

Table 2. Cosmetic Product Uses and Concentrations for Hyaluronic Acid, Sodium Hyaluronate, and Potassium Hyaluronate

Product Category	Ingredient Uses[52]	Use Concentrations[53](%)
Hyaluronic Acid		
Bath products		
Other	-	0.001
Eye makeup		
Eye shadow	15	0.02
Eye lotions	5	-
Eye makeup remover	2	0.001
Other	11	0.07
Fragrance products		
Powders	1	-
Noncoloring hair care products		
Conditioners	2	-
Permanent waves	1	-
Tonics, dressings, etc	1	-
Hair coloring products		
Rinses	1	-
Makeup		
Blushers	7	0.02
Face powders	5	0.00005
Foundations	24	0.002
Lipsticks	-	0.01
Makeup bases	22	-
Other	-	0.001
Nail care products		
Cuticle softeners	1	0.001
Other	1	0.01
Personal hygiene products		
Other	1	-
Shaving products		
Aftershave lotions	1	-
Shaving creams	3	0.3
Skin care products		
Skin cleansing creams, lotions, liquids, and pads	6	0.001
Face and neck creams, lotions, powders, and sprays	8	0.1
Body and hand creams, lotions, powders, and sprays	12	0.001-1
Foot powders and sprays	1	-
Moisturizers	37	0.001-0.1
Night creams, lotions, powders, and sprays	17	0.02
Paste masks/mud packs	13	0.001
Other	23	0.001
Suntan products		
Suntan gels, creams, liquids, and sprays	1	-
Indoor tanning preparations	1	0.001
Total uses/ranges for hyaluronic acid	223	0.00005-1
Sodium Hyaluronate		
Baby products		
Shampoos	-	0.5
Lotions, oils, powders, and creams	-	0.5
Other	-	0.5
Bath products		
Oils, tablets, and salts	-	0.5
Soaps and detergents	1	0.001-0.5
Bubble baths	-	0.001-0.5
Capsules	-	0.5

(continued)

Distributed for Comment Only -- Do Not Cite or Quote

Table 2. (continued)

Product Category	Ingredient Uses[52]	Use Concentrations[53](%)
Other	-	0.001-0.5
Eye makeup		
Eyebrow pencils	3	0.5
Eyeliners	4	0.001-0.5
Eye shadow	11	0.0001-0.5
Eye lotions	6	0.001-0.7
Mascara	9	0.0001-0.5
Other	16	0.0001-0.5
Fragrance products		
Perfumes	1	0.5
Powders	1	0.5
Sachets	-	0.5
Other	-	0.0002
Noncoloring hair care products		
Conditioners	5	0.001-0.5
Sprays/aerosol fixatives	-	0.5
Straighteners	1	0.5
Permanent waves	-	0.5
Rinses	-	0.001-0.5
Shampoos	6	0.001-0.5
Tonics, dressings, etc	-	0.02-0.5
Wave sets	-	0.5
Other	-	0.5
Hair coloring products		
Dyes and colors	-	0.5
Tints	-	0.5
Rinses	-	0.5
Color sprays	-	0.5
Lighteners with color	-	0.5
Bleaches	-	0.5
Other	2	0.5
Makeup		
Blushers	20	0.001-0.5
Face powders	15	0.0005-0.5
Foundations	27	0.001-0.5
Leg and body paints	1	0.5
Lipsticks	96	0.0002-0.5
Makeup bases	15	0.002-0.5
Rouges	10	0.0001-0.5
Makeup fixatives	3	0.05-0.5
Other	17	0.0001-0.5
Nail care products		
Basecoats and undercoats	-	0.5
Cuticle softeners	-	0.01-0.5
Creams and lotions	-	0.5
Nail extenders	-	0.5
Nail polishes and enamels	-	0.5
Nail polish and enamel removers	-	0.5
Other	-	0.5
Personal hygiene products		
Underarm deodorants	-	0.5
Feminine deodorants	-	0.001
Other	-	0.5
Shaving products		
Aftershave lotions	6	0.001-0.5

(continued)

Distributed for Comment Only -- Do Not Cite or Quote

Table 2. (continued)

Product Category	Ingredient Uses[52]	Use Concentrations[53](%)
Beard softeners	-	0.5
Men's talcum	-	0.5
Preshave lotions	-	0.5
Shaving creams	-	0.001-0.5
Shaving soaps	-	0.5
Other	2	0.5
Skin care products		
Skin cleansing creams, lotions, liquids, and pads	20	0.000001-0.5
Depilatories	-	0.5
Face and neck creams, lotions, powders, and sprays	48	0.005-1
Body and hand creams, lotions, powders, and sprays	25	0.0001-2
Foot powders and sprays	-	1
Moisturizers	151	0.000001-1
Night creams, lotions, powders, and sprays	11	0.0001-1
Paste masks/mud packs	12	0.005-0.5
Skin fresheners	10	0.05-0.5
Other	38	0.001-1
Suntan products		
Suntan gels, creams, liquids, and sprays	4	0.000001-1
Indoor tanning preparations	1	0.001-0.5
Other	3	0.001-0.5
Total uses/ranges for sodium hyaluronate	601	0.000001-2
Potassium Hyaluronate		
Skin care products		
Face and neck creams, lotions, powders, and sprays	6	-
Moisturizers	4	-
Paste masks/mud packs	1	-
Total uses/ranges for potassium hyaluronate	11	None reported

size and density. The parameter most closely associated with this regional deposition is the aerodynamic diameter, d_a, defined as the diameter of a sphere of unit density possessing the same terminal setting velocity as the particle in question. These authors reported that the mean aerodynamic diameter of respirable particles is 4.25 ± 1.5 μm. This value may be compared with diameters of anhydrous hair spray particles of 60 to 80 μm (typically, <1% are below 10 μm) and pump hair sprays with particle diameters of ≥80 μm.[58]

Noncosmetic Use

General Medical

Hyaluronic acid has been used to simulate neutrophil function in patients with extreme susceptibility to bacterial infections.[59,60] Hyaluronic acid is also used in ophthalmic surgery, treating joint inflammation in racehorses, drug delivery, orthopedics, cardiovascular aids, and wound healing.[6,61]

Hyaluronic acid has been tested as a treatment for lipodystrophy in AIDS patients.[62] Injections of hyaluronans is considered effective in relieving the pain and effects of osteoarthritis (OA).[63,64] Hyaluronic acid application also has potential in facilitating the regrowth of severed nerves.[65]

Eye. Hyaluronic acid at 0.1% provides therapeutic benefit in dry eyes, which is because hyaluronic acid has a long residence time in the conjunctival sac and prolonged contact with the cornea.[66,67] It also has been suggested for use in glaucoma and other eye surgery techniques.[68-71]

Surgery. Hyaluronic acid has also been used in cochlear implant surgery to lubricate the electrode on insertion as well as tympanic membrane repair and otosurgery.[72-74] Hyaluronic acid also has been used in urological surgery, particularly for vesicoureteral reflux Krauss.[75]

Distributed for Comment Only -- Do Not Cite or Quote

Table 3. Summary of Drug Delivery Applications of Hyaluronic Acid[277]

Route	Role of Hyaluronic Acid	Therapeutic Agents
Ophthalmic	Increased ocular residence of drug, which can lead to increased bioavailability	Pilocarpine, tropicamide, timolol, gentamycin, tobramycin, arecaidine polyester, (S) aceclidine
Nasal	Bioadhesion resulting in increased bioavailability	Xylometazoline, vasopressin, gentamycin
Pulmonary	Absorption enhancer and dissolution rate modification	Insulin
Parenteral	Drug carrier and facilitator of liposomal entrapment	Taxol, superoxide dismutase, human recombinant insulin-like growth factor, doxorubicin, paclitaxel (Rosato et al[278])
Implant	Dissolution rate modification	Insulin
Gene	Dissolution rate modification and protection	Plasmid DNA/monoclonal antibodies

Sodium hyaluronate may be the solution best suited to provide a sufficiently thick mucosal layer by endoscopic injection for endoscopic mucosal resection resulting in fewer complications because the raised effect is maintained longer.[76] However, hyaluronic acid may stimulate cell growth of any residual tumor cells.[77] Hyaluronic acid has potential use in anesthesia because its presence prolongs the effects of tetrodotoxin blocks on rabbit nerves.[78]

Respiratory Function. Inhalation of hyaluronic acid (at respirable particle sizes) by persons with pulmonary emphysema may improve the elastic properties of the lungs thus improving respiratory function.[79]

Drug Delivery. Hyaluronic acid is used as a drug delivery agent.[80] Hyaluronic acid may be used as a skin substitute delivering antibiotics to burns or skin dressing and to accelerate healing.[81,82] The uses of hyaluronic acid in drug delivery are shown in Table 3.

FDA Approvals. Deflux (Q-Med Scandinavia Inc, Uppsala, Sweden) is used to treat children who have vesicoureteral reflux, an abnormal condition in which urine flows backwards from the bladder to the kidneys.[83] This condition causes repeated, severe urinary tract infections, which can harm the child's kidneys. This treatment should not be used in patients who have one kidney that does not work normally, an abnormal pouch in the bladder wall, an extra ureter, a urinary tract infection, or abnormal urination. Deflux should never be injected into blood vessels.

Healon GV (Abbott Medical Optics, Inc, Abbott Park, IL) is indicated for use in anterior segment ophthalmic surgical procedures.[83] Healon GV creates and maintains a deep anterior chamber to facilitate manipulation inside the eye with reduced trauma to the corneal endothelium and other ocular tissues. Healon GV also can be used to efficiently maneuver, separate, and control ocular tissues.

Synvisc (Genzyme Corporation, Cambridge, MA) and Nuflexxa (Savient Pharmaceuticals, Inc., East Brunswick, NJ) are indicated for the treatment of pain in osteoarthritis of the knee in patients who have failed to respond adequately to conservative nonpharmacological therapy and simple analgesics, for example, acetaminophen.[83,84]

Restylane is used in cosmetic surgery for the correction of wrinkles and facial sulci, including perioral and periorbital wrinkles, wrinkles in the genal region, lower lip elevation, and drooping of the nasogenian sulcus. It is also used in lip contour and augmentation as well as correcting depressed scars. It is contraindicated in patients with severe allergies manifested by a history of anaphylaxis, history or presence of multiple severe allergies, or allergies to gram-positive bacterial proteins.[85]

Hyaluronic acid's use in correction of glabella wrinkles (ie, between the eyebrows, above the nose) is controversial because this region is crisscrossed with relatively large blood vessels near the skin surface and can lead to the possibility of severe complications, including blindness.[86] Current and potential noncosmetic uses for hyaluronic acid are listed in Table 4.

General Biology

Natural Occurrence

Hyaluronic acid naturally occurs in the human body in the avascular body compartments like the synovial fluid and vitreous humor.[67] It is also abundant in tendon sheaths and bursae and found in the small amounts of fluid in the "serous" cavities (pleura, pericardium, and peritoneum) and in the less well-

Distributed for Comment Only -- Do Not Cite or Quote

Table 4. Current and Potential Noncosmetic Applications of Hyaluronic Acid

Application	Reference
Ophthalmic	
Comfort eye drops	Tan et al[6]
Cataract surgery to protect endothelium and maintain anterior chamber dome during surgery	Tan et al[6]
Vitreous substitute - Healon	Tan et al[6]
Penetrating keratoplasty (corneal transplantation)	Goa and Benfield[279]
Trabeculectomy in glaucoma management	Goa and Benfield[279]
Retinal reattachment	Goa and Benefield[279]
Trauma surgery	Goa and Benefield[279]
Orthopedic	
Joint mobilization during bone setting	Tan et al[6]
Arthroscopic surgery	Tan et al[6]
Surgical	
Ear surgery (eg, provides support to allow hearing of perforated ear drums)	Tan et al[6]
Nose and throat surgery	Tan et al[6]
Antiadhesive applications after abdominal surgery and in hand tendon surgery	Tan et al[6]
Spacing applications (eg, fallopian tube surgery)	Tan et al[6]
Encapsulate cells for implantation	Laurent[280]
Osteoarthritis	
Possible human use based on proven effects in horses	Tan et al[6]
Rheumatoid arthritis	Goa and Benfield[279]
Wound healing	
Silver hyaluronate provides slow release of Ag^+ (microbiocidal) into wounds	Tan et al[6]
Hyaluronic acid applied topically or in dressing	Goa and Benfield[279]
Other	
Drug delivery vehicle - skin patch	Tan et al[6]
Drug delivery vehicle - topical	Brown et al[145]; Brown and Jones[277]
Drug delivery vehicle - nasal spray	Surendrakumar et al[153]
Allergy response prevention	Scuri[152]
Vocal cord implants	Laurent[280]

defined planes of tissue movement such as those between muscle bodies and skin. Hyaluronic acid persists between the individual fibers, spindles, and septa in skeletal and cardiac muscle.[87]

The largest amount of hyaluronic acid (7-8 g per average adult human, ~50% of the total in the body) resides in skin tissue, where it is present in both the dermis (~0.5 mg/g wet tissue) and the epidermis (~0.1 mg/g wet tissue).[4] The actual concentrations of hyaluronic acid in the matrix around the cells in the epidermis (estimated to be 2-4 mg/mL) are an order of magnitude higher than in the dermis (estimated to be ~0.5 mg/mL).

Based on this information, the CIR Expert Panel calculated that approximately 0.6 mg/g hyaluronic acid resides in the skin. Approximately 15% of a 60 kg (132 lb) woman is skin (CTFA 2006), yielding a total of approximately 9000 g of skin. The average woman has a total surface area of 16 900 cm^2.[88] Dividing the weight of skin by the area of skin yields a weight per area of skin: 9000 g / 16 900 cm^2 = 0.53 g skin/cm^2. By using the estimate of hyaluronic acid in skin by weight of approximately 0.6 mg/g, the amount of hyaluronic acid in skin by area is estimated to be the following: 0.6 mg hyaluronic acid/g × 0.53 g/cm^2/1 g skin = 0.318 mg hyaluronic acid/cm^2.

HMW hyaluronic acid, in the range of millions of daltons, is present in cartilage, in the vitreous of the eye, and in synovial fluid joints.[89] These authors noted that HMW hyaluronic acid inhibits the growth of blood vessels, while LMW hyaluronic acid fragments are highly angiogenic and are potent stimulators of blood vessel growth.

Biosynthesis

Synthesis of hyaluronic acid increased during mitosis.[90] When synthesis was blocked by an inhibitor, the cells were arrested in mitosis. The authors concluded that hyaluronic acid was required for the

Distributed for Comment Only -- Do Not Cite or Quote

detachment of the cells from their substrate before they could divide. Hyaluronic acid is biosynthesized in the outer cell membranes.[19]

In the skin, hyaluronic acid is synthesized primarily by dermal fibroblasts and by epidermal keratinocytes.[91] In fibroblast cultures, the rate of hyaluronic acid biosynthesis is regulated in part by cell density. At low cell densities, biosynthesis is high, and cell motility and cell proliferation are also high. At high cell densities, cell proliferation is low, and hyaluronic acid biosynthesis is shut down.[92]

Biological Functions

Biological functions involving hyaluronic acid and its salts include water retention in the matrix, tissue hydration, water homeostasis, lubrication, solute transport, cell migration, neutrophil adhesion, cell interaction, cell division, bone resorption, fetal wound healing, wound healing, development, and red blood cell aggregation and adhesion.[11,12,74,93-115]

Chondrocytes are dependent on a hyaluronic acid coat for the deposition of the cartilage matrix.[116] In egg fertilization, a layer of hyaluronic acid must be penetrated by the sperm, accomplished by virtue of hyaluronidase on the sperm head.[117] Sliwa suggested that hyaluronic acid plays a part in the attraction of sperm to the ovum.[118]

Hyaluronic acid in the extracellular matrix binds to cells through specific cell-surface receptors, including CD44 and the receptor for hyaluronan-mediated motility (RHAMM), leading to intracellular signaling and modification of cell behavior.[119]

Role in Development

Hyaluronic acid plays a role in mammalian development. For example, the concentration of hyaluronic acid is high during the morphogenetic phase of development and removed by hyaluronidase during differentiation.[120,121]

Possible functions for hyaluronic acid during development include promoting the detachment of cells from a substrate, disruption of intercellular junctions permitting cell migration, reduction of the effect of fibronectin on spreading and motility of neural crest cells, increased cell motility, vascularization control, cell protection, and embryo implantation.[122-129] Hyaluronic acid has been shown to be important to the development of the cornea, neural tube, mammary ducts, placenta, and epidermis.[130-134]

Immunological Effects

There are divergent reports on the effect of hyaluronic acid on macrophages.[2] Relatively high concentrations of HMW hyaluronic acid inhibit the movement and phagocytosis of macrophages, presumably due to the viscosity of the medium, while lower concentration enhance their phagocytosis and pinocytosis.[101,135-137]

Suppression of the formation of humoral precipitating antibodies to certain major classes of proteins present in the peritoneal fluid from a patient with Wilm's tumor was reported.[138] Their findings were interpreted to suggest that hyaluronic acid can interfere with both the elicitation of a complete antibody response and the formation of "normal" patterns of antigen-antibody precipitates in laboratory tests. The authors stated that their results support the possibility that hyaluronic acid may play an immunoregulatory role by masking potential immunogens.

Hyaluronic acid functions in U937 macrophage proliferation and death.[139] Hyaluronic acid inhibited U937 macorphage proliferation by impeding cell proliferation and inducing apoptosis.

Hyaluronic acid plays an important role in mucosal host defense in the lungs.[140] Hyaluronic acid retains and regulates enzymes important for homeostasis at the apical mucosal surface of the lungs, releasing them and activating some of them into the airway lumen at times of insult to the epithelium when hyaluronic acid is degraded. Because LMW hyaluronic acid increases ciliary action in the lungs, simultaneous with the release of enzymes, smaller hyaluronic acid fragments stimulate ciliary beating (through its interaction with RHAMM) and hence the clearance of foreign material from mucosal surfaces.

Infection Control

Pericellular hyaluronic acid of human synovial cells can interfere with infection by the Newcastle disease virus.[141] The authors hypothesized that cell-bound hyaluronic acid occurs as a dense gel and would be expected to impede the access of viruses to the plasma membrane.

Distributed for Comment Only -- Do Not Cite or Quote

Table 5. Compositions of Skin Models Used by Trommer et al[143]

System	Lipids	Concentration (μM)	Manufacturing Process
Simple system (A)	α-Linolenic acid	100	Shaking for 120 min
Complex system (B)	α-Linolenic acid	100	Liposomes prepared by the thin layer method
	Dipalmitoyl Phosphatidylcholine	200	
	Cholesterol	100	
Complex system with ceramide III (C)	α-Linolenic acid	100	Liposomes prepared by the thin layer method
	Dipalmitoyl Phosphatidylcholine	200	
	Cholesterol	100	
	Ceramide III	100	
Complex system with ceramide IV (D)	α-Linolenic acid	100	Liposomes prepared by the thin layer method
	Dipalmitoyl Phosphatidylcholine	200	
	Cholesterol	100	
	Ceramide IV	100	

Cytotoxicity

The cytotoxicity of hyaluronan-based hydrogels to vascular smooth muscle cells harvested from the thoracic aorta of Sprague-Dawley rats were assessed.[142] Sodium hyaluronate was dissolved in Dulbecco's Modified Eagle Medium (DMEM) to form a 1% viscous mixture to form HyA. Dextran-tetramethyl-rhodamine and dextran were mixed in a 0.04% (w/w) ratio (dex-rhod/dextran) to form Dex 15-rhod. HyA was incorporated into a 20% solution of methacryloyl-dex in DMEM with photoinitiators to form Dex 15-HyA. HyA was combined with methacryloyl in an aqueous environment then glycidyl methacrylate (GMA) was added to the solution for a theoretical substitution of HyA60. The resulting powder was dissolved in DMEM to form a 1% (w/v) solution that was free-radical photopolymerized to form HyA60. The solutions/gels were placed in indirect contact with rat cells with nutrient supplement for 48 hours. Cells exposed to HyA60 were significantly less viable than cells from the control groups. None of the other solutions/gels had toxic effects.

Photoreactivity

The effects of hyaluronic acid and its degradation products on irradiation-induced lipid peroxidation were tested.[143] Liposomal skin lipid models of increasing complexity were used to quantify the effects of hyaluronic acid and hyaluronic acid fragments (from *S zooepidemicus*) under ultraviolet exposure in the presence of iron ions. The molecular weights of the 5 batches of hyaluronic acid, determined by laser light scattering, were 1×10^6 g/mol and 6, 3.3, 3.2, and 2.2×10^5 g/mol. The 4 model skin preparations were produced with the composition shown in Table 5. Ten millimolars ferrous sulfate was added to the samples as an electron donator and catalyst of the Haber-Weiss reaction to initiate reactive oxygen species (ROS) generation via a Fenton type reaction. Samples of each composition were irradiated with a UV-B dose of 0.25 J/cm^2, which corresponds to 2 to 3 times the human minimal erythemal dose with and without hyaluronic acid. A thiobarbituric acid (TBA) assay was used to determine the amount of malondialdehyde (MDA) in each exposed sample. The concentration of the TBA reaction products was lower for all of the samples treated with hyaluronic acid preparations ($P < .05$) than the untreated samples for all skin model systems. The authors reported that the mass spectrometry ion signals demonstrated photodegradation of hyaluronic acid. Electron paramagnetic resonance (EPR) spectroscopy was used to determine the effects of hyaluronic acid on ROS and on stable free radicals. There was no difference in the EPR signal intensities between the samples with the hyaluronic acid or its fragments and the reference. The authors concluded that neither hyaluronic acid nor its enzymatically degraded fragments are able to reduce stable free radicals. The authors suggested that exclusive topical administration of hyaluronic acid in cosmetic and pharmaceutical semisolid formulations could be protective for lipids within the skin.

Distributed for Comment Only -- Do Not Cite or Quote

Absorption, Distribution, Metabolism, and Excretion

Absorption/Dermal Penetration

Hyaluronic acid from human umbilical vein (1350-4500 Da) was applied in a citric buffer to a 1 × 6 cm area of the shaved backs of 11 male Sprague-Dawley rats.[144] The citric buffer was applied to a similar area elsewhere on the back as a control. The rats were treated twice daily for 5 days and killed 3 days after the final treatment. The skin was excised and processed for histology, and the number of blood vessels per unit length was calculated. The addition of hyaluronic acid to rat skin had no adverse effect on the morphology of the skin. The overall mean blood vessel number per millimeter was significantly higher in treated skin than untreated skin. The hyaluronic acid penetrated to a maximum depth of 136 μm beneath the epidermis. These authors also applied radiolabeled hyaluronic acid (^{3}H-glucosamin; 10 μCi/mL) in a citrate buffer to a 1 cm^{2} circle shaved on the back of one young adult Spraque-Dawley rat under general anesthesia. After 4 hours, the shaved area was excised to the depth of muscle and processed for frozen sectioning. Sections, 40-μm thick, were cut from the fatty side upwards, dissolved in NaOH, and counted. Radiolabeled hyaluronic acid was found to penetrate rat skin to a maximum depth of approximately 800 μm. The highest values, in excess of 400 dpm, were evident at a depth of 500 to 700 μm, deeper that the maximum depth examined in the blood vessel experiment above.

Autoradiography was used to detect the dermal penetration of hyaluronic acid, in the form of [^{3}H]hyaluronan, using mouse skin. SKh/1 hairless mice, aged 3 to 6 months, were used.[145] One group of 4 was treated with radioactive gel and the other with nonradioactive gel. In the first part of the experiment, the mice were treated every 12 hours for 3 or 12 applications of approximately 50 mg (range, 44.7-63.1 mg) that was gently rubbed on a marked area of 5 to 6 cm^{2} on the dorsum of the trunk. Twelve to 16 hours after the last application, the mice were killed and the skin fixed and examined autoradiographically. Radiolabel was found mainly in the dermis, from the outermost layer down to the lymphatic and blood vessels just above the platysma muscle. Counterstaining showed that silver grains were clearly confined between collagen bundles. There were grains aggregated at hair follicles and the keratinous layers of the epidermis. The authors stated that these findings were an indication that there was failure to penetrate these areas, more rapid transit through them, or degradation within these areas.

In a second experiment using 10 mice, radiolabel was found in the same distribution within the dermis at the 2-hour time point, with the radiolabel concentrated within cell boundaries in the basal epidermis, the dermal matrix, and in the lining cells of the lymphatic sinuses.[145] Grains were also found in the keratinized layer of the skin and in the rudimentary hair follicles. The authors stated that in both of the mouse studies the fraction of hyaluronic acid recovered from the skin surface was small from 8 hours onward. Because the quantity of hyaluronic acid recovered from within the skin was high after 30 minutes but was similar to control after 1 to 8 hours, the authors suggested that levels equilibrated at approximately 1 hour. In the bloodstream, there was a significant macromolecular content as early as 30 minutes after gel application, which the authors identified as hyaluronic acid. Because the molecular weight profile of hyaluronic acid in the bloodstream (3.6 × 10^{5} Da) was only slightly lower than that applied to the skin (4 × 10^{5} Da), the authors concluded that passage through the skin was not restricted to smaller polymers. From 4 to 8 hours after application, increased amounts of radioactivity were found, but mainly as the metabolites of the labeled acetyl group of hyaluronic acid. As in serum, relatively little hyaluronic acid was found intact after 4 hours, but the authors stated that there was clear evidence that hyaluronic acid had been absorbed and its metabolic degradation had begun within 1 to 2 hours after application. Mice receiving one or several applications did demonstrate lower proportion of [^{3}H] in the skin after 12 to 16 hours.

Distribution

The transfer of radioactivity into the fetuses of pregnant rats injected intravenously with ^{14}C-SL-1010 sodium hyaluronate was examined.[146] SD series pregnant rats were injected on day 17 of pregnancy (number of rats not provided). Autoradiograms of the entire body were taken at 1, 4, 24, 48, and 72 hours after the injection. The dams were killed by freezing. The brain, Harder's gland, lungs, heart, liver, spleen, kidneys, adrenal gland, placenta, and amniotic fluid were collected from the dams. Three to 5 whole

Distributed for Comment Only -- Do Not Cite or Quote

Table 6. Tissue Concentration of Radioactivity After Intravenous Administration of ^{14}C-SL-1010 (A Formulation of Sodium Hyaluronate) to Rats on Day 18 of Pregnancy[146]

Sample	Radioactivity (μg equivalent of SL-1010/g wet tissue)		
	1 h (n = 2)	4 h (n = 3 or 4)	24 h (n = 3 or 4)
Dam			
Plasma	211.791	117.343 ± 29.934	3.082 ± 0.209
Blood	126.571	90.098 ± 19.890	2.071 ± 0.098
Brain	2.797	2.314 ± 0.212	0.633 ± 0.141
Harder gland	6.839	20.462 ± 3.530	29.072 ± 6.048
Heart	12.217	7.966 ± 2.749	1.624 ± 0.108
Lung	26.396	16.915 ± 2.910	3.135 ± 0.121
Liver	31.667	35.159 ± 2.208	13.256 ± 1.190
Kidney	20.529	29.369 ± 4.214	3.932 ± 0.184
Spleen	21.166	24.712 ± 1.687	17.103 ± 2.048
Adrenal	14.753	13.970 ± 0.552	6.500 ± 1.108
Placenta	22.195	18.271 ± 2.962	3.267 ± 0.173
Amniotic fluid	0.219	0.870 ± 0.438	0.185 ± 0.008
Fetus			
Whole body	0.608	2.141 ± 0.443	3.432 ± 0.499
Fetal liver	0.913	4.347 ± 1.339	6.427 ± 1.028

fetuses per dam were homogenized, and 3 to 5 livers were collected from other fetuses in the litters. The radioactivity of the fetuses increased over time until 24 hours after the injection and was maintained until 48 hours then reduced after 72 hours. Radioactivity was distributed throughout the body of the dams 1 to 4 hours after administration and decreased in the tissues of the entire body over time.

The authors repeated the above injection to pregnant rats on day 18 of pregnancy.[146] At 1, 4, and 24 hours after the injection, the dams were killed, and the plasma was obtained. The distribution of radioactivity in the tissues is shown in Table 6 and follows the same general pattern seen in the first experiment. Lactating rats (n = 3) were mildly ether-anesthetized on the 15th to 17th days after giving birth 1, 4, and 24 hours after administration of ^{14}C-SL-1010 sodium hyaluronate (10 mg/kg) to the caudal vein. Milk was collected (16-200 μL), and blood from the ocular fundus vein was collected at the same time. Radioactivity in the milk increased with time until 24 hours. The radioactivity in the plasma was highest at 1 hour and decreased with time. The authors stated that sodium hyaluronate was nontoxic to the dams and offspring.

In another experiment, the authors collected milk from 2 lactating rats and divided the fractions into total casein, casein fraction, calcium (Ca) binding casein fraction, hyaluronic acid fraction, and supernatant fraction after binding with cetylpyridinium chloride, and measured the radioactivity of each.[146] Total casein contained 77% to 84% of the radiolabel. A subset of that, the casein fraction, contained 56% to 63% of the radiolabel and the Ca binding casein fraction of that contained around 21% of the radiolabel. The hyaluronic acid fraction was 0.2% to 0.5% (almost background level). Approximately 20% of the radioactivity was in the supernatant fraction.

Metabolism

The turnover of hyaluronic acid was tested by injecting it into the anterior chamber of the eyes of female New Zealand White rabbits, weighing 2.7 to 3.4 kg.[147] The test material was purified; [^{3}H]hyaluronic acid was dissolved in phosphate-buffered saline containing 1% dimethyl sulfoxide. The weight-average molecular weight was 920 000 and a number average of 80 000. [^{3}H]Hyaluronic acid (3.8 mL) was mixed with 1.93 g of commercial hyaluronic acid (Healon), then concentrated until the original volume of commercial hyaluronic acid was reached. The hyaluronic acid concentration of the mixture was determined by radioassay to be 10 mg/mL. Two rabbits had 0.055 mL of aqueous humor removed and replaced by the labeled hyaluronic acid mixture, and 2 more had 0.2 mL replaced. In addition, 1 rabbit was injected in the anterior chamber with a trace amount of [^{3}H]hyaluronic

Distributed for Comment Only -- Do Not Cite or Quote

acid that had not been mixed with the commercial hyaluronic acid; 1 rabbit was injected intramuscularly in the thigh with 0.1 mL with the mixture, and 1 other rabbit was injected subcutaneously in the back of the neck with 0.1 mL of the mixture. Two-milliliter blood samples were collected before the injections and approximately 1, 2, 4, 8, and 24 hours after the injections. Blood samples were then collected daily for 4 days then every other day for 9 to 13 days. ^{3}H radiolabel was detectable 2 to 3 hours in blood after injection into the eye. It reached its maximum within 2 days and then decreased exponentially. The half-life of the [^{3}H]hyaluronic acid with the 0.2 mL injections was approximately 14 hours and, with the 0.055 mL injections, was approximately 8 hours. The subcutaneously injected [^{3}H]hyaluronic acid had a half-life of approximately 50 hours, and the intramuscularly injected material had a half-life of approximately 30 hours. The authors stated that these times should be adjusted for an approximate 45-minute delay due to metabolism of hyaluronic acid in the liver.

The time it takes for excess hyaluronic acid to clear the blood of sheep at normal levels and at increased levels was investigated.[148] Ten Merino ewes, weighing between 20 and 38 kg, were fitted with venous and arterial catheters for infusion and blood sampling in the right jugular vein and the right carotid artery, respectively. A baseline blood sample was taken; then a 20 μg intravenous tracer dose of [^{3}H]hyaluronic acid was given. Arterial blood samples were drawn at 2, 4, 6, 8, 10, 15, 20, and 30 minutes after injection to determine half-life. Three to 4 hours later, a 2 mg intravenous bolus dose of unlabeled hyaluronic acid was given immediately followed by a 40-minute intravenous infusion of additional hyaluronic acid at a rate of approximately 125 μg/min (2 mL/min). This dosage was estimated to give a steady-state plasma concentration of approximately 1 μg/mL to 2 μg/mL; the normal level is 0.12 ± 0.05 μg/mL. Arterial blood samples were taken at baseline, 5, 10, and 15 minutes after the bolus dose. Twenty micrograms of [^{3}H]hyaluronic acid were then given, and arterial blood samples were taken 2, 5, 20, and 40 minutes later. The hyaluronic acid infusion was stopped, and blood samples were taken 1, 5, 10, 20, and 40 minutes postinfusion. [^{3}H]Hyaluronic acid half-life was 5.3 ± 1.1 minutes (range, 3.3-6.5 minutes). The authors stated that both elimination curves obtained fitted well to a linear, 1-compartment, kinetics model. After bolus injection, the mean plasma concentration of hyaluronic acid was 2.42 ± 0.48 μg/mL. The maximum metabolic rate was 0.062 ± 0.009 μg/mL/min or 3.32 ± 0.30 μg/min/kg body weight. The elimination of [^{3}H]hyaluronic acid at the plateau level occurred with a half-life of 26.9 ± 7.0 minutes (range, 18.2-43.5 minutes). The authors reported that there were no adverse effects or raised hyaluronic acid levels in the bloodstream of the sheep.

Laurent and Fraser repeated the experiment of Laurent et al on cynomolgus monkeys.[149] Hyaluronic acid labeled with ^{3}H in the acetyl group of the N-acetyl-glucosamine was produced and purified. The weight-average molecular weight was 4.2×10^6, which was reproducible after 7 months showing stability. Unlabeled hyaluronic acid with a concentration of 10 mg/mL was mixed as in the previously mentioned experiment. Twelve cynomolgus monkeys of both sexes, weighing between 2.3 and 4.9 kg, were used. Injections were through the peripheral cornea into the anterior chamber. Aqueous humor (100 μL) was withdrawn, and 50 μL of [^{3}H]hyaluronic acid was injected into the eye of 5 monkeys. The procedure was repeated using 75 μL on 3 monkeys, and 4 additional monkeys were treated with 50 μL of [^{3}H]hyaluronic acid with the addition of pilocarpine, 10 μL at 4%, dropped into the eye at 1, 8, and 24 hours after injection. The authors reported that ^{3}H in the blood could be detected 2 to 3 hours after injection. It reached a maximum at 2 to 3 days in the animals not given pilocarpine and at 1 day in the monkeys treated with pilocarpine. After reaching maximum concentration, the [^{3}H] in plasma decreased exponentially. The authors stated that the mean half-life for the 8 monkeys without pilocarpine treatment was 21 hours and 9.5 hours for the pilocarpine-treated animals. The researchers found about the same time for the half-life from the eye when both volumes were used (average of 21 ± 3 hours) but showed a lag phase of a few hours followed by a more rapid turnover during the following 24 hours and often a slower rate later.

[^{3}H]Hyaluronic acid was injected into the pleural space of 6 adult New Zealand White rabbits weighing 2.2 to 2.7 kg. Injections ranged from 21.1 to 46.3 μg, and the molecular weight was 6×10^6.[150] Blood samples were taken from an ear vein at 0, 2, 4, 6, 8, 12, and 24 hours after injection. Blood samples were also collected every day after 4 days and every other day for the next week. The authors stated that when metabolized, the ^{3}H from the hyaluronic acid

Distributed for Comment Only -- Do Not Cite or Quote

appears as [^{3}H]H_2O in the blood and is proportional to the loss of hyaluronic acid from the pleural space. The half-life for pleural hyaluronic acid varied with the amount of [^{3}H]hyaluronic acid injected and was in the 8- to 15-hour range.

Excretion

A polyethylene tube was inserted into the bile duct of male rats under ether anesthesia.[146] After confirming the excretion of bile approximately 1 hour after treatment, ^{14}C-sodium hyaluronate (SL-1010) was administered at 10 mg/kg into the caudal vein. Bile was collected every 2 hours for 10 hours and then at 24 hours. The cumulative excretion rate into the bile up to 24 hours was 0.4% of the administered dose. The bile was analyzed using gel filtration column chromatography and the hyaluronidase digestion method. A major peak eluted at a LMW region centered on the fraction number 51. The lower peak that eluted in the others from the fraction numbers 20 to 45 was confirmed to be hyaluronic acid because it disappeared with the addition of hyaluronidase.

Effect on Penetration of Other Chemicals

Brown and Jones, in a review article, compared dermal penetration studies of hyaluronic acid and proposed that hyaluronic acid's influence as a drug delivery system may depend on the species of animal.[277] They noted that in mice, the drug reservoir forms in the deeper layers of the skin (dermis) compared with humans where it forms in more shallow layers (epidermis).

Hyaluronic Acid Produced During Inflammation

The effect of intraperitoneal infection on hyaluronic acid presence and turnover in the intraperitoneal cavity was investigated.[151] Nineteen New Zealand White rabbits, male and female, with an average weight of 2.9 kg, were injected with irritating agents to induce peritonitis. The irritant was made up of suspensions or solutions of 0.1% latex beads, 3% thioglycolate, and 2% starch in phosphate-buffered saline (PBS). The authors used a peritoneal lavage with 100 mL of PBS at 1, 2, 3, 4, or 5 days. An additional 5 control rabbits, male and female, average weight of 2.9 kg, were lavaged without previous injection. The sham lavage was made up of PBS. One rabbit died within 24 hours after the injection, but the remaining animals had no clinical signs of distress. All the injected irritants caused a 10- to 1000-fold increase in hyaluronic acid concentrations in the lavage fluid on days 2 and 3, with large individual variations.

Animal Toxicology

Acute Toxicity

Oral

No deaths in ICR mice orally administered >1200 mg/kg hyaluronic acid were produced by fermentation.[31] No further details were provided.

Peritoneal

Denlinger and Balazs injected 0.5 mL of buffer, sodium hyaluronate (n = 12), or highly purified sodium hyaluronate (n = 10) into the peritoneal space of random-bred white male rats.[21] Animals were killed and blood collected at 12, 24, 48, 72, and 96 hours after injection. The peritoneal cavity was injected with 10 mL of buffer. The abdominal wall was then gently massaged, and after the skin was removed from a large part of the abdominal wall, the injected fluid was extracted. The leukocyte count in the peritoneal wash reached its peak at 24 hours after sodium hyaluronate injection ($55 \pm 4 \times 10^6$ cell/animal). The count decreased at 48 hours (count not provided). The count returned to control level in the sodium hyaluronate at 96 hours. The highly purified sodium hyaluronate leukocyte count peaked at $36 \pm 3 \times 10^6$ cell/animal and was not significantly different from the control level. The authors concluded that highly purified sodium hyaluronate can be used as a surgical tool in the eye without any adverse effects.

Pleural Space

Allen et al injected 21.1 μg to 46.3 μg of [^{3}H]hyaluronic acid into the pleural space of 6 adult New Zealand White rabbits weighing between 2.2 kg and 2.7 kg.[150] After 15 days, the animals were killed. All 6 rabbits tolerated the pleural injections well and manifested no evidence of pneumothorax, such as respiratory stress. No toxicity was noted by the researchers.

Distributed for Comment Only -- Do Not Cite or Quote

Inhalation

Sheep allergic to human neutrophil elastase (HNE) were used to test hyaluronic acid as a way to block bronchoconstriction.[152] Using a disposable medical nebulizer, the sheep inhaled a placebo then the HNE (dissolved in PBS) 30 minutes later to establish a baseline. The sheep later inhaled 3 or 15 mg LMW hyaluronic acid (150 kD) or HMW hyaluronic acid (300 kD) dissolved in PBS. After either 0.5, 4, or 8 hours, the sheep were then challenged with HNE. Inhaled HNE in untreated sheep caused a short-lived bronchoconstriction reaching its peak quickly (0-5 minutes) after challenge and resolved within 30 minutes. Aerosolized LMW hyaluronic acid blocked the HNE-induced airway response for the 0.5- and 4-hour time period. The 8-hour time period was only partially effective at the 3 mg dose but was completely effective at 15 mg. There were no ill effects reported from the hyaluronic acid. In another set of trials, 6, 7.5, or 15 mg HMW hyaluronic acid was administered 8 hours before the HNE challenge. The 15 mg of HMW hyaluronic acid was completely effective while the 6- and 7.5-mg doses were less effective in blocking bronchoconstriction. There were no toxicities reported.

Short-Term Toxicity

Inhalation

Male Beagle dogs were used to test the effectiveness of using a mixture of hyaluronic acid and recombinant human insulin in dried powder form as an inhaled drug delivery system for insulin.[153] Hyaluronic acid formulations containing insulin (10% w/w) were found to extend the mean residence time in blood and terminal half-life when compared to spray dried pure insulin. There were no toxicities reported.

Implantation

Four samples of Restylane (20 mg/mL nonanimal stabilized hyaluronic acid) and 4 samples of high density polyethylene reference standard were implanted into the paravertebral muscles in each of 3 anesthetized rabbits.[154] After the rabbits were observed for 4 weeks, they were killed, and implant sites were examined macroscopically and microscopically. A white focus (possibly a deposit of hyaluronic acid) was seen at 3 of the 12 Restylane implant sites. Encapsulation, with a thickness of 1 mm, was seen around one of the 12 control implants. Microscopic examination revealed that there was minimal to slight chronic inflammatory cell infiltration at both types of implant sites. The fibrous membrane was graded minimal to slight around the control and minimal to marked around the Restylane implants.

Chronic Toxicity

Implantation

A study conducted by Q-Med AB of Sweden on Deflux was reported.[155] The product is composed of microspheres of crosslinked dextan suspended in a carrier gel of nonanimal, stabilized hyaluronic acid. A 2-year implant study of Defulx was conducted using 22 rabbits. The objectives of this study were to determine the biocompatibility and migration potential of Deflux when implanted into the rabbit bladder submucosa. In each rabbit, 1 g of the test article was injected submucosally into each of the following bladder sites: right and left bladder neck and right and left bladder wall. Follow-up evaluations were conducted at 1 week and 1, 3, 6, 12, and 24 months. The urinary bladder, pancreas, kidney, liver, lung, draining lymph nodes, and brain were removed and examined histologically for inflammation, infection, irritation, foreign body responses, tissue necrosis, and scarring. Other organs were examined for gross abnormalities. The microspheres induced a fibrous tissue reaction around each microsphere without any adverse inflammatory reaction.

Another 2-year implant study of Deflux was reported.[155] Twelve dogs were implanted with 2.5 g of the test article in the following sites: right and left urinary bladder neck and right and left urinary bladder wall. The follow-up evaluations were conducted at 2 weeks and 3, 6, 12, and 24 months. In each animal, the urinary bladder, pancreas, kidney, liver, lung, draining lymph nodes, and brain were removed and examined histologically for inflammation, infection, irritation, foreign body responses, tissue necrosis, and scarring. Other organs were examined for gross abnormalities. The microspheres remained in the tissue in all injection sites for at least 2 years without causing any adverse foreign body reaction. There were no inflammatory reactions.

Ocular Toxicity

A highly purified special fraction of sodium hyaluronate was used in an experiment testing the amount of

Distributed for Comment Only -- Do Not Cite or Quote

inflamation caused when using hyaluronic acid to replace the liquid vitreous in the eyes of owl monkeys (*Aotus trivirgatus*) and rhesus monkeys (*Macaca mulatta*).[21] Sodium hyaluronate from 2 sources of rooster combs and from human umbilical cord were also tested. The molecular weight varied between 1.2 and 3.0×10^6, and the concentration was 10.0 ± 2.0 mg/mL. Sodium hyaluronate was dissolved in a physiological balanced salt solution that was also used as the control substance. Sixty-seven owl monkeys and 15 rhesus monkeys were used in this experiment. In owl monkeys, 1 mL of the liquid vitreous, representing approximately half of the total, was slowly withdrawn from 1 eye and replaced with the test solution. The same was done with the rhesus monkeys except that only 0.5 mL vitreous liquid was replaced. Evaluation for inflammation by visual examination and leukocyte count was carried out 48 hours after surgery and at day 7. The leukocyte count in the aqueous humor varied between 0 and 115 cells/mm^3 in owl monkey eyes and between 0 and 85 cells/mm^3 in rhesus monkey eyes. The mean values were 28 ± 7 and 38 ± 13 cells/mm^3, respectively. The difference between these values was not significant ($P \approx .4$). Only 2 eyes had slight turbidity and slight haziness in the vitreous in the owl monkeys. Only 2 rhesus monkeys had slight turbidity and haze. In both species, these reactions completely disappeared within 72 to 96 hours after injection.

These authors also injected the highly purified sodium hyaluronate into the eyes of an undisclosed number of owl monkeys that had previous reactions to concanavalin A, endotoxin, and a less purified sodium hyaluronate after the eyes had been clear of the reactions for 1 month.[21] The highly purified sodium hyaluronate implantations did not cause any significant inflammatory reaction in the previously inflamed eyes. The leukocyte counts after a first and second implantation of sodium hyaluronate were not significantly different from normal owl monkey aqueous after highly purified sodium hyaluronate was injected 1 or 2 times.

These authors repeatedly, up to 6 times, injected sodium hyaluronate into owl monkey eyes.[21] The experiment started with 76 eyes and finished with 11. The average time between each injection was 5.5 months. The leukocyte count 48 hours after the first injection in 102 eyes varied between 0 and 200 cells/mm^3. The mean and standard error of the mean were 20 ± 3 cells/mm^3. In 71% of these implantations, the cell count was between 0 and 24. Repeated implantations of sodium hyaluronate did not increase the severity of the haze and flare or any immunogenic response.

In a continuation of the above studies, highly purified HMW factions of sodium hyaluronate were injected into 6 eyes of 5 owl monkeys.[21] Each received 2 to 4 injections over 5.5 years in 2 eyes, 6.5 years in 2 eyes, and 9 years in 2 eyes. All eyes were completely normal, having no pathology in the anterior segment, lens, vitreous, retina, or choroid.

Akasaka et al reported a "negative" result of an eye stimulus test based on the standard Draize test of hyaluronic acid produced by the fermentation.[31] No further details were provided.

Skin Irritation

Hyaluronic acid did not cause irritation in a single-stimulus skin test using Japanese rabbits and Hartley guinea pigs.[31] No further details were provided.

Skin Sensitization

Potassium hyaluronate prepared from human umbilical cords was tested for antigenicity using rabbits.[156] Potassium hyaluronate was purified by repeated extraction with 90% phenol solution and by repeated precipitation from aqueous solution at pH 9 to 10 by 1.25 vol of ethanol saturated with potassium acetate. Potassium hyaluronate was then centrifuged after treatment with *p*-nitrobezyl bromide and acidified to hyaluronic acid. The aminobenzyl ether of the hyaluronic acid was coupled to horse-serum albumin and whole rabbit serum (3.6 g protein to 1 g compound). Three rabbits were immunized with the horse serum–hyaluronic acid preparation by injection at 30 mg, 60 mg, and 120 mg subcutaneously at weekly intervals for the first course. The rabbits were bled 1 week postinjection. The second course consisted of 4 intravenous injections of 20 mg at weekly intervals. These animals were bled (second time) 10 days postinjection. Precipitin tests were carried out in bulk. Complement fixation was carried out using 2 minimal hemolytic doses of complement. Agglutination was tested on a washed suspension of cells from a young culture of a capsulated group C *Streptococcus* whose capsules were known to be dissolved by hyaluronidase. A positive agglutination reaction was reported with the horse serum albumin but not to test substances that contained rabbit serum. The presence or

Distributed for Comment Only -- Do Not Cite or Quote

absence of hyaluronic acid did not change the reaction. No adverse effects from the injections were reported.

The possibility of an antigenic response to hyaluronic acid derived from different sources was tested.[157] Hyaluronic acid was prepared from 2 different extractions from human umbilical cords and NY 5 strain of *Streptococcus*. The Streptococcal-derived hyaluronic acid was free of protein. The bacterial antigens were derived from 3 strains of group A beta hemolytic streptococci belonging to serological types 1, 4, and 14 and a strain of hemolytic *Streptococcus aureus*. Young adult rabbits weighting between 5.5 and 6.5 kg were inoculated with the antigens either by injection intravenously in the marginal ear vein, intramuscularly, or subcutaneously (n = 3 for each route). For the intravenous injections, each rabbit received 1 injection of 0.5 mL of antigen daily for 3 days during the first week (1 mg umbilical cord hyaluronic acids or 1.3 mg streptococcal hyaluronic acid adjusted to 1 mg after the first week). They were allowed to rest 4 days, then received 1 mL daily for 3 days then allowed to rest for 4 days. This continued for 7 more weeks for a total of 27 injections except that the doses of the seventh week were administered subcutaneously in a dose of 0.5 mL. Each rabbit was bled from the ear before treatment and after the injections of the third, sixth, and eighth day after the ninth week of injections. The serum was separated, merthiolate added, and stored at 4°C to 8°C. For the intramuscular and subcutaneous injections, the hyaluronic acid content was 1 mg/mL; the dose was 0.5 mL 3 times per week for an average of 23 injections. Each rabbit was bled before treatment, at 2-week intervals and 10 days after the last injection. The sera from the rabbits in each treatment were tested for the presence of antibodies against the group-specific C carbohydrate and for antitype-specific M protein. None of the rabbits had any noticeable physical reactions following injections of antigens. None of the rabbits developed precipitating antibodies against either umbilical cord or streptococcal hyaluronic acid.

These authors performed skin sensitization tests on rabbits with the same hyaluronic acid preparations as above.[157] Each rabbit was skin-tested with 0.1 mg in 0.1 mL of the antigen that it received: 2 types of umbilical cord hyaluronic acid or the streptococcal hyaluronic acid. Injection sites were observed immediately and at 24, 48, and 72 hours for erythema and induration. A test for nonprecipitating, skin-sensitizing antibodies was performed with the sera of rabbits that had received streptococcal or umbilical cord hyaluronic acid. Test sites were injected with 0.1 mL of each rabbit's serum intradermally into a normal rabbit. Forty-eight hours later, each prepared site was injected with 0.01 mL of the same hyaluronic acid, which had been employed to vaccinate the animal supplying the serum. The results were read at 30, 45, and 60 minutes. The skin test revealed slight erythema lasting for 2 days about the injections site in one rabbit, which had received *Streptococcus* strain 1, and in one, which had received hyaluronic acid from *S aureus*. A large area of erythema, fading gradually in 3 days, was observed about the injection site in one rabbit, which had received umbilical cord hyaluronic acid, and a small area of erythema lasting 1 day was in another rabbit, which had received the same antigen. A small raised area of induration, lasting for 2 days, and a similar reaction, plus a small amount of erythema, was observed in 2 rabbits, which received streptococcal hyaluronic acid. Erythema fading in 3 days was observed in one rabbit, which received streptococcus A-1 plus hyaluronic acid, and in one, which received *S aureus* plus hyaluronic acid. Erythema was observed at 1 hour in 2 rabbits, which received crude extract plus streptococcus A-4. At 24 hours, the area of erythema was reduced, and there were small areas of induration. The induration remained for several days. The results of the tests for nonprecipitating, skin-sensitizing antibodies were entirely negative during the 1-hour observation. The authors did not report any findings for the 24-, 48-, and 72-hour observations.

The antigenicity of streptococcal-derived hyaluronic acid was tested on rabbits.[158] Streptococcal-derived hyaluronic acid was from 5 sources (isolated by other researchers). Each rabbit was immunized with 1 mL (2 mg/mL isotonic saline) of streptococcal-derived hyaluronic acid emulsified with 1 mL Freund's complete adjuvant. Injections were given in multiple sites (intramuscular, subcutaneous, and intradermal). One month later, the rabbits were injected again with 1 mL (1 mg/mL) hyaluronic acid without adjuvant. Preimmunization and postimmunization sera were compared. The sera were analyzed by double diffusion studies using antiserum to streptococcal-derived hyaluronic acid. The authors stated that the findings indicated that the rabbits formed precipitating antibodies to the streptococcal hyaluronic acid, which, they speculated,

Distributed for Comment Only -- Do Not Cite or Quote

may indicate that the streptococcal hyaluronic acid crossreacts with proteoglycan from cartilage.

The immunogenicity of purified hyaluronic acid was evaluated using rabbits.[159] Hyaluronic acid derived from rooster combs and human umbilical cords was used. Hyaluronic acid preparations contained 0.28% to 0.57% of protein or peptides. Rabbits received 4 intramuscular injections at 4-weekly intervals using 1 of the following formulas: human umbilical cord hyaluronic acid, 500 μg/dose; human umbilical cord hyaluronic acid, precipitated with cetylpyridinium chloride (CPC), 500 μg/dose; human umbilical cord hyaluronic acid–albumin conjugate, precipitated with CPC, 500 μg/dose; and rooster comb hyaluronic acid at dose levels of 5, 50, and 500 μg. Rabbit sera were tested before, during, and after immunization for passive cutaneous anaphylaxis (PCA) reactive antibodies in guinea pigs. No formation of PCA reactive antibodies against any of the 4 hyaluronic acid preparations was observed during or after immunization.

The effect of hyaluronic acid on antibody responses to egg albumen, dog albumin, and birch pollen proteins was tested.[160] Hyaluronic acid derived from rooster combs (10 mg/mL) was used. In the first experiment, 1 μg egg albumen in Freund's complete adjuvant was subcutaneously injected into hooded Lister and BNW/FU rats of both sexes. Three weeks later, egg albumen was injected in the solutions (1) coprecipitated with hyaluronic acid (n = 8), (2) adsorbed to $Al(OH)_3$ (n = 9), (3) admixed with hyaluronic acid (n = 9), and (4) denatured with ethanol (n = 8). Passive hemaglutination testing was used for estimation of antibodies. Egg albumen adsorbed to $Al(OH)_3$ and coprecipitated with hyaluronic acid produced comparable secondary antibody responses, which were stronger ($P < .001$) than those obtained by egg albumen admixed with hyaluronic acid or by ethanol denatured egg albumen. An enhanced secondary response was also obtained ($P < .01$) by coprecipitation of dog albumin and hyaluronic acid. Enhanced, although less pronounced, antibody responses were obtained by an admixture of hyaluronic acid to dog albumin. In the same experiment with birch pollen as antigen, there was no enhancement of hyaluronic acid compared with saline. Adsorption to $Al(OH)_3$ resulted in moderately enhanced secondary antibody responses in the experiments with birch pollen protein.

In a second experiment, the authors tested the effect of systemic hyaluronic acid on antibody response in the rats.[160] Hyaluronic acid (1.1 mg/kg) or saline was injected 1 or 3 days before the first injection of dog albumin or 1 day before a second antigen injections. Hyaluronic acid administered 1 to 3 days prior to the priming injection of dog albumin enhanced the secondary response markedly compared with that seen using dog albumin suspended in saline ($P < .01$), independent of the time of administration.

In a third experiment, hyaluronic acid was simultaneously injected with the first dose of dog albumin (0.044 mg/kg) at doses of 0.044 mg/kg, 0.44 mg/kg, and 4.440 mg/kg at separate skin sites.[160] A booster dose of dog albumin was given 14 days later without additional hyaluronic acid. The various doses of hyaluronic acid induced an enhanced immune response to dog albumin. This was independent of the hyaluronic dose level within the range of 0.044 to 4.44 mg/kg. The enhancing effect was discernible ($P < .05$) both in the primary and the secondary response. There were no reactions in a maximization test.[31] No details were provided.

Rabbits were used to investigate severe acute inflammatory reactions to Hylan G-F 20.[31] Three groups of rabbits (n = 4) were immunized subcutaneously at 4 separate sites per rabbit at weeks 0, 1, 4, 12, 18, and 24. They were immunized with either sodium hyaluronate, Hylan G-F 20, or a crude rooster comb preparation. Serum samples were collected before each immunization and before the rabbits were killed at week 29. A heterogeneous chicken comb protein preparation was prepared and purified. The resultant chicken protein preparation and a purchased purified hyaluronic acid were used as antigens to coat enzyme-linked immunoassay plates for a direct binding assay to detect antibodies. None of the preparations elicited a significant hyaluronic acid–specific antibody response. All 4 rabbits immunized with the positive control, crude rooster protein, and 3 of the 4 rabbits immunized with Hylan G-F 20 exhibited an anti-chicken protein response. Two of 4 of this group responded after only 3 injections, and the anti-chicken protein titer was sustained over a period of several weeks. The authors stated that the lower maximum optical density readings obtained with sera from the hylan-immunized rabbits, compared with those obtained with sera from the rabbits immunized with the crude rooster protein, probably represented reactivity to only a subset of proteins contained in the heterogeneous crude rooster protein. None of the rabbits immunized with sodium hyaluronate had a detectable response to chicken protein.[31]

Distributed for Comment Only -- Do Not Cite or Quote

Table 7. Treatment Groups of Guinea Pigs to Compare the Sensitization of Noncrosslinked (SupArtz) and Crosslinked (Synvisc) Hyaluronic Acid[161]

Group	Test Substance	Dosage (mg/kg)	Concentration (mg/mL)	Volume (mL/kg)
1	Synvisc	0.5	0.5	1
2	Synvisc	2.5	2.5	1
3	Synvisc + CFA[a]	2.5	2.5	2a
4	SupArtz	2.5	2.5	1
5	SupArtz + CFAa	2.5	2.5	2a
6	No injection	NA	NA	NA
7	Egg albumen + CFA	1 mg/animal	2	1 mL/animal

NA, not applicable.
[a] Equal amount of complete Freund's adjuvant (CFA) and test substance.

Hartley guinea pigs were used to compare the sensitization by noncrosslinked (SupArtz, Smith and Nephew, London, England) and crosslinked (Synvisc, Genzyme Corporation, Cambridge, MA) hyaluronic acid.[161] In the first part of the experiment, the guinea pigs (n = 8) were anesthetized with isoflurane, weighed, and subcutaneously injected with hyaluronic acid or egg albumin according to the doses in Table 7. Injections were administered on days 0, 7, and 14. Controls were not given injections. Sera were prepared from blood collected from the medial saphenous vein 12 days after the final sensitization injection. A skin allergy test was performed on the sensitized guinea pigs. Fourteen days after the last sensitizing injection, each abdomen was shaved and 2 injections of phosphate buffered saline (PBS) were given. This was followed by 2 injections of 0.1 mg hyaluronic acid sensitizing agent that the animal was sensitized to or 0.01 mg egg albumen if the animal was sensitized to egg albumen. The guinea pigs were observed at 3, 24, and 48 hours after the injections. Changes in skin conditions were measured and reactions recorded as follows: <1 mm, –; 1.0 to 5.0 mm, +; 5.1 to 10.0 mm, ++; 10.1 to 15.0 mm, +++; and >15.0 mm, ++++. At 3 hours, 7 of the 8 animals in the group treated with 0.5 mg/mL crosslinked (Synvisc) hyaluronic acid had reactions of ++ grade and 1 animal had a reaction of +. Six of the 8 animals treated with 2.5 mg/mL crosslinked (Synvisc) hyaluronic acid had reactions of ++ and 2 had a reaction of +. Three animals treated with 2.5 mg/mL noncrosslinked (SupArtz) hyaluronic acid had – grades, 3 with + grades, and 2 animals with ++ grade reactions. Six of the animals receiving 2.5 mg/mL noncrosslinked (SupArtz) hyaluronic acid + CFA had + grades, and 2 of these animals had ++ grade reactions. Two of the nonsensitized animals were treated with SupArtz and both had + grade reactions. At 24 hours, 6 of the animals treated with 0.5 mg/mL SupArtz had + grade reaction and 1 had a +++ grade reaction. Four of the animals treated with 2.5 mg/mL Synvisc had a + grade, 1 had a ++ grade, and 3 had – grade reactions. All 8 of the animals in the 2.5 mg/mL noncrosslinked (SupArtz) hyaluronic acid had – grade reactions. Seven of the animals in the group receiving 2.5 mg/mL noncrosslinked (SupArtz) hyaluronic acid + CFA had – grade reactions, and 1 had a + grade reaction. Of the animals treated with egg albumen, 6 had a ++ grade reaction, and 2 had a +++ grade reaction. Two control animals were treated with crosslinked (Synvisc) hyaluronic acid, and both had ++ grade reactions. Two other control animals treated with noncrosslinked (SupArtz) hyaluronic acid had – grade reactions. Forty-eight hours after the injections, 4 animals treated with 0.5 mg/mL noncrosslinked (SupArtz) hyaluronic acid had – grade reactions, 3 had a +, and 1 had a ++ grade reaction. Five animals treated with 2.5 mg/mL crosslinked (Synvisc) hyaluronic acid had + grade reactions, 1 had a –, and 2 had ++ grade reactions. Four of the animals treated with 2.5 mg/mL crosslinked (Synvisc) hyaluronic acid + CFA had ++ grade reactions, 2 had +, and 2 had – grade reactions. All of the SupArtz treated group had – grade reactions. Seven animals treated with egg albumen had +++ grade reactions, and 1 had a ++++ grade reaction. Two of the nonsensitized animals were treated with crosslinked (Synvisc) hyaluronic acid; 1 had a + grade reaction, and the other had a ++ grade reaction. Two nonsensitized animals treated with noncrosslinked (SupArtz) hyaluronic acid had – grade reactions. Eighteen days after the final sensitizing injection, the animals were anesthetized, weighed, and given an

Distributed for Comment Only -- Do Not Cite or Quote

intravenous injection into the medial saphenous veins of 3 mL/kg crosslinked (Synvisc) hyaluronic acid, 3 mL/kg noncrosslinked (SupArtz) hyaluronic acid, or 1 mL/kg egg albumen, matching the substance to what the animals were sensitized (without the CFA). The animals were observed for 3 hours for anaphylactic symptoms then observed again at 24 hours for additional symptoms.

Twenty addtional guinea pigs were used to perform an antibody titer on the sera from the sensitized animals.[161] The guinea pigs received 8 intradermal injections each: (1) 0.1 mL saline; (2) undiluted sera of a nonsensitized guinea pig; (3) and (4) sera in a 5-fold dilution; (5) and (6) sera in a 25-fold dilution; and (7) and (8) sera in a 125-fold dilution. Eighteen hours after the intradermal injections, the guinea pigs were anesthetized, weighed, and injected intravenously in the medial saphenous vein with 0.4 mL/kg Evans Blue (1% solution with saline) and 5 mg/kg crosslinked (Synvisc) hyaluronic acid for animals that received sera from Synvisc-sensitized animals or 5 mg/kg noncrosslinked (SupArtz) hyaluronic acid for animals that received sera from SupArtz-sensitized guinea pigs. Animals that received sera from egg albumen + CFA-sensitized animals received an intravenous injection of 0.4 mL/kg Evans Blue and 1 mg/animal egg albumen. Thirty minutes after the intravenous injections, the guinea pigs were killed, and the diameter of the blue spots on their skin was measured and photographed. The guinea pigs that received sera that were diluted 5-, 25-, and 125-fold also received the same intravenous injection. Hyaluronic acid IgG production in the guinea pig serum was determined by competitive ELISAs. Neither noncrosslinked (SupArtz) hyaluronic acid or crosslinked (Synvisc) hyaluronic acid caused a passive cutaneous anaphylactic response in guinea pigs at any concentration tested. The injected egg albumen did elicit a strong passive cutaneous anaphylactic response. Synvisc caused the production of higher numbers of hyaluronic acid–specific immunoglobins ($P = .0005$) when compared with the "native" form.

Ototoxicity

Twenty adult pigmented Sprague-Dawley rats were used to test the ototoxicity of hyaluronic acid.[162] Hearing tests were performed on the animals then the bulla tympanica was completely filled with 1.9% hyaluronic acid. This application was repeated twice at 2-day intervals. Five days after the last application, a new hearing test was performed. Fifteen of these animals were killed, and their middle ears were evaluated microscopically for the presence of hyaluronic acid. Hearing tests were given to the last 5 animals at 1 and 3 months after the last application. Five days after the last application of hyaluronic acid, various amounts of hyaluronic acid were observed in the middle ear. The round window niche was filled with viscous material. All perforations of the tympanic membrane were closed. One month after the application, there was some viscous material under the tympanic membrane. Two of the 5 middle ears examined still exhibited viscous material in the round window niche after 3 months. After application of hyaluronic acid, the auditory brainstem response thresholds dropped, but all thresholds had returned to normal 3 months after the last application. The authors concluded that hyaluronic acid was not ototoxic to the inner ear.

The toxicity of hyaluronic acid administered into the ear cavity of guinea pigs was examined.[163] Twenty (10 of each sex) young healthy albino Guinea pigs of the Duncin-Hartley strain, weighing between 450 and 800 g, were anesthetized. Each animal was shaved behind the right ear, and the tympanic bulla was opened by drilling a hole through its wall. The middle ear was filled with hyaluronic acid (average molecular weight of 3.4×10^6; 19 mg/mL), giving 200 to 300 µL as a single dose. The left ear was left intact. In a control group of another 20 animals, a similar experimental procedure was performed for access to the right middle ear, but nothing was administered in the tympanic bulla. The wounds were sutured, and after 14 days, the animals were killed by an overdose of pentothal sodium. In the control group, 18 animals had a slight amount of exudate in the right middle ear cavity, 1 had none, and 1 had a large quantity. In the test group, 3 animals had a small quantity of exudate, three had a large amount of colorless or yellow viscous exudate, and 14 had the right ear filled with viscous exudate. There was no exudate in any of the animal's left ears of either the test or control groups. There was no sign of missing outer hair cells caused by the hyaluronic acid except in one animal where the area of outer hair cell loss was greater in the treated ear. The authors concluded that hyaluronic acid administered into the middle ear of the guinea pig did not cause destruction of the cochlear sensory cells, strongly suggesting that it may not be harmful to administer hyaluronic acid in the middle ear of humans.

Distributed for Comment Only -- Do Not Cite or Quote

The hearing of 20 (10 of each sex) young, healthy albino Duncan-Hartley strain guinea pigs was tested while under anesthesia.[164] They weighed between 340 and 730 g. After half of the animals were tested, their middle ear was slowly filled from the bottom via a thin catheter with 200 to 300 μL of hyaluronic acid (average molecular weight of 3.4×10^6; 19 mg/mL). The hearing test was readministered immediately and again after 28 days. The other half, the control group, was also tested again after 28 days. The mean auditory thresholds shifted for the animals that were administered hyaluronic acid in their middle ear. Histologically, 3 of the control animals had a small amount of brownish material at the stapes and/or on the mucosal membrane of the right middle ear cavity, which was interpreted to a sequella of the surgery. Six of the test group animals had a small amount of brown viscous fluid in a recess in the right middle ear cavity. One of these also had a small amount of a similar fluid at the footplate of the stapes. Macroscopic examination of the middle ear cavity revealed that hyaluronic acid was almost completely eliminated from the cavity 28 days after its administration. Scanning electron microscopy revealed no morphological alterations of the hair cells that could be related to the hyaluronic acid.

Sodium hyaluronate was used as a lubricant in the implantation of Silastic silicone rubber (Dow Corning Corp, Midland, Michigan) electrodes with 4 platinum bands into the ears of cats.[72] One ear had one drop of sodium hyaluronate instilled through the round window, and a drop was spread on the probes before insertion. This was repeated in the other ear with sodium chloride solution as a control. There were no ill effects of the procedure or of sodium hyaluronate noted over the next 4 months. The effect of sodium hyaluronate on the hearing threshold was inconclusive in that 2 cats had higher hearing thresholds in the treatment ear, 3 cats had higher thresholds in the control ear, and no difference was detected in the sixth cat.

Neurotoxicity

The acute neurotoxicity of hyaluronic acid was tested on 20 New Zealand White male rabbits weighing between 2.0 and 3.0 kg.[165] An epidural injection of 0.2 mL/kg normal saline or hyaluronic acid (Hyruan, LG Life Sciences, Seoul, Korea) (10 mg/mL; molecular weight, 1100 kD; pH, 6.3-8.3) was administered (n = 10 per group). No signs of any motor or sensory change or any behavioral change were noted during the 3-week period after the epidural injection. One saline injected rabbit had decreased appetite, activity, and body weight. This animal also had wound inflammation at necropsy. The hyaluronic acid group had no pathological abnormalities by light microscopy, whereas 2 rabbits in the saline group had abnormal findings (localized or diffuse meningeal inflammation, focal inflammation, and degenerative myelopathy in the white matter). No structural changes of neurons or glial cells were detected by electron microscopy. The blood–brain barrier, nuclei of neurons, nucleoplasm, cytoplasm, astrocytes, oligodendrocytes, and basal lamina of capillaries were normal. The authors state that hyaluronic acid administered epidurally to rabbits was not found to cause any sensory-motor dysfunction, behavioral change, or neurotoxicity by either light or electron microscopy.

Reproductive and Developmental Toxicity

Rats

Several similar reproductive and developmental toxicity studies on hyaluronic acid and its salts were performed using rats. One study[166] is described in detail in this section and summarized in Table 8 with 10 other studies.

A multigenerational study of the effects of sodium hyaluronate derived from cockscombs on pregnant Sprague-Dawley rats and their offspring was performed.[166] In all cases, a 1% solution of hyaluronic acid in physiological saline solution was used. After weighing, male and female 12-week-old rats were housed together in pairs. From day 17 of pregnancy to day 20 after parturition, sodium hyaluronate was administered by subcutaneous injection in the back area to the dams at 7 mg/kg (0.7 mL/kg), 20 mg/kg (2 mL/kg), or 60 mg/kg (6 mL/kg). The control group was administered 6 mL/kg physiological saline solution (n = 21 or 22). Body weights were determined daily during pregnancy, and dams were examined on delivery day. Food consumption was measured on days 3, 6, 9, 12, 15, and 19 after parturition. Females nursed the pups for 21 days during which the pups were observed for abnormalities (physical and behavioral) and weighed 3 times per week. The dams were killed on day 21, bled, and dissected so to observe the organs. Implantation marks in the uteri were also counted to calculate the live

Distributed for Comment Only -- Do Not Cite or Quote

Table 8. Summary of Reproductive and Developmental Studies of Subcutaneously Injected Hyaluronic Acid Using Rats

Study	Source/ Treatment	Notes on F_0	Notes on F_1 – Notes on F_2	NOAEL
Furuhasi et al[166]	Rooster combs/subcutaneous injection from day 17 of pregnancy to day 20 after parturition	Unabsorbed residue of hyaluronic acid at injection sites; no effect on weight or food and water consumption; no effect on fertility	No neonatal abnormalities found[a]	60 mg/kg d^{-1}
Ota et al[281]	Rooster combs/subcutaenous injection to females from day 17 of pregnancy to day 21 after delivery	Unabsorbed residue of hyaluronic acid at injection sites; no effect on weight or food and water consumption; no effect on fertility; no effect found at necropsy after delivery	Neonatal development normal; unabsorbed residue of hyaluronic acid at injection sites; no effect on weight or food and water consumption; no effect on fertility; no effect found at necropsy after delivery; no neonatal abnormalities found	50 mL/kg d^{-1}
Tanaka et al[282]	Bacteria/subcutaneous injection 2 weeks before mating and 1 week after mating	Unabsorbed residue of hyaluronic acid at injection sites; no effect on weight or food and water consumption; no effect on fertility; no effect found at necropsy after delivery	No neonatal abnormalities found[a]	50 mL/kg d^{-1}
Tanaka et al[283]	Bacteria/subcutaneous injection to females on days 7 to 17 of pregnancy	Unabsorbed residue of hyaluronic acid at injection sites; no effect on weight or food and water consumption; no effect on fertility; no effect found at necropsy after delivery	Neonatal development normal; unabsorbed residue of hyaluronic acid at injection sites; no effect on weight or food and water consumption; no effect on fertility; no effect found at necropsy after delivery; no neonatal abnormalities found	50 mL/kg d^{-1}
Ono et al[284]	Rooster combs/subcutaneous injection to females from day 7 to day 17 of pregnancy	Unabsorbed residue of hyaluronic acid at injection sites; no effect on weight or food and water consumption; no effect on fertility; no effect found at necropsy after delivery	Neonatal development normal; unabsorbed residue of hyaluronic acid at injection sites; no effect on weight or food and water consumption; no effect on fertility; no effect found at necropsy after delivery[a]	50 mL/kg d^{-1}
Ono et al[285]	Rooster combs/subcutaneous injection to females 9 weeks prior to copulation and first week of primary copulation	Unabsorbed residue of hyaluronic acid at injection sites; no effect on weight or food and water consumption; no effect on fertility; no effect found at necropsy after delivery	No neonatal abnormalities found[a]	50 mL/kg d^{-1}
Ono et al[286]	Rooster combs/subcutaneous injection to females from day 17 of pregnancy to day 21 after delivery	Unabsorbed residue of hyaluronic acid at injection sites; no effect on weight or food and water consumption; no effect on fertility; no effect found at necropsy after delivery	Neonatal development normal; reproductive ability normal; neonatal development normal	50 mL/kg d^{-1}
Matsuura et al[287]	Bacteria/intraperitoneal injection to females day 7 to day 17 of pregnancy	Unabsorbed residue of hyaluronic acid at injection sites; no effect on weight or food and water consumption; no effect on fertility; no effect found at necropsy after delivery	No neonatal abnormalities found; neonatal development normal	64 mg/kg d^{-1}
Matsuura et al[288]	Bacteria/intraperitoneal injection to females day 7 to day 17 of pregnancy	Unabsorbed residue of hyaluronic acid at injection sites; no effect on weight or food and water consumption; no effect on fertility; no effect found at necropsy after delivery	No neonatal abnormalities found; reproduction ability normal; neonatal development normal	64 mg/kg d^{-1}

(continued)

Distributed for Comment Only -- Do Not Cite or Quote

Table 8. (continued)

Study	Source/ Treatment	Notes on F_0	Notes on F_1 – Notes on F_2	NOAEL
Hattori et al[289]	Not stated/daily subcutaneous injection to males and females for 8 weeks during 2 weeks of mating	Unabsorbed residue of hyaluronic acid at injection sites; no effect on weight or food and water consumption; no effect on fertility; no effect found at necropsy after delivery	No neonatal abnormalities found; reproduction ability normal[a]	40 mg/kg d^{-1}
Kumada et al[290]	Not stated/subcutaneous injection to females from day 7 to day 17 of pregnancy	Unabsorbed residue of hyaluronic acid at injection sites; no effect on weight or food and water consumption; no effect on fertility; no effect found at necropsy after delivery	Neonatal development normal; unabsorbed residue of hyaluronic acid at injection sites; no effect on weight or food and water consumption; no effect on fertility; no effect found at necropsy after delivery; no neonatal abnormalities found	40 mg/kg d^{-1}

[a] F_2 observation not reported.

Distributed for Comment Only -- Do Not Cite or Quote

birth index. The newborn survivor and morbidity indexes were determined at hour 16 for the F_1 generation. Weight, sex, and malformations were noted. On day 4, the F_1 newborn pups were divided into litters of 10 (5 male, 5 female). Individual body weights were measured on days 4, 7, 14, and 21. Morbidities and general symptoms were recorded every day. Other measurements included auricle separations (days 2, 3, and 4), fur growth (days 8 through 12), dentition (days 10 through 14), and opening of eyes (days 16 and 17).

During week 3, the pups culled from the litters were killed and treated with 70% ethanol and their skeletal structures examined. On day 21, the F_1 generation was subjected to motor function test (corneal reflex, righting reflex, and avoidance reaction). Three males and 3 females from each group were killed and dissected. The remaining pups were weighed weekly and kept until 10 weeks of age. Sexual development was noted at week 3 and 4 for males and weeks 5 and 6 for females. A motor test (rotating rod, vertical, and diagonal board) was performed at week 4. At week 7, the electrical shock avoidance aptitude test was administered. At week 10, the remaining pups were mated.

Two-thirds of the dams were killed on the 20th day of pregnancy, weighed, measured, and dissected. The number of surviving fetuses was counted. The remaining third of the dams was allowed to give birth. The litters were grouped into 10 each as before. After 21 days of nursing, the live birth index, viability, and nursing indexes were determined.

There was no morbidity among the control or experimental dams. The weights of the dams in the 60 mg/kg group were higher than the control group, and the relative weights of the heart and lungs were lower on the 20th day of pregnancy. The 7 mg/kg group increased food consumption on day 4 after delivery. There were no other macroscopic differences observed during pregnancy or nursing. A gelatinous residue at the site where the 60 mg/kg injections were made was noted histologically. Nodular hyperplasia of reticular zone cells was present in the adrenal gland for 1 of 3 of the 7 mg/kg group, for 2 of 3 in the 20 mg/kg group, and 3 of 3 in the 60 mg/kg group. The severity of adrenal gland effects ranged from slightly sporadic and reduced nodular foci (most cases) to one case of severe pervasive nodular foci in the 60 mg/kg group. No other abnormalities were found.

There were no differences between treated and controls for the number of implantations, mean gestation length, number of newborns, sex ratio, live birth index, viability index, or external malformations compared with controls. There were no differences in the timing of separation of auricle, appearance of abdominal hair, odontiasis, eye opening, descent of testes, or vaginal opening in treated when compared with controls.

There was no difference in the change of body weight through day 70 for male and female F_1 pups compared with controls. There were no differences in absolute or relative organ weights at day 21 compared with controls. The relative weight of the epididymis in the male 7.0 mg/kg group was lower than the control group at day 70 ($P < .05$). At 70 days, the thymus and uterus weights of the 7.0 mg/kg group were less than the control group ($P < .05$); the relative weight of the bled carcass was less than control ($P < .05$), and the relative and absolute weights of the ovaries were less than the control group ($P < .05$).

The skeletal examinations of the F_1 pups showed no malformations nor differences between the groups with dams administered sodium hyaluronate and the control group. The motor function tests of day 21 showed no abnormalities. Balance on the diagonal board was maintained at a steeper angle for all the male experimental groups and the female 20.0 mg/kg group, compared with the control group ($P < .05$). Of the F_1 offspring, there was no difference in the copulation and fertility index between treated and control animals.

When examining the F_1 generation and their fetuses (F_2), the authors reported no difference in the number of corpora lutea, number of implantations, number of live fetuses, percentage of resorptions, percentage of macerated fetuses, percentage of dead fetuses, fetus body length or weight, or adhesion of placenta compared to controls. The 7.0 mg/kg group had a lower male/female sex ratio than the control, and the female placenta weight was higher ($P < .05$). The 60.0 mg/kg group had a longer male tail length ($P < .05$). When comparing the newborns of the F_2 generation, the authors stated that the mean gestation length was longer for all treatment groups. There were no differences in the number of implantations, number of newborns, sex ratio, live birth index, viability index, lactation index, body weight at birth on day 21, or external malformations compared with controls. In the postnatal development of the F_2 generation, there were a higher number of pups that had separation of auricle on day 3 in the 7.0 mg/kg and the 20.0 mg/kg groups when compared with the control group ($P < .05$); there

Distributed for Comment Only -- Do Not Cite or Quote

were no differences on day 2 or 4 for these 2 groups and no difference at all for the 60.0 mg/kg group compared with the control group. There were a higher number of pups with the appearance of abdominal hair on day 10 in the 20.0 mg/kg group ($P < .05$) compared with the control group but not with any other group or on any other day. There were more pups with odontiasis in the 20.0 mg/kg group on day 11 when compared with the control group ($P < .05$); there was no difference on any other day for all 3 groups. On day 17, a higher number of pups had opened their eyes in the 60.0 mg/kg group when compared with the control ($P < .05$). There were no differences in the other groups. The authors concluded that there were no adverse prenatal or postnatal effects due to hyaluronic acid in rats; the NOAEL was reported to be 60 mg/kg. As noted in Table 8, there was a slightly higher NOAEL in rats of 64 mg/kg d^{-1} and a lower NOAEL in rats of 40 mg/kg d^{-1}.

Rabbits

A reproductive and developmental toxicity study was conducted using KBL: Japanese white rabbits to determine the effects of SL-1010 sodium hyaluronate (average molecular weight of 1.78 million, concentration not reported) in isotonic sodium chloride solution.[167] After 3 weeks of acclimation when the females were 4 months old and the males were over 5 months old, the nulliparous females and males were mated. When a pair had been observed to mate twice, that day was considered day 0 of pregnancy. Four groups of pregnant rabbits (n = 13 or 14) were grouped in a stratified random sampling method by body weight based on the weights on day 0 of pregnancy. Sodium hyaluronic acid solution, 0.5, 15, and 50 mg/kg d^{-1}, was subcutaneously administered to the dams once per day from day 6 to day 18 of pregnancy. The locations of the injections were rotated between 6 sites: the left and right sides of the neck, chest, and lumbar. The doses were control (isotonic sodium chloride solution), 5 mg/kg d^{-1}, 15 mg/kg d^{-1}, and 50 mg/kg d^{-1}. The dams were observed for general condition and health before and after each injection. Body weight was measured on day 0, days 6 to 19, day 23, and day 28 of pregnancy. Food consumption was measured every other day from day 1. The dams were killed on day 28 of pregnancy. The uteri and ovaries were removed and the main organs observed macroscopically. The corpora lutea were counted. The number of implantations, dead embryos and fetuses, and viable fetuses were recorded. Body weight and placental weight of the live fetuses were measured. Then they were sexed and examined for external anomalies and intrathoracic and interperitoneal abnomalities. The intrathroracic and interperitoneal organs of the fetuses were fixed, stained, and microscopically examined for anomalies, including dilation of the ventricle system. The skeletons were stained and examined for skeletal anomalies or variations. The number of sacral and caudal vertebra with ossification was counted as an indicator of progression of ossification. There were no observed changes in health or general condition of the treated dams during the pregnancy period. No miscarriages were observed. There was no body weight difference between the control group and the 5 mL/kg d^{-1} group throughout the pregnancy. From day 15 to day 17, the mean weight of the 15 mL/kg d^{-1} group was higher than the control group ($P < .05$). The mean body weight gain for day 6 to day 19 was also more than the control group ($P < .05$). There was no difference in mean body weights for this group for the remainder of the pregnancy period. On day 12, the mean body weights of the 50 mL/kg d^{-1} group was higher than the control group ($P < .05$); this continued through day 19 ($P < .01$). The weight gain for day 6 to day 19 was higher ($P < .01$) and lower from day 19 to day 28 ($P < .01$). There were no differences between the treated and control groups during the remainder of the pregnancy period. The increased body weight was attributed to the unabsorbed sodium hyaluronate accumulating in each dam; there were no differences in food consumption for the pregnancy period. There were no differences in any of the measured reproduction parameters or external anomalies in their fetuses. There were no differences in skeletal abnormalities found. There were no differences in the visceral observations of fetuses.

A developmental toxicity study was performed of sodium hyaluronate using New Zealand White rabbits.[168] The rabbits were grouped into control, 8 mg/kg d^{-1}, 20 mg/kg d^{-1}, and 50 mg/kg d^{-1} and mated (n = 16 of each sex). In all groups but the 50 mg/kg d^{-1} group, 13 females became pregnant. In the highest dose group, 15 females became pregnant. The injections were made on the 6th through the 18th day of gestation. Body weights of the dams were measured on day 0, 6, 7, 8, 9, 12, 15, 24, and 29 of gestation. Food intake was measured every day of pregnancy. The dams were killed on the 29th day

Distributed for Comment Only -- Do Not Cite or Quote

of gestation with pentobarbital sodium and necropsied. The ovaries and the uterus were removed from each animal, and the number of corpora lutea, implantations, live fetuses, and dead fetuses were counted. Body weight and placental weight of the fetuses were measured. The live fetuses were sexed and observed for external and visceral abnormalities. Skeletal specimens were processed and examined for abnormalities. There were no deaths and no changes in the general condition of the animals. In the 20 mg/kg d^{-1} and the 50 mg/kg d^{-1} groups, there was protrusion around the periphery of the injection site containing a gelatinous/foamy material retention, which the authors interpreted to be unabsorbed sodium hyaluronate solution. The 50 mg/kg d^{-1} group had increased body weights on the 15th through the 24th day of pregnancy compared with controls, which the authors suggested was related to retention of sodium hyaluronate, but was not an indication of toxicity. This was also true of the 19th day of pregnancy for the 8 mg/kg d^{-1} group. The 50 mg/kg d^{-1} group had increased placental weights compared with controls. Otherwise, there were no differences observed between the experimental groups and the control group. There were occasional external, visceral, and skeletal anomalies in each group, but there was no statistical pattern, and they were thought to be due to spontaneous generation by the authors. The NOAEL was 50 mg/kg d^{-1}, the highest dose tested.

The effects of HMW sodium hyaluronate (NRD101; molecular weight, 1.9 million) were studied on organogenesis using Japanese white rabbits (SPF).[169] After 4 weeks of acclimation, 12-week-old males were paired with 25- to 26-week-old females or 56- to 57-week-old females (the 2 age groups were not separated in the results of this experiment). The pregnant females were placed into 1 of 4 groups (n = 17) and housed individually. Based on a subacute toxicity test on rats, the test dosages were set at 40 mg/kg d^{-1}, 20 mg/kg d^{-1}, and 10 mg/kg d^{-1}, delivered subcutaneously on days 6 to 18 of pregnancy. The general conditions of the dams were observed daily. Body weights were measured on days 6 to 18 and on days 20, 22, 24, 26, and 28 of pregnancy. Food consumption was measured on day 2 of pregnancy before treatment and on days 6 to 18, 20, 22, 24, 26, and 28 of pregnancy. The dams were killed, exsanguinated, and necropsied. The brain, pituitary, thymus gland, lungs, heart, liver, spleen, adrenal gland, kidneys, uterus, and ovaries were weighed. The uterus and ovaries were removed from each animal. The corpea lutea, absorbed embryos, dead fetuses, and surviving fetuses were counted. The surviving fetuses were examined for external abnormalities, and body weights and weights of placenta were measured. One-third of the fetuses were fixed and necropsied. The remainder was used for skeletal examination. There was no maternal toxicity in any treatment group. There were no body weight or food consumption differences that could be attributed to the sodium hyaluronate, and none of the measurements taken at necropsy demonstrated an adverse effect. There were no teratological effects demonstrated. Sodium hyaluronate did not affect survival, sex ratios, or any of the other parameters examined in the fetuses.

Genotoxicity

Hyaluronic acid produced by the bacterial fermentation method was inactive in mutagenicity tests. No details were provided.[31]

A reverse mutagenicity test of bacterial sodium hyaluronate (1%) on *Salmonella typhimurium* (TA98, TA100, TA1535, and TA1537) and *Escherichia coli* (WP2uvr A) was performed.[170] The preincubation method was used with and without metabolic activation (S9 derived from phenobarbital and 5,6-benzoflavone induced Sprague-Dawley rat livers). Sodium hyaluronate was tested at 31.5 µg/plate, 62.5 µg/plate, 125 µg/plate, 250 µg/plate, 500 µg/plate, and 1000 µg/plate at 2 plates/test concentration. The positive controls were 2-(2-furyl)-3-(5-nitro-2-furyl)-acrylamide, N-ethyl-N'-nitro-N-nitrosoguanidine, and 1,3-propanediamine-N-(2-chloroethyl)-N'-(6-chloro-2-methoxy-9-acridinyl)-dihydrochrolide in the absence of S9 Mix and 2-aminoanthracene in the presence of S9 Mix. The negative control was purified water. There was no increase in the mutation frequency in any test strain at any concentration.

The genotoxicity of sodium hyaluronate was in an in vivo micronucleus test using CD-1 (ICR) male mice (8 weeks of age).[171] The test solution of sodium hyaluronate (molecular weight of 2.4 million) was 1% in phosphate buffer. Phosphate buffer was used as the negative control, and mitomycin C was used as the positive control. The experimental groups were injected twice into the abdominal cavity (24 hours apart) with 0, 75 mg/kg (7.5 mL/kg), 150 mg/kg (15.0 mL/kg), or 300 mg/kg (30 mL/kg)

Distributed for Comment Only -- Do Not Cite or Quote

of the sodium hyaluronate solution. The positive control group received 10 mL/kg mitomycin C in distilled water injected once. Bone marrow cells were sampled from 5 mice from each treatment group 24 hours, 48 hours, and 72 hours after the last injection. Mice were killed by cervical dislocation and marrow extracted from the thigh bone using fetal calf serum. The samples were fixed and stained. The number of blood cells with micronuclei among 1000 polychromatic erythrocytes per subject and the number of polychromatic erythrocytes in 1000 blood cells were counted. No signs of toxicity were noted while the mice were alive, and there were no deaths during the treatment period. The positive control produced an increase in micronuclei. There was no difference found between the treatment groups and the control groups in regard to the number of polychromatic erythrocytes.

A reverse mutation test using 1% sodium hyaluronate in phosphoric acid (2 lots with molecular weights averaging 2.12 million and 2 million) was conducted.[172] Histidine-requiring strains of *S typhimurium* (TA98, TA100, TA1535, and TA1537) and 5 tryptophan-requiring strains of *E coli* WP2*uvr*A were used. S9, fractionated from rodent livers, was used for metabolic activation. Hyaluronic acid doses were 31.3 μg/plate, 62.5 μg/plate, 125 μg/plate, 250 μg/plate, 500 μg/plate, and 1000 μg/plate. Isotonic sodium chloride solution was used as the negative control. The positive control for TA98 and TA100 was 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide; *N*-ethyl-*N*'-nitro-*N*-nitrosoguanidine was used for the positive control for TA1535 and WP2*uvr*A, and 9-aminoacridine was used for TA1537. The tests were run for both 24 hours and 48 hours. There was no difference in the number of reverse mutations between any of the test groups and the negative controls. These authors also performed an in vitro chromosomal aberration test using cultured Chinese hamster fibroblast cells with and without metabolic activation. The dosages were 250 μg/mL, 500 μg/mL, and 1000 μg/mL of 1% sodium hyaluronate. Isotonic sodium chloride solution was used as the negative control, and 5 μg/mL of *N*-ethyl-*N*'-nitro-*N*-nitrosoguanidine and 20 μg/mL of benzo[a]pyrene were used as positive controls. Petri dishes received 5 mL of medium in which 4×10^3/mL cells were dispersed. On the third day, the test substances, positive controls, or negative controls were added. Incubation continued for either 24 hours or 48 hours. Positive control groups produced expected results, but there was no difference in chromosomal aberrations between treatment and negative control groups.

Multiple mutagenicity tests were performed on HMW sodium hyaluronate (trade name NRD101; molecular weight, 1.9 million).[173] An Ames test used *S aureus* strains TA100, TA98, TA1535, and TA1537 and *E coli*. Sodium hyaluronate was provided by the manufacturer (0.25%, 0.5%, and 1% aqueous solutions) and diluted with distilled water to yield 312.5, 625, 1250, 2500, and 5000 μg/plate. Distilled water was the negative control. The positive controls were 2-aminoantracene, 9-aminoacridine hydrochloride, sodium azide, and N-ethyl-N'-nitro-N-nitrosoguanidine disolved in dimethylsulfoxide. The test solutions were tested with and without metabolic activation. Positive control groups produced expected results, but there were no differences in the number of reverse mutation colonies between any test culture and the negative control.

In an in vitro chromosomal aberration test using Chinese hamster lung fibroblasts, sodium hyaluronate was tested at 62.5 μg/mL, 125 μg/mL, 250 μg/mL, 500 μg/mL, and 1000 μg/mL for a treatment time of 24 and 48 hours with and without metabolic activation.[173] The controls were the same as above. Two hundred metaphase cells were observed for each slide for gaps, breaks, exchanges, and other chromosomal abnormalities. Positive control groups produced expected results, but there were no differences between the treatment groups and the negative controls.

Sodium hyaluronate was tested in a micronucleus assay using 8-week-old ICR (Crj: CD-1) mice, treated with 90, 180, and 360 mg/kg once a day for 1 day or for 4 consecutive days.[173] Bone marrow specimens were collected 24, 48, and 72 hours after the final injection or the single injection after killing the mice by cervical dislocation. The number of polychromatic erythrocytes with micronuclei among 1000 erythrocytes was counted. There were no differences between any of the treatment groups and the negative controls.

Carcinogenicity

Tumor Cells

McBride and Bard used several types of cultured fibrosarcoma cells, lymphoblastoid cells, mammary carcinoma cells, VERO and BHK-21 cells, and

Distributed for Comment Only -- Do Not Cite or Quote

Table 9. Metatstic Potential of HA-L and HA-H Cells Following Intravenous Injection of B16-F1 Mouse Melanoma Cell Lines Into C57BL/6 Mice[176]

Cell Type	No. of Cells Injected($\times 10^5$)	Lung Nodules/Lung	
		Mean $\pm$ SD	Range
HA-L	2.5	3 $\pm$ 1.6	0-5
	10	115 $\pm$ 35	80-150
HA-H	2.5	172 $\pm$ 66.5[a]	90->200
	10	>200[b]	>200

[a] $P < .01$ compared with HA-L.
[b] $P < .05$ compared with HA-L.

fibroblasts from 14-day-old mouse embryos and human skin to study the hyaluronic acid halo produced by these cells.[174] Cells of each type were placed on cover slips or in 96-well microtiter plates at a density of 10^4 cells/cm^2. C2Hf/Bu fibrosarcoma (FSA) cells displayed translucent halos ranging in diameter up to 17 μm, averaging 8.8 μm. The halos were not penetrated by spleen cells added to the culture. Motile cells had very prominent barriers around their trailing edge, but the halo tended to be much less pronounced at their leading edge. The exclusion phenomenon was seen with particles other than normal spleen cells. Halos also excluded immune spleen cells, lymph node cells, thymocytes, normal peritoneal exudate cells, and erythrocytes. When fixed with 1% formal saline or 2.5% glutaraldehyde, the halos remained. When dehydrated, the halos were no longer visible.

The same results were achieved with 8 types of FSA cells from mice; 3 types of mammary carcinoma cells from mice; adenocarcinomas 1, IM, and 2 from mice and rats; 3T3 G and 3T3 S cells from mice, BHK-21 hamster cells; mouse embryo fibroblasts and adult human skin fibroblasts.[174] The exception was the 7 lymphoblastoid cells lines tested. Vero cells also appeared not to have the protective halo. The authors note that halos <2 μm could not be detected. When bovine or ovine testicular hyaluronidase (10 IU/mL) was introduced, the barrier was removed, and the spleen cells could approach the cell membrane. This was also the result with fungal (0.1-10 IU/mL) hyaluronidase. After removal of the enzyme, the cells regenerated their ability to repel spleen cells within 2 hours.

The production of hyaluronic acid by 3 human malignant mesothelioma cell lines (Mero-25, Mero-14, Mero-82) and 9 primary human mesothelial cell types were measured.[175] The mesothelioma cell lines produced small amounts of hyaluronic acid (<0.1 μg/10^6 cells/48 hours) compared with mesothelial cells (10 to 72 μg/10^6 cells/48 hours). When placed in conditioned media from the mesothelioma cells, fibroblast and mesothelial cell production of hyaluronic acid increased; a concentration of 50% of 10-fold concentrated conditioned medium in relation to culture medium (v/v) induced a near maximal effect in mesothelial cells and 70% of the maximal effect in fibroblasts.

The metastatic potential of tumor cells expressing different levels of cell surface hyaluronic acid were examined.[176] Flow cytometry was used to isolate B16-F1 mouse melanoma cell lines expressing either high (HA-H) or low (HA-L) hyaluronic acid on their surfaces. HA-H had approximately 32 times more cell surface hyaluronic acid than HA-L cells. After removal of hyaluronic acid from the cell surfaces with testicular hyaluronidase, it was found that HA-H produced hyaluronic acid approximately 15 times faster than HA-L; hyaluronic acid levels were restored within 20 hours in both cell lines. The 2 cell lines had similar growth rates in vitro; when injected into syngeneic mice, the 2 cell lines gave rise to tumors that grew at similar rates. HA-H or HA-L cells were injected into the tail veins of syngeneic C57BL/6 mice (2.5×10^5 or 1×10^6 cells /mouse; n = 10; experiment was run 3 times). Mice were killed 14 days later, and the lungs were fixed. Visible lung tumor nodules were assessed under a dissecting microscope. Another set of mice were allowed to live to determine the mortality from HA-H and HA-L. The HA-H cells caused a greater number of lung metastases that were larger than the HA-L cells as shown in Table 9. In general, the nodules of the HA-L cells were smaller (0.1-0.5 mm) than the HA-H cells (0.1-8 mm). The mortality rate was 50% at day 25 and day 32 for the HA-H and HA-L cells, respectively.

Distributed for Comment Only -- Do Not Cite or Quote

Human mesothelioma cells (Mero-25) with and without transfected HAS2 gene (the gene controlling the ability to produce hyaluronic acid) were compared.[177] The cells were plated (5×10^4 cell/dish), incubated for 24 hours, and then overlaid with 1×10^7 formalin-fixed erythrocytes. The cells were observed through an inverted microscope and the size of the hyaluronic acid halos measured. Halos around the HAS2 transfected cells were larger than the halos around the unmodified cells ($P < .05$). The HAS2 transfected cells produced 59.6, 103.8, and 187.2 ng/10^4 cells/24 hours hyaluronic acid compared with 14 ng/10^4 cells/24 hours from unmodified cells. The peaks in the size range of hyaluronic acid was 3.9×10^6 kD. Proliferation of both types of cells was compared by counting the cell number at 1, 3, 6, and 9 days after subculturing. The modified cells had about a 2-fold higher proliferative capacity than the unmodified cells.

These authors analyzed the cell cycle profiles by placing both types of cells in culture in starvation medium, centrifuged, and subjected to DNA content analysis and cell cycle kinetics using a flow cytometer for cell cycle analysis.[177] After serum stimulation, HAS2-transfected cells had a higher proportion of cells in S phase (26.6%) compared with unmodified cells (18.9%). The cells were grown in soft agar for 4 weeks, and colonies larger than 0.5 arbitrary units were counted by microscope and their area measured for a growth assay. The hyaluronic acid–producing cells formed 2- to 3-fold larger colonies in soft agar than unmodified cells. The shape of the colonies formed by the modified cells was more irregular than unmodified cells. Increased synthesis of hyaluronic acid was found to lead to an increased proliferation rate and to anchorage-independent growth in soft agar.

The HAS2 gene was inserted into a murine astrocytoma cell line (SMA560) using the murine mHAS2/pCIneo plasmid.[178] SMA560 cells and HAS2-modified SMA560 cells (3 strains) were suspended in PBS (10^6 cells/100 μL) and injected subcutaneously into the flanks of syngeneic VM/Dk mice (n = 10). When a tumor became palpable, it was measured in 3 dimensions using calipers. When the tumors reached 1 cm^3, the mice were killed and the tumors excised and frozen in embedding medium. Overexpression of HAS2 caused a reduction in tumor growth rate; the onset of tumor formation was similar to unmodified cells. One strain of modified cells formed cysts with few tumor cells surrounding necrotic tissue, while the other 2 strains formed distinct tumors that were histologically similar to the controls.

The authors injected each of the above cell lines (10^5 cells in 10 μL PBS) into the caudate nucleus of syngeneic mice (n = 10) to induce intracranial tumors.[178] The mice were killed when they became moribund or after 1 month; the brains were excised and frozen in embedding medium. The tumors and brains were sectioned for microscopic examination. The experiment was repeated and extended to 2 months. Unmodified SMA560 cells formed large intracranial tumors within 18 days. The mice injected with the modified cells did not result in any obvious disease within 8 weeks. Upon examination, it was observed that individual tumor cells were observed at the injection site but no tumors. Northern blot analysis was performed on human glioma cell lines (D54, D270, D645, U373 MG, and U251 MG). It was observed that U251 MG and D270 expressed HAS2 at high levels; U373 MG, U87 MG, and D645 expressed HAS2 at lower levels; and D54 cell did not express HAS2 at all. The size of the hyaluronic acid halo, in general, corresponded with the level of HAS2 gene expression in D270 cells. However, U251 MG cells had only a small hyaluronic acid halo.

Balb/c nu/nu male mice or A/Jax mice were used to test the effects of hyaluronic acid on the growth of injected LX-1 human lung carcinoma cells or TA3/St cells.[179] ALZET osmotic pumps were inserted under the skin in the dorsal region of the mice loaded with either PBS or hyaluronic acid (approximate molecular weight, 2.5×10^3; n = 5). Pumps delivered the treatments at approximately 0.5 μg/0.05 μL/h. On the day after insertion, 0.5 to 1.0×10^6 of LX-1 or TA3/St tumor cells (in PBS) were injected in front of the pump. After 7 or 14 days of treatment, the mice were killed and the tumor's growth was measured by weight. Hyaluronic acid inhibited LX-1 tumor growth by approximately 50% to 80% and TA3/St tumor growth by approximately 60% to 65%. In additional experiments, animals were injected with LX-1 cells and were untreated for 7 days. They were then treated with hyaluronic acid for 14 days. This experiment was run twice. The growth inhibition was between 40% and 75%. When the treatment regime was reversed, treatment for 14 days and no treatment for 7 days, inhibition was 68%. When treated for 7 days followed by no treatment for 14 days, inhibition was 52%. In a soft agar

Distributed for Comment Only -- Do Not Cite or Quote

Table 10. Growth Characteristics of the Transplantable Tumors[180]

Transplantable Clone	n	Tumor Grafts		
		Weeks of Growth	Wet Weight (g)	Tumor Growth[a]
Mock	4	14.0 ± 0.8	4.2 ± 0.6	0.30
Has2-b	4	16.5 ± 2.0	4.2 ± 0.7	0.25
Has2-d	11	7.9 ± 0.5	3.4 ± 0.4	0.43
Hyal1-f	3	17.3 ± 3.0	2.5 ± 1.0	0.14
Hyal1-h	4	16.5 ± 2.5	2.6 ± 0.3	0.16

[a] Wet weight/weeks of growth.

assay, inclusion of 100 μg/mL hyaluronic acid in the agar inhibited colony formation by LX-1 human lung carcinoma, HCT116 human colon carcinoma, and TA3/St murine mammary carcinoma cells by 80%, 68%, and 72%, respectively.

The effects of inserting hyaluronan synthase 2 (Has2) and hyaluronidase 1 (Has1) genes into non-hyaluronic acid–producing rat colon carcinoma cells (PROb, a subclone of the cell line DHD-K12) were tested.[180] The genes for the synthase and the hyaluronidase were inserted separately into the cancer cells using pCl-neo producing the Has2-b, Has2-d, Hyal1-f, and Hal1-h cell lines. Has2-b and Has2-d synthesized about 2.5 and 12 μg hyaluronic acid per 1×10^6 cells/24 hours; Hyal1-f and Hal1-h exhibited hyaluronidase activity of about 360 and 220 mU/mL, respectively. No hyaluronic acid or hyaluronidase activity was detected in the wild-type PROb cells or in mock transfected cells. Transfected cells (5×10^6 cells in 50 μL PBS) were injected subcutaneously into the right shoulder of BD-IX rats. The subsequent tumors were measured with a caliper and allowed to grow to approximately 1 cm in diameter. The rats were killed and tumors were excised, cleaned of nontumor tissue, and the wet weight was determined. The tumors were then snap-frozen in liquid nitrogen. Tumor growth was determined by dividing the wet weight by the number of weeks of growth. Tumor production rates were different for each group as shown in Table 10. The tumors of mock transfected cells grew to 4.2 g in 14 weeks, similar to the low hyaluronic acid–producing Has2-b–derived tumors. The higher hyaluronic acid–producing Has2-d tumors reached 3.4 g in 8 weeks. The growth rates of the Hyal1-f and Hyal-h cells were lower compared with tumors of mock transfected cells; a wet weight of 2.5 g was reached after 17 weeks. The growth rate of tumors from the Has2-d transfectants was higher (P = .038), and the growth rate of tumors from Hyal1-f and Hyal1-h transfectants was slower (P = .029) compared with mock transfectants. The authors stated that the expression of Has2 enhanced tumor growth, whereas expression of Hyal1 delayed tumor development. The amount of hyaluronic acid in the tumors was determined by microtiter-based assay. Tumors from the high hyaluronic acid–producing clone contained an average of 150% more extractable hyaluronic acid compared with tumors derived from mock and Hyal1 transfectants (P = .006). The mean vessel area, boundary length, and diameter did not differ among the tumor types examined, leading the authors to suggest a similar vascular phenotype. The authors concluded that Has2 overexpression suppresses vascularization of the viable tumor fraction.

The effects of the 3 different genes controlling hyaluronic acid synthesis on tumor cell metastasis were tested.[181] Aneuploid human breast adnocarcinoma cell line MDA-MB-231 was selected based on its production of HAS2. Nontransformed rat cells were transfected with HAS1, 2, and 3. In the MDA-MB-231 cell line, overexpression of each HAS isoform promoted the formation of a hyaluronic acid coat where HAS2 produced a larger matrix than HAS1 or HAS3. These authors investigated the importance of HAS2 expression in highly invasive breast cancer (MDA-MB-231) by characterization of the antisense inhibition of HAS2 (ASHAS2). ASHAS2 resulted in a 24-hour lag in proliferation that was concomitant to transient arrest of 79% of the cell population in G_0 to G_1. ASHAS2 did not alter the expression of the other HAS isoforms, whereas hyaluronidase and the hyaluronic acid receptor, CD44, were downregulated. The antisense inhibition of HAS2 cells accumulated greater amounts of HMW hyaluronic acid (>10 000 kDa) in the culture medium, whereas mock and parental cells liberated less hyaluronic acid of 3 distinct molecular weights (100, 400, and 3000 kDa).

Distributed for Comment Only -- Do Not Cite or Quote

Five-week-old CBA nude mice were used to generate parental, mock, and antisense inhibition of HAS2 tumors.[181] The tumor cells were harvested in the logarithmic growth phase by scraping, resuspended (final concentration, 2×10^6), and injected into the mammary fat pad of the same mice. Tumor growth was measured twice weekly. After 84 days, the mice were killed and the primary tumor, liver, kidneys, brain, and lungs removed at necropsy and examined. Mice inoculated with parental or mock-transfected MDA-MD-231 established primary tumors with comparable growth over the experiment. Mice inoculated with ASHAS2 tranfectants did not establish primary tumors. Metastasis in animals inoculated with parental and mock-tranfected cells was most prevalent in the brain and lung but also detected in kidney and liver transfectants. Mice injected with MDA-MB-231 ASHAS2 did not exhibit metastasis to any organs.

Additional mice were injected in the left ventricle with 1×10^5 cells.[181] Animals inoculated with parental and mock-transfected cells had prevalent spread of the cancer to the brain, liver, kidneys, lung, and bone. Mice injected with MDA-MD-231 ASHAS2 did not exhibit metastasis to any organs. Mice inoculated with parental or mock-transfected MDA-MB-231 cells had a shorter survival period (72 and 77 days, respectively), compared with ASHAS2 animals (124 days; $P = .0001$). The authors stated that, collectively, these results strongly implicated the central role of HAS2 in the initiation and progression of breast cancer, potentially highlighting the codependency between HAS2, CD44 (hyaluronic acid receptor), and hyaluronidase 2 expression.

The mechanism that human pancreatic carcinoma cells (MIA PaCa-2) use to create LMW (~10-40 polymers) hyaluronic acid that induce angiogenesis, enhance CD44 (hyaluronic acid receptor) cleavage, and promote the migration of tumor cells in a CD44-dependent manner was researched.[182] MIA PaCa-2 cells show CD44 cleavage in the absence of any exogenous stimulation at a readily detectable level; therefore the possibility that these tumor cells may generate CD44 cleavage inducible hyaluronic acid oligosaccharides by expressing hyaluronic acid–degrading enzymes was tested. To prepare the MIA-PaCa-2 culture supernatant, MIA PaCa-2 cells were cultivated in a flask (3×10^5 cells/flask) overnight. The culture medium was collected, centrifuged, and concentrated 50-fold. It was then filtered (0.22-μm pore filter). Reverse transcriptase-PCR was used to detect hyaluronidases HYAL1 and HYAL2 transcript expression. Enzyme-linked immunosorbent (ELISA)–like assay was used to detect hyaluronidase levels. Western blotting and SDS-PAGE was used to detect hyaluronidase proteins. Hyaluronic acid levels were measured by ELISA. Size profiling and purification of MIA PaCa-2 hyaluronic acid was done by gel filtration chromatography. For the CD44 cleavage assay, MIA PaCa-2 cells were plated (5×10^4) and cultured overnight, then incubated with 10 μM MG132 for 30 minutes to inhibit secondary cleavage of the CD44 intracellular domain. The cells were incubated with various samples of hyaluronic acid for 1 hour then lysed. Samples were separated by electrophoresis under reducing conditions and transferred to a polyvinylidene difluoride filter. The filter was incubated with HRP-conjugated anti-rabbit IgG to detect the anti-CD44cyto pAb, or with HRP-conjugated anti-mouse IgG to detect the anti-β-tubulin mAb. The secondary antibodies were detected using ECL Western blotting detection reagents. Immunofluorescence microscopy was performed. To perform a migration assay, 12-well Costar Transwell (Corning, Inc., Lowell, MA) chambers containing polycarbonate filters with a 12-μm port size were used. Both sides of the filter were coated with 500 μg/mL 1000-kD hyaluronic acid. MIA PaCa-2 cells (2×10^5 cells/mL) were added to the upper compartment and incubated for 3 hours with or without BRIC235 or mouse IgG. Hyaluronic acid was added to the upper compartment at a final concentration of 50 μg/mL and incubated for an additional 15 hours. The cells on the upper side of the filters were wiped off. The filters were fixed in methanol, stained, and mounted on glass slides. Migrated cells were counted under a light microscope. The MIA PaCa-2 culture supernatant was found to contain hyaluronic acid–degrading enzymes, which digested hyaluronic acid in a pH-dependent manner with the optimal pH of 4.0. The presence of hyaluronic acid–degrading activities in the culture supernatant was confirmed by a substrate-gel electrophoresis analysis. MIA PaCa-2 cells expressed 2 of the known hyaluronidases, Hyal-1 and Hyal-2, at both the mRNA and protein levels and secreted both of these proteins into the culture supernatant. The researchers stated that other studies noted that Hyal-1 and Hyal-2 expression was detected in a human prostate cancer cell line LNCaP and in a human breast cancer cell line,

Distributed for Comment Only -- Do Not Cite or Quote

MDA-MB231, respectively. In this study, high levels of hyaluronic acid were detected in the supernatant; the cells generated hyaluronic acid ranging from approximately 10 to 40 saccharide pairs, which are similar in size to those that have been shown to enhance Cdrr cleavage in CD44-expressing tumor cells. The addition of MIA PaCa-2 culture supernatant induced the upregulation of CD44 cleavage in MIA PaCa-2 cells, as evidenced by an increase in the membrane-bound 25-kD cleavage product in Western blotting analysis. This cleavage was strongly inhibited by the Fab fragment of the anti-CD44 neutralizing monoclonal antibody BRIC235, indicating the upregulated CD44 cleavage was because of the interaction between CD44 and its ligand. The culture supernatant also enhanced the migration of MIA PaCa-2 cells in the Transwell migration assay; this migration was almost completely inhibited by the anti-CD44 monoclonal antibody BRIC235 but not by mouse IgG, indicating that the enhanced tumor cell motility was also dependent on the CD44–hyaluronic acid interaction.

Tumor Treatment

The use of testicular hyaluronidase (PH-20) to reduce the presence/production of hyaluronic acid in cancer treatment was tested.[183] Homozygous, female ICR SCID mice were injected with 5×10^6 human breast carcinoma cells (MDA435) into the mammary foot pad. Hyaluronidase activity is expressed in relative turbidity reducing units (rTRUs). The mice were injected intravenously with 75 rTRU hyaluronidase on days 0, 2, 4, and 6 or were administered a single injection of 300 rTRU hyaluronidase (day not specified). Control animals were administered saline. Tumors were measured every other day. After 4 days in both treatment regimes, the tumor volume decreased by 50%; the tumors in the controls continued to grow. After 1 month without any further treatment, major differences continued to be observed between tumors in the treated and nontreated animals. There were no apparent toxic effects or changes in the behavior of the mice during the experiment.

The effectiveness of the application of hyaluronic acid to drug-resistant cancer cells was tested.[184] MCF-7/Adr drug (doxorubicin)–resistant human mammary carcinoma cells were grown in culture for 24 hours in 24-well plates. Controls were the nonresistant parental MCF-7 cells. Various concentrations of chemotherapeutic agents were added and the cells incubated for an additional 72 hours. At that point, 10 μg/mL hyaluronic acid (mixture of 3 to 8 repeating disaccharides in length) was either added or not to the medium, and the cells were incubated for another 24 hours. Cells were harvested, and viable cells were counted. The hyaluronic acid caused approximately 55-fold sensitization increase in the MCF-7Adr cells but had little effect on the non-drug-resistant cells.

The authors tested a range of concentrations for effectiveness and found that up to 250 μg/mL there was little or no effect of hyaluronic acid alone.[184] In combination with doxorubicin, concentrations of 10 μg/mL were effective on the drug-resistant cancer cells. The authors repeated this experiment using other drug-resistant cells and reported that hyaluronic acid increased the sensitivity of cells resistant to taxol by approximately 12-fold, 1,3-bis(2-chloroethyl)-1-nitrosurea (BCNU) by approximately 78-fold, and to vincristine by approximately 10-fold. Hyaluronic acid had little effect on the non-drug-resistant cancer cells. Hyaluronic acid treated MDA-MB231 human mammary carcinoma cells, resistant to the folate analog methotrexate, were 133-fold more sensitive compared with nontreated cells.

The authors stimulated hyaluronic acid production in drug-sensitive MCF-7 cells by infection with a recombinant adenovirus driving expression of HAS2.[184] In 3 runs, modified MCF-7 cells produced 2.5- to 4-fold more hyaluronic acid than untreated cells or control cells infected with recombinant β-galactosidase andenovirus. The increased hyaluronic acid production induced a 10- to 12-fold increase in resistance to doxorubicin, the opposite effect of continuous treatment with hyaluronic acid. This experiment was repeated with emmprin, a member of the immunoglobulin (Ig) superfamily that is enriched on the surface of most malignant cancer cells and promotes tumor progression and regulates hyaluronic acid production. MCF-7 cells infected with recombinant emmprin adenovirus were approximately 10-fold more resistant to doxorubicin treatment than controls. The effect of emmprin was reversed by treatment with hyaluronic acid oligomers. The authors stated that this finding confirmed that emmprin increases drug resistance with hyaluronic acid.

The authors tested the effects of hyaluronic acid oligomers on the phoshoinositide 3-kinase (PI 3-kinase)/Akt cell survival pathway in MCF-7/Adr

Distributed for Comment Only -- Do Not Cite or Quote

cells.[184] Hyaluronic acid suppressed phosphorylation of Akt (protein kinase B) and stimulated expression of protein tyrosine phosphatase (PTEN) in the presence of doxorubicin, taxol, vincristine, and BCNU. PI 3-kinase activity was also inhibited, but there were no effects on total levels of Akt. BAD is a member of the family of apoptosis-regulating proteins. The authors expected hyaluronic acid treatment to lead to phosphorylation of BAD at serine residue 136 (BAD136), the site of Akt-mediated phosphorylation. However, in MCF-7/Adr cells, there was little phosphorylation of BAD136 in the presence or absence of the drugs or hyaluronic acid.

The use of hyaluronic acid in the endoscopic mucosal resection (EMR) procedure to remove tumors was tested.[77] Mice (n = 20) were injected into the right back with hyaluronic acid (0.2 mL; 5%) or saline (0.2 mL). A wound was inflicted by using a surgical knife to remove a 5-mm circle of skin, which was sutured closed. The mice treated with hyaluronic acid were then injected with transplantable adenocarcinoma cell line colon 26 (1×10^6 tumor cells) in 0.1 mL 0.5% hyaluronic acid. The controls were injected with the same cells in 0.1 mL PBS. Tumors were measured every 3 days. After 2 weeks, the tumors were removed, weighed, measured, and examined histopathologically. The hyaluronic acid group had larger tumors than the controls on days 9, 12, and 14 ($P = .001$, $P = .0001$, $P = .0001$, respectively). The tumor weights were also greater for the hyaluronic acid group on day 14 ($P = .001$). On day 14, the proliferating cell nuclear antigen-labeled index in cancer cells was higher in the hyaluronic acid group than in the control group ($P = .0001$). CD44 expression on the surface of the cancer cells was enhanced in the hyaluronic acid group compared with the control group. Western blot analysis also revealed that CD44 protein expression was higher in the hyaluronic acid group compared with the control group.

The use of hyaluronic acid in conjunction with cisplatin for the treatment of cisplatin-resistant head and neck squamous cell carcinoma (HNSCC) was investigated.[185] Two cell lines were used: SCC-4 and HSC-3, both from primary oral tongue squamous cell carcinomas. Various doses of cisplatin along with 50 μg/mL hyaluronic acid were used to treat plated cells (3000/plate), and the IC_{50} was calculated. Cisplatin alone inhibited tumor cell growth; the addition of hyaluronic acid resulted in a 5-fold reduced ability of cisplatin to cause HNSCC cell death. Hyaluronic acid plus anti-CD44 antibody did not inhibit tumor cell growth. For HSC-3 cells, the IC_{50} for cisplatin alone was 4 μM and 20 μM with hyaluronic acid. For SCC-4 cells, the IC_{50} for cisplatin alone was 20 μM and 100 μM with hyaluronic acid.

Yin et al[186] explored the inhibitory effects of hyaluronic acid with paclitaxel using tumor metastasis and ascites formation. Female mice (strain no. 615) were inoculated IP with U14 cells cervical tumor cells (2.5×10^6 cells/mouse) then treated daily for 5 days with saline, 30 mg/kg IP hyaluronic acid alone, 10 mg/kg IP paclitaxel alone, 30 mg/kg hyaluronic acid plus 10 mg/kg paclitaxel, or 15 mg/kg hyaluronic acid plus 5 mg/kg paclitaxel (n = 10 each group). Survival was recorded for 40 days; all mice were killed after 40 days. This experiment was repeated except that chemotherapy was started on day 2 after inoculation of tumor cells and continued for 3 days. Nine days after IP implantation of tumor cells, the mice were killed to observe for ascites formation. Paclitaxel alone and both the lower and higher dose combinations of paclitaxel and hyaluronic acid improved survival up to 40 days compared with saline control by 108.8%, 135.3%, and 135.3%, respectively ($P < .01$). Both the combinations improved life span when compared with paclitaxel alone ($P < .05$). Hyaluronic acid alone did not improve life span. Paclitaxel alone and both the lower and higher dose combinations of paclitaxel and hyaluronic acid reduced ascites formation inoculated with U14 cervical tumor cells ($P < .05$).

These authors also implanted 7-week-old female C57BL/6 mice with Lewis lung carcinoma (LLC) cells in the footpad (5×10^6 cells/mouse).[186] After 12 days, the mice were anesthetized and the tumor-injected foot removed surgically. The mice were grouped and treated daily for 5 days as above with saline, paclitaxel, and/or hyaluronic acid (n = 10 per treatment group). On day 40, the mice were killed and the lungs removed and fixed. Lung metastases were counted and measured under microscopy. Tumor metastases were reduced with hyaluronic acid alone (23.1%) and paclitaxel and hyaluronic acid at 1:1 (19.2%) and 1:3 (36.3%; $P < .05$). Blood samples were taken and analyzed for gene expression. The combination treatment upregulated the expression of vitamin D_3 binding proteins, which is a macrophage-stimulating activator.

Distributed for Comment Only -- Do Not Cite or Quote

Clinical Assessment of Safety

Dermal Absorption

Autoradiography was used to detect the dermal penetration of hyaluronic acid, in the form of [^{3}H]hyaluronan, using human skin.[145] The authors made 2 applications of 56.3 mg and 56.4 mg of [^{3}H]hyaluronic acid gel in a 1.8-cm^2 area of 1 forearm (number of subjects not given). This was repeated 12 hours later. The same was done to the opposing forearm with nonradioactive hyaluronic acid gel. Seven hours after the second application, the skin was swabbed as previously described, and samples were taken from the treated and control areas with a diagnostic trephine of 3-mm diameter. Adherent subcutaneous fat was removed before fixation. ^{3}H activity removed before fixation was measured in each case. The density of the grains was less intense, but there was still similar aggregation of grains in the keratinized layer, epidermis, and clear penetration activity to the deeper dermis with concentration at the level and just beneath the epidermis when examined autoradiographically. The authors concluded that hyaluronic acid penetrates normal epidermis to accumulate at least briefly in the dermis before its disposal and degradation via known metabolic pathways. The transit is rapid, and because there is no inward movement of extracellular fluid at this point, its passage must be mediated by extracellular diffusion, active transport through the cells, or combinations thereof.

Laugier et al[187] studied the dermal penetration of hyaluronic acid and hyaluronidase using human and synthetic skin. Synthetic skin and skin excised from the cadaver of a 42-year-old woman were used. Hyaluronidase was obtained from bacterium *S hyaluroniliticus* and purified. Applications of 40 μL volumes of hyaluronidase and hyaluronic acid (at 0.001, 0.010, and 0.100 mg/mL for synthetic skin and 0.01, 0.10, and 1.00 mg/mL for human skin samples) were placed on the skin surface. Samples were incubated for 24 hours in a humidified 5% CO_2 atmosphere. A punch biopsy was sampled from each experimental area and immediately placed into an acid-alcohol-formalin solution. Samples were fixed for 2 hours for the synthetic skin and 12 hours for the cadaver skin. The amount of hyaluronic acid and CD44 receptors (the cell surface protein that recognizes hyaluronic acid) in human and synthetic skin decreased in those treated with hyaluronidase. The decrease was concentration dependent. There was no significant change in the amount of hyaluronic acid or CD44 expression in either type of skin at any concentration. The authors concluded that hyaluronic acid does not penetrate the skin.

Natural Occurrence/Distribution

Some hyaluronic acid accumulates in the spleen, lymph nodes, and bone marrow.[188] An adult human male has hyaluronic acid entering general circulation at 10 to 100 mg/24 hours.[189]

According to Fraser et al,[87] hyaluronic acid is naturally present throughout the human body, particularly in the eyes and connective tissues. It is produced in the peripheral tissues where most of the turnover takes place in situ. A small amount is carried by lymph to the lymph nodes where it is metabolized.[188,190] The kidneys extract about 10% but excrete only 1% to 2% in urine.[87,188,190]

Some cells, such as chondrocytes in cartilage, actively synthesize and catabolize hyaluronic acid throughout the lifetime of the tissue.[4] The authors estimated that almost a third of the total hyaluronic acid in the human body is metabolically removed and replaced during an average day.

Metabolism

In humans, hyaluronic acid has a half-life of 2.5 to 5.5 minutes in blood. The mean amount of hyaluronic acid in blood is 30 to 40 μg/L.[189,191,192] From the blood, it is mostly taken up by the liver where catabolism takes place in the endothelial cells in the sinusoids.[191,193-195]

Hyaluronic acid breaks down into acetate and lactate.[193] Hyaluronic acid is broken down by hyaluronidases, β-glucuronidase, and β-N-acetylglucosaminidase, or by exposure to oxygen-free radicals.[195-197] Reactive oxygen species produced by keratinocytes are probably involved in the catabolism of epidermal hyaluronic acid.[198]

The level of hyaluronic acid in the blood is increased in people with liver cirrhosis where uptake and degradation are impaired.[189,199-201] This is also seen in rheumatoid arthritis and scleroderma.[199,202]

The half-life of hyaluronic acid in cartilage is normally 1 to 3 weeks.[4] Reticuloendothelial cells lining the lymphatics actively remove almost 90% of the hyaluronic acid before the remainder reaches the vascular system. The half-life of hyaluronic acid is

Distributed for Comment Only -- Do Not Cite or Quote

12 hours in the skin. The cells in the dermis actively synthesize more hyaluronic acid than they catabolize, much of which escapes only to be rapidly captured by receptors on reticuloendothelial cells in lymph nodes and liver, which internalize them for subsequent catabolism in lysosomes.

Injected hyaluronic acid and its derivatives undergo local degradation.[203] The metabolites are then further catabolized by the liver into carbon dioxide and water.

Effect on Penetration of Other Chemicals

Hyaluronic acid facilitates penetration of other substances through the human stratum corneum because a hydrated epidermis is more permeable.[204] The effects of hyaluronic acid on the in vitro diffusion and deposition of diclofenac within the skin were tested.[205] Human skin samples were obtained directly after abdominoplasties and surgery from male and female donors aged 30 to 50 years. Split thickness or epidermal sheet sections were used rather than full-thickness skin. The fat layer was removed. The skin was placed on a cork dissection board, and the epidermis was gently teased off the dermis using forceps and mounted in a Franz cell.

The receptor compartment was filled with previously sonicated Sørensen's buffer (pH 7.0). The area of exposed skin was 2.27 cm^2. The applied solution was either ^{14}C-labeled diclofenac (0.75 MBq/mg) mixed with Sørensen's buffer or ^{14}C-labeled diclofenac (0.75 MBq/mg) mixed with [^3H]hyaluronic acid (4.9 Mbq/mg). As a function of time after application of the labeled solution, 0.5 mL samples were removed from the sampling port in the receptor chamber and replaced with pre-equilibrated buffer.

Diffusion of ^{14}C-labeled diclofenac in buffer reached 10% in 12 hours, while it took ^{14}C-labeled diclofenac in the hyaluronic acid formula over a week to reach the same level. The buffer solution permeated the epidermal sheet relatively rapidly in the first 100 hours so that approximately 30% was in the receptor chamber, then leveled off. The hyaluronic acid solution maintained a steadier rate over the week of the experiment, and at 100 hours, only about 3% of the diclofenac had diffused through to the receptor chamber. Approximately 20% of the solution had diffused through the epidermal sheet by the end of the week-long experiment.[206] Brown et al showed that hyaluronic acid minimized the percutaneous absorption of diclofenac, indicating the formation of a reservoir of drug in the epidermis, which was confirmed using autoradiography.[205]

Lin and Maibach demonstrated that hyaluronic acid delivered twice the diclofenac to the epidermis over 24 hours compared with an aqueous control and sodium carboxymethyl cellulose.[207] Similar effects have been found with ibuprofen, clindamycin phosphate, and cyclosporin.[208-212]

The development of Solaraze (PharmaDerm, Melville, NY), 3% diclofenac in 2.5% hyaluronic acid gel, is used for the treatment of actinic keratosis .[213] The authors stated that hyaluronic acid enhanced the partitioning of diclofenac into human skin and its retention and localization in the epidermis when compared with an aqueous control, other glycosaminoglycans (ie, chondroitin sulphate), and commonly used pharmaceutically acceptable gelling agents (ie, sodium carboxymethyl cellulose) at either molar or rheologically equivalent concentrations.

Dermal Irritation

A "negative" result of a closed skin patch test of hyaluronic acid produced by fermentation was reported. No details were provided.[31]

Immunogenicity

General

Hyaluronic acid binds to monocytes and lymphocytes in inflammatory diseases such as ulcerative colitis and reportedly participates in other inflammatory conditions as well such as rheumatoid arthritis, scleroderma, and psoriasis.[202,214-218] Increased blood levels of hyaluronic acid have been reported in patients with sepsis.[219] Physical activity, which enhances the lymph drainage, also leads to a temporary increase in the serum hyaluronic acid level.[217]

Hyaluronic Acid

In tests for the stimulatory function of hyaluronic acid on polymorphonuclear leukocytes (PMNs), 5 to 10 mg of hyaluronic acid was subcutaneously injected into 6 healthy subjects and 10 persons with decreased resistance to bacterial infections and impaired phagocytic activity.[220] Heparinized venous blood was collected every or every other day, and the phagocytic rate of PMN was measured. PMNs were stimulated in all healthy subjects. The stimulation was evident 1 day after injection, maximized after

Distributed for Comment Only -- Do Not Cite or Quote

2 to 4 days, and lasted about a week. Leukocytosis or fever was not observed, and there was no local reaction at the injection site. The neutrophils of all the immunocompromised subjects responded by increased rates of phagocytosis of both IgG- and serum-opsonized particles. Peak phagocytic activities were seen 2 to 6 days after injection. An increased intracellular content of ATP and enhanced chemiluminescence of isolated PMN were also found after the hyaluronic injections.

Small fragments of hyaluronic acid may stimulate an inflammatory response.[221] In skin pathology, for example, they suggest that the accumulation of fragmented hyaluronic acid molecules in dermal papillae supports the growth of psoriatic lesions by stimulating the growth of capillaries and attracting inflammatory cells.

Sodium Hyaluronate

Immunogenicity of sodium hyaluronate was tested.[222] Nine of 10 healthy subjects (25-44 years of age; 50-73 kg; 163-183 cm tall; 8 females, 2 males) completed the study. For the skin prick test, the volar side of the forearm was pricked with Coca's solution (negative control consisting of 5 g of NaCl and 2.5 g of $NaHCO_3$/L), histamine chloride 1:10 000 (positive control), or sodium hyaluronate (10 mg/mL in a 2-mL disposable syringe; pH 7.3). Examination took place at 15 minutes and 2, 6, and 24 hours after pricking. For the immunization test, 2 subcutaneous injections of 1 mL sodium hyaluronate were administered to the upper arm at an interval of 1 week. In a microprecipitation test, 2-mL samples were taken from the subjects before and after the subcutaneous injections. They were divided into 2 samples, and 0.5 mL of either 5 or 50 μg/mL sodium hyaluronate was added to each. The protein content was estimated by the Folin test. In a complement analysis, sera and EDTA-plasma samples were collected on 4 occasions: before immunization, 24 hours and 6 days after starting immunization, and 2 weeks after the second injection. Sera was tested for total hemolytic complement titer. Plasma samples were tested for conversion products of factor C3 by immunoelectrophoresis in agarose. No skin reactions were observed at sites challenged with sodium hyaluronate or Coca's solution at 2, 6, and 24 hours after skin pricking. There was no increase in protein measured in the microprecipitation test after immunization for either the 5 or 50 μg/mL sodium hyaluronate. None of the sera showed a significant decrease in total hemolytic activity. No conversion products of factor C3 were seen in plasma samples collected before and after immunization.

Osteoarthritis Treatment

Hyaluronic acid and sodium hyaluronate have been used in clinical studies to evaluate their effectiveness in treating osteoarthritis (OA). Three such trials (the most recent) are described in detail; other trials and their safety results are summarized in Table 11 and discussed below.

The effectiveness of an injection of non-animal-stabilized hyaluronic acid on sufferers of OA was tested.[223] Subjects received a subcutaneous injection of 3 mL of either 60 mg hyaluronic acid in buffered sodium chloride (0.9%; pH 7; n = 172) or the identical buffered sodium chloride vehicle (n = 174). Seventy-four subjects did not finish the study. After 26 weeks, there was no significant difference between the control and treatment groups in the response to the treatment; the pain, stiffness, and physical function scores in both groups decreased over the study period. The safety evaluation included all recruited patients (n = 347). A total of 513 adverse effects (AEs) were reported by 227 patients (65.4%) over the study period. The majority of AEs (79.3%) were classified as mild/moderate. The number of patients reporting treatment-related AEs was 22 (12.8%) in the hyaluronic acid group and 14 (8.0%) in the saline group. The most common treatment-related AE was arthralgia, reported by 11 patients (6.4%) and 5 patients (2.9%) in the hyaluronic acid and saline groups, respectively. The majority of treatment-related AEs (>70%) were reported within 2 days of injection in both treatment groups. Treatment withdrawal attributable to AEs occurred in 13 hyaluronic acid and 6 control patients in the 2 groups; 5 and 4 of these events were considered related to treatment, respectively. Of the 9 treatment-related AEs leading to withdrawal, 7 reported general knee pain, 1 reported worsening OA pain in the knee (hyaluronic acid group), and 1 reported knee synovitis (placebo group). Ten of the patients withdrawing from treatment (7 in the hyaluronic acid group and 3 in the saline group) reported serious AEs (not defined), all of which were assessed by the investigator as being unrelated to the study treatment.

Distributed for Comment Only -- Do Not Cite or Quote

Table 11. United States and International Placebo-Controlled Trials Evaluating the Safety of Hyalgan,[1] Snyvisc,[2] and Supartz[3]

Study	Country: Regimen	No. of Patients	Results
Hyalgan[a] studies			
Carrabba et al[291]	Italy: placebo- and arthrocentesis-controlled, 1, 3, or 5 weekly injections with a 6-month follow-up	100	All regimens well tolerated; AEs mild and transient; no serious AEs
Bragantini et al[292]	Italy: saline-controlled, 3 weekly injections	55	Well tolerated; no serious AEs
Grecomoro et al[293]	Italy: vehicle-controlled, 3 weekly injections	36	Well tolerated; no reported AEs
Dixon et al[294]	UK: 0.2 mg sodium hyaluronate–controlled, up to 11 injections given at 1, 2, 3, 5, 7, 9, 11, 15, 19, and 23 weeks	63	Well tolerated; 3 reports of local joint reaction in Hyalgan patients
Dougados et al[295]	France: vehicle-controlled, 4 weekly injections	110	Well tolerated; no serious AEs; = control
Henderson et al[296]	UK: saline-controlled, 5 weekly injections	91	More poorly tolerated than placebo; local pain/swelling in 47% Hyalgan vs 22% placebo patients
Corrado et al[297]	Italy: buffered saline-controlled, 5 weekly injections	40	Decreased inflammatory effusion in Hyalgan patients
Listrat et al[298]	France: no injection-controlled, 3 courses of 3 weekly injections over 1 year	39	Well tolerated; 8 reports of injection pain limited to moment/few moment of injection
Altman and Moskowitz[299]	US: saline- and nonsteroidal anti-inflammatory drug–controlled, 5 weekly Hyalgan injections	456	Gastrointestinal AEs higher in naproxen group; local injection site pain higher in Hyalgan vs control
Huskisson and Donnelly[300]	UK: saline-controlled, 5 weekly injections	100	Well tolerated; = control; injection site reactions similar in placebo and active groups
Synvisc[b] studies			
Wobig et al[301]	Germany: placebo-controlled, 3 weekly injections	110	No serious AEs; only local AEs
FDA[83] Premarket Approval Application Study #5	US: arthrocentesis-controlled, 5 weekly injections	94	No significant differences in numbers or types of AEs between Synvisc and control
Supartz[c] studies			
Puhl et al[302]	Germany: vehicle-controlled, 5 weekly injections	195	Well tolerated; no clinical abnormalities
Dahlberg et al[303]	Sweden: vehicle-controlled, 5 weekly injections	52	Well tolerated; no serious AEs; similar injection-site pain with Supartz and control
Lohmander et al[304]	Sweden: vehicle-controlled, 5 weekly injections	240	Well tolerated; no serious AEs; greater severity of injection-site AEs in control patients ($P = .041$)
Wu et al[305]	Republic of China: saline-controlled, 5 weekly injections	90	Well tolerated; no clinical abnormalities

AE, adverse event.
[a] Hyalgan - sodium hyaluronate. Fidia Pharmaceutical Corporation, Washington, DC. Approved for marketing in US in May 1997. Molecular weight, 500-730 kDa; 1% protein.
[b] Synvisc - Hylan G-F 20. Sodium hyaluronate chemically crosslinked with formaldehyde and vinylsulfone to increase molecular weight. Biomatrix Inc, Ridgefield, New Jersey. Approved for marketing in US in August 1997. 80% Molecular weight, 6000 kDa; 20% molecular weight, indeterminate; 1% protein.
[c] Supartz - sodium hyaluronate. Seikagaku Corporation, Tokyo, Japan. Approved for marketing in US in January 2001. Molecular weight, 620-1710 kDa; 1% protein.

Hyaluronic acid was tested for effectiveness and safety as a treatment for OA of the knee. Seventy-six patients (92 knees) with moderate to severe OA received injections of 20 mg of sodium hyaluronate into the knee joint (intra-articular) at weekly intervals for 5 weeks.[224] There were no placebos. Clinical assessments were carried out at baseline and 6, 12, and 24 months. Monitoring for possible treatment-related undesirable or AEs was carried out at each clinical assessment. Seventy-two percent achieved a >50% improvement for 1 year or longer. No systemic effects were noted during the follow-up period. The AEs were minor and infrequent. They included brief

Distributed for Comment Only -- Do Not Cite or Quote

postinjection pain, minor bruising at the injection site, rare headache, and nausea.

Another approach to the evaluation of hyaluronic acid and its derivatives was taken.[225] Six knees in 5 patients that received a series of 3 intra-articular injections of hylan G-F 20 viscosupplementation underwent surgical procedures because of persistent symptoms of OA. No patient had a history of ongoing evidence of infection, evidence of immunocompromise, or a history of long-term use of immunosuppressive medications. No patient had an allergy to chicken or egg products. Two patients had received prior injections of corticosteroids to the knee joint. Previous surgical procedures on the knee included open reduction and internal fixation of a patellar fracture in 1 patient and arthroscopic debridement in 2. Routine histological examination of arthroscopic shavings from the latter procedures revealed no abnormalities. Each patient had pain, swelling, and warmth in the knee following viscosupplementation that developed within 48 hours after injection. This peaked at 4 to 5 days and gradually resolved in approximately 1 to 2 weeks following final injection. No patient had a fever or erythematous reaction. All patients managed the pain with nonsteroidal anti-inflammatory medications, but relief was minimal. No patient had evidence of peripheral leukocytosis on routine preoperative evaluation, and all had a normal C-reactive protein level. Knee aspriation was performed in 4 knees; no frank purulence or cloudy aspirate was found, and no organisms were seen on Gram staining. All 4 aspirates had a white blood cell count $<10\,000/mm^3$ ($<10.0 \times 10^9/L$). There were fewer than 5 neutrophils per high-power field, and cultures were negative. Two knees underwent arthroscopic debridement, one at 2 months and one at 6 months after last injection of hyaluronic acid (hylan G-F 20). Specimens were collected with use of a specimen trap connected to the arthroscopic shaver and preserved in 10% buffered formalin. Four knees underwent total knee arthroplasty between 5 and 9 months after final injection of hylan G-F 20. This resulted in the collection of tibial plateau, femoral condyle, shaved bone fragments, meniscus, and capsular tissue. Soft-tissue sections from each case revealed similar histological findings. Chronically inflamed synovium and adipose tissue containing numerous areas of histiocytic and foreign-body giant-cell reaction surrounding acellular, amorphous, pink fluid-like material were noted. Use of special stains for microorganisms had negative results. No birefringent crystalline material was observed under polarized light microscopy. The acellular material was stained with alcian blue, a stain for hyaluronic acid, which disappeared after hyaluronidase digestion. None of the bone fragments revealed granulomatous inflammation.

Vesicoureteral Reflux Treatment

Q-Med AB research on their product Deflux Injectable Gel, which consists of microspheres of cross-linked dextran suspended in a gel of nonanimal, stabilized hyaluronic acid to be used for children with vesicoureteral reflux (VUR).[155] Deflux is injected submucosally in the urinary bladder in close proximity to the ureteral orifice. Dextranimer microspheres are gradually surrounded by the body's own connective tissue, which provided a bulking effect. Thirty-nine subjects were treated with Deflux, and 21 were treated with antibiotic prophylaxis. They also treated a total of 170 subjects in 2 nonrandomized studies and followed them for 12 months. There were 14 urinary tract infections total in the 3 studies. In the first study, 2 patients had nausea, vomiting, and abdominal pain following injection procedure. This complication resolved in both cases. None of these problems were attributed to the hyaluronic acid.

Tissue Augmentation

Duranti et al[24] treated 158 patients with facial intradermal implant of hyaluronic acid gel for augmentation therapy of wrinkles, folds, acne scars, as well as lip augmentation and recontouring. All patients were white women with a mean age of 36.8 years (range, 26-68). Patients requiring further implants ($n = 11$) or extensive follow-up ($n = 4$) were excluded from the study. Patients were examined at time 0 and 1, 2, 4, and 8 months after the injection. Hyaluronic acid (Restylane; derived from bacterial sources) was placed into the mid-dermis using 27- or 30-gauge needles. Common antiseptic solutions were used to prepare the skin. There were 12.5% (34 cases) who experienced immediate AEs that were localized and transient. The most commonly reported were bruising, tenderness, discomfort, edema, and erythema at the treatment site. Most events lasted less than 3 days and resolved spontaneously. One erythema and swelling case lasted 5 days. Thirteen patients complained, particularly after lip augmentation, of

Distributed for Comment Only -- Do Not Cite or Quote

an intermittent swelling of the implanted material. There were 7 cases of erythema, 5 of edema, 5 of discomfort, 3 of tenderness, 3 of bruising, 1 of itching, and 1 of pain. There was no clinical evidence of major systemic side effects nor of acute or chronic hypersensitivity. No blood chemistry data were available.

These authors carried out a histological study on 5 volunteers (aged 26-54 years) for 52 weeks.[24] Prior to inclusion in the study, they were examined for skin diseases and double-tested for possible sensitivity to hyaluronic acid gel and collagen. The treatment consisted of spot injections of 0.05 mL of each product at 4 sites alternating between the products on the volar surface of the left and right forearm. Each person received a total of 8 implants. Biopsies were taken at weeks 4, 12, 24, and 52 after a physical evaluation of the sites. The biopsies were examined blind by 2 experienced pathologists who reached a consensus on each biopsy with respect to inflammation, foreign body reaction, and fibrosis. Hyaluronic acid implants maintained their spot size between the 12th and 24th week. At week 52, 4 of the implants were still clearly visible under the skin. Staining for hyaluronic acid revealed the presence of such material but with a significantly more watery appearance than at earlier biopsies. All of the specimens were free of fibrosis and severe foreign body reaction but often presented a slight inflammatory reaction. There were no differences in the presence of cells around each of the implants over time throughout the 52 weeks of observation.

The use of hyaluronic acid (Restylane; stabilized hyaluronic acid), for tissue augmentation, was tested.[226] One hundred thirteen patients (106 females and 7 males) were recruited, each receiving treatment in up to 3 sites, including glabellar lines, nasolabial folds, mouth angle wrinkles, and other facial lines. A total of 285 sites were treated. All patients were monitored for at least 30 minutes after treatment for erythema, swelling, local pain, redness, itching, and tenderness. All patients were evaluated at weeks 0, 1, 12, and 26. Twenty patients were randomly chosen to come back after 52 weeks for additional assessment. Additional injections were given to 66% of the 113 patients who were deemed to be in need of a "touch up." Nineteen (6.6%) of the sites showed redness, red spots, and/or swelling. Four sites (1.4%) developed dark areas. Three of these developed at week 1 and one at week 2. One patient reported slight pain at week 2. All of these events were resolved within 1 week.

A test on the use of stabilized hyaluronic acid for dermal augmentation was reported.[41] This was an open-label, 12-month study conducted at 6 sites. Investigators were all experienced plastic surgeons and dermatologists. A total of 216 patients were enrolled in the study, 191 female and 25 male, between 25 and 76 years old. All patients received at least 1 treatment, 30% received a second injection, and 17% received a third. The mean total volume of hyaluronic acid injected was 0.32 mL, ranging up to 1.60 mL. A total of 177 patients completed the 1-year study. Two of those who did not complete the study left because of dissatisfaction with their treatment and 3 because of localized discomfort or reactions associated with treatments. The authors stated that reactions were as expected and included transient and mild erythema, itching, swelling, and pain. Related or probably related adverse reactions occurred in less than 2% of all treatments and included persistent erythema, acne papule formation, and ecchymotic changes (blood from ruptured blood vessels leaking into subcutaneous tissue). No antigenic or immunogenic responses were observed.

A prospective study on the use of hyaluronic acid (Restylane) for lip tissue augmentation was reported.[154] This hyaluronic acid product was injected into the upper lips of 192 women aged 24 to 77 years (average age, 46 years). Of these patients, 88% had previously received collagen treatments. Two percent of the patients were allergic or had some adverse reactions to collagen. One patient had Hashimoto's thyroid disease, and 1 had rheumatoid arthritis. All patients received an initial treatment. Second and third treatments were at the mutual discretion of the patient and the investigator. Treatments were spread over 4 to 6 weeks. All patients were anesthetized with lidocaine cream 5%, and endobuccal anesthesia of the troncular type was administered to the most sensitive patients (2% adrenaline/lidocaine). Each patient received between 0.7 and 1.1 mL of hyaluronic acid. Swelling was noted in 86% of the patients during the first 24 hours and was noticeable at 5 days in 14% and 1% at 10 days. Redness was noted during the first 24 hours in 52% of the patients and in 36% of the patients the following day, and 12% on the third day. There was 1 case of a delayed effect. At the fifth week, 1 woman experienced inflammation that disappeared within 10 days with no treatment. The authors speculated that her trip to Africa with extra exposure to the sun in the fourth week may have been the cause.

Distributed for Comment Only -- Do Not Cite or Quote

A study of the use of hyaluronic acid from 2 sources for tissue augmentation were conducted: Restylane produced by microbiologic engineering techniques and Hylaform extracted from rooster combs.[227] The authors stated that both products contain varying amounts of hyaluronin-associated protein and therefore a theoretic risk for sensitivity reactions existed. Patients (677 women and 32 men) were treated, 438 with Hylaform and 271 with Restylane without prior skin testing. The patient's ages ranged from 25 to 75 years. The filler was administered with an intradermal, horizontal tunneling injection. Repeated treatments were performed on 180 of the Hylaform patients and 56 of the Restylane patients. No abnormal skin reactions were observed at the time of injection other than mild transient erythema. Delayed inflammatory reactions developed 6 to 8 weeks after the injection at some of the injection sites in 3 of the 709 patients (0.42%). These patients had not had tissue augmentation injections before this procedure. Also, 3 other patients from outside the study were referred for evaluation after injections of hyaluronic acid and included in this report. Four patients (3 with Hylaform and 1 with Restylane) developed induration and inflammation at the injection sites. They all developed an abscess in the nasolabial region. On palpation, these areas were firm, tender, edematous, and erythematous. Resolution was achieved in 6 to 24 weeks. Three patients required treatment to reduce the induration and inflammation.

These authors performed retrospective skin tests on 5 of the patients with delayed reactions.[227] Aliquots (0.1 mL) of both fillers were injected intradermally into the forearm skin. Four of the 5 patients tested developed discernable skin reactions ranging from mildly inflammatory nodules to inflammatory reactions with an abscess in 1 patient. Both Hylaform and Restylane caused reactions in 3 patients. Hylaform (extracted from rooster combs) reacted in 1 patient, and 1 patient had no reaction. These reactions appeared between 5 and 7 weeks. All reactions resolved in 2 to 12 weeks.

The authors also performed a 3-mm punch biopsy on one patient's nasolabial area about 6 weeks after the original inflammatory reaction.[227] The biopsy showed a normal epidermis with major changes confined to the dermis and subcutaneous fat. The upper dermis showed elastic degeneration, which was consistent with actinic damage. The mid and lower dermis showed a moderate infiltration of lymphocytes and plasma cells with few scattered macrophages containing hemosiderin pigment. Eosinophils were not seen, and there were no foreign body giant cells. Organizing fibrosis was most prominent in the lower dermis. All changes extended into the subcutaneous fat.

The data collected by Q-Med Esthetics, the manufacturer of a hyaluronic acid gel, for use in soft-tissue augmentation were examined.[228] They found that out of 144 000 possible treatments there were 222 adverse reactions reported, including localized hypersensitivity reactions, swelling, erythema, and induration at the transplant site, in 1999. There were no reports of systemic symptoms or anaphylaxes. In 2000, there were 144 reported adverse reactions out of 262 000 treatments. These included redness, edema, tenderness, injection site inflammation, erythema, swelling, pain, itching, discoloration, and temporary palpable lumpiness. Most of these problems resolved within 2 weeks. There were rare reports of localized granulomatous reactions, bacterial infection, and acneiform and cystic lesions. Two cases of injection site necrosis in the glabelar area a few days after injection were observed, which the authors considered likely secondary to compression of vascular supply from excessive use of product. The authors stated that the results may be skewed because the "treatment" number was deduced from the number of preloaded syringes sold, not by the number of patients; there is the possibility of doses not being used or multiple doses applied to the same person.

Physicians in European countries that use hyaluronic acid produced by Q-Medical for soft-tissue augmentation were surveyed.[229] A total of 12 344 syringes were sold by the company to these physicians. A total of 4320 patients were treated and evaluated in this survey. A total of 34 cases of hypersensitivity were reported between 1997 and 2001. Sixteen cases were immediate reactions. Fourteen of these resolved within 3 weeks, 1 lasted 6 weeks, and 1 lasted 2 months, resolving with the use of corticosteroid cream. One abscess was reported and was resolved with several evacuations. There were 18 delayed reactions. Most of these appeared a few weeks later, but 1 case appeared 6 months after injection with a streptococcal infection. There were 3 cases of delayed abscess. The author attributed 2 cases of reticular, livedoid reactions of the nose to an intravascular injection. These 2 cases lasted for 2 weeks before resolving without any sequela and/or scarring. Skin testing was not recommended by the author.

Distributed for Comment Only -- Do Not Cite or Quote

Cancer and Other Diseases

Elevated levels of hyaluronic acid are associated with tumor progression and cancer migration, an effect postulated to result from the influence of hyaluronic acid on cell division and attachment as well as its stimulation of angiogenesis.[230,231] Tumor cells have been found to increase production of hyaluronic acid; the cells in vitro are seen to have a halo of the substance around them in the growth medium.[232]

The levels of hyaluronic acid in neoplastic epithelial cells may be predictors of malignancy of gastric, breast, and ovarian cancer tumors.[231,233,234] This is also true of atherosclerotic cardiovascular disease.[235]

LMW hyaluronic acid (3-12 disaccharide units) has been shown to inhibit tumor growth.[236] Increased concentration of hyaluronic acid stimulates cell-survival signaling and reportedly stimulates drug resistance in drug-sensitive cancer cells.[184,237]

Increased hyaluronic acid levels in the blood interfere with platelet function, and the patients have disturbances in blood coagulation mimicking acquired von Willebrand's disease.[238] It has been shown that nephroblastomas produce platelet-derived growth factor, and it is known that this growth factor can activate hyaluronic acid synthesis, as did epidermal, basic fibroblast, and transforming growth factors.[239,240]

Increased amounts of hyaluronic acid have been found in the serum of patients suffering from Wilm's tumor.[241-245] Another factor, which they called "hyaluronic acid stimulating activity" (HASA) in serum and urine of Wilm's tumor patients, was reported.[246] This glycoprotein is synthesized by the fetal kidney and circulated in fetal blood. These authors suggested that Wilm's tumor occurs when the transformed rests of the fetal kidney retains the ability to produce HASA. Through HASA, the tumor cells can presumably induce other cells to produce hyaluronic acid.

If the hyaluronic acid in the serum is of high molecular weight (2×10^6 Da), as in Wilm's patients, there is hyperviscosity in the serum.[247,248] If the polysaccharide is of a low molecular weight, the angiogenic activity could be the underlying cause of the metastasizing power of patients with bone metastasizing renal tumors.[247,248]

Mesothelioma has been shown to have elevated hyaluronic acid.[249,250] High hyaluronic acid levels have been shown to be an indicator of a poor prognosis.[251,252] The patients who responded to treatment had decreased serum levels.[252] When studied in culture, mesothelioma cell lines produce negligible hyaluronic acid, in contrast to normal mesothelial cells. This is an analogous situation to HASA in Wilm's tumors.[175]

Hyaluronic acid significantly inhibited the active E rosette forming T lymphocytes in vitro.[253] In many cases, the level of hyaluronic acid that surrounds a cancer correlates with tumor aggressiveness.[254] Anttilla et al reported that stromal hyaluronan (hyaluronic acid) accumulation may be a powerful enhancer of tumor progression and, as such, provides a novel, independent prognostic marker and potential target for therapy.[234] Hyaluronic acid used to facilitate tumor excision surgery may stimulate the cell growth of any residual tumor cells after endoscopic mucosal resection.[77]

In discussing the relationship between tumors and hyaluronic acid, Laurent et al stated that, except for studies of Wilm's tumor and mesothelioma, there are several reports in which patients with various types of cancer have been screened for serum hyaluronic acid.[48,97,255-257] Increased levels have sometimes been noted in a few patients, while the majority of the cases have normal values; the high values have not been correlated with any particular tissue or metastatic involvement.[255]

Two studies have specifically assayed serum from patients with breast cancer and arrive at different conclusions. While Delpech et al[258] found a significant increase in serum hyaluronic acid especially in patients with metastases, Ponting et al[259] could not demonstrate any correlation with a number of prognostic factors. However, both groups concluded that serum hyaluronic acid is of no prognostic value.

An increase of collagen peptides and an increase of serum hyaluronic acid in patients with multiple myeloma were noticed, and researchers speculated that it was caused by myeloma activity in close proximity to periosteum or joints.[260] The same properties of hyaluronic acid that facilitate growth and motility during fetal development and tissue formation are utilized by cancers to promote their own growth.[176,261]

Cancer Patients

Serum hyaluronic acid was sampled from 57 women with breast cancer and 26 without and compared them to 50 patients with benign breast lesions

Distributed for Comment Only -- Do Not Cite or Quote

(controls).[258] Hyaluronic acid was increased in the sera of metastatic patients compared with nonmetastatic patients ($P < .0001$) as well as the control sera ($P < .01$). The difference was not related to the number of metastatic sites. Lower concentrations of hyaluronic acid were observed in patients after 3 months who were responding to chemotherapy. The initial concentrations of hyaluronic acid had no predictive value.

The level of hyaluronic acid in the sera of 238 women with breast cancer, measured by radiometric assay, showed no increase when compared to 120 healthy women (controls).[259] Predictive properties of hyaluronic acid were examined for stage of disease, lymph node involvement, tumor size, histology, and presence of estrogen or progesterone receptors in the tumors. There was no correlation with any of these parameters.

The cellular expression of hyaluronic acid in 215 stage I to IV gastric cancer patients was measured using a specific biotinylated probe.[233] Of the tumors, 93% were stained for hyaluronic acid in the parenchyma, and all had hyaluronic acid in the stroma inside and around the tumor. Hyaluronic acid expression was compared to clinical and histological features of the tumors. Strong staining intensity in the tumor parenchyma was associated with deep tumor invasion and with mixed types of classifications (diffuse, intestinal, mixed, unclassified). Hyaluronic acid–positive cells were associated with deep tumor invasion ($P < .0001$), nodal metastasis ($P < .07$; $F = 4.2$), positive lymphatic invasion ($P < .002$), poor differentiation grade ($P < .006$), and inferior prognosis in univariate survival analysis ($P < .0025$) (but not with multivariate analysis).

The relationship between hyaluronic acid and epithelial ovarian cancer tumors was examined.[234] Samples and histories were collected from 309 patients with adequate archival tumor material. A biotinylated affinity probe specific for hyaluronic acid was applied to histological sections of the samples and 45 matched metastatic lesions. The staining was scored for the percentage of area of strong hyaluronic acid signal (peritumoral and intratumoral stroma) as low (<35%), moderate (35%-75%), or high (>75%).

Levels of stromal hyaluronic acid were 95 (31%) low, 116 (37%) moderate, and 98 (32%) high. The high stromal hyaluronic acid level was associated with poor differentiation ($P < .0005$), serous histological type ($P < .05$), advanced stage ($P < .03$), and large primary residual tumor (>2 cm; $P < .03$) but not correlated with high CD44 expression. High amounts of hyaluronic acid in cancer cells were associated with poor differentiation of the tumor ($P < .002$). Low levels of stromal hyaluronic acid were associated with early FIGO stage ($P < .008$) and mucinous histological type ($P < .05$).

The 5-year survival of the disease decreased with increasing stromal hyaluronic acid levels for both overall (45% vs 39% vs 26%; $P = .002$) and recurrence-free (66% vs 56% vs 40%; $P = .008$) survival. High levels of stromal hyaluronic acid were more frequent ($P = .0001$) in metastatic lesions than in primary tumors.[234]

Samples were collected from 143 human breast carcinoma patients.[231] The localization and signal intensity of hyaluronic acid were analyzed in the paraffin-embedded tumor samples using a biotinylated hyaluronic acid–specific probe. In the immediate peritumoral stroma, hyaluronic acid signal was moderately or strongly increased in 39% and 56% of the cases, respectively. Normal ductal epithelium showed no hyaluronic acid, but in 57% of the tumors, at least some of the carcinoma cells were hyaluronic acid positive. Hyaluronic acid in the malignant cells was located on the plasma membrane in 77 of 143 (54%) cases, in the cytoplasm in 65 of 143 (45%) cases, and in the nucleus in 21 of 143 (15%) cases. The intensity of the stromal hyaluronic acid signal and the presence of cell-associated hyaluronic acid were both related to poor differentiation of the tumors ($P < .003$), axillary lymph node positivity ($P < .015$), and decreased survival of the patients.

The levels of CD44 (hyaluronic acid receptor) and hyaluronic acid associated with disease progression and survival of cutaneous melanoma were studied.[231] A series of 292 clinical stage I cutaneous melanomas were analyzed by immunohistochemistry using an anti-CD44H antibody (clone 2C5). Hyaluronic acid was demonstrated histochemically using a biotinylated hyaluronic acid–specific affinity probe (bHABC). CD44 was positively associated with cellular hyaluronic acid. Decreasing levels of cancer cell–associated CD44 and hyaluronic acid were related to increasing Breslow thickness ($P < .000\,05$ and $P < .001$, respectively), increasing Clark level ($P < .000\,05$ and $P < .000\,05$, respectively), and increasing pT category ($P < .000\,05$ and $P < .000\,05$, respectively); this trend was evenly distributed within all 3 categories. Decreasing CD44 and hyaluronic acid levels also associated with bleeding

Distributed for Comment Only -- Do Not Cite or Quote

($P = .024$ and $P = .011$, respectively) and recurrent disease ($P < .00005$ and $P < .01$, respectively). Stromal hyaluronic acid intensity was not correlated with CD44 or any of the clinicopathological variables. Stromal hyaluronic acid intensity was not related to overall survival or recurrence-free survival.

The uses of 3 serum markers (tissue polypeptide antigen (TPA), hyaluronic acid, and cancer antigen 125) were explored in following the progress of mesothelioma.[262] Historical blood profiles were examined for 11 patients over 1 to 63 months with 1 to 18 samples. Correspondence between initial TPA levels and survival were better than either hyaluronic acid or cancer antigen 125 markers. Five patients had increasing serum levels of all 3 markers as the mesothelioma progressed (according to CT scans). In 3 patients, stable disease was followed by a decrease in all 3 serum marker levels.

Paraffin-embedded sections of 114 basal cell carcinomas (BCC), 31 in situ carcinomas (ISC), and 35 squamous cell carcinomas (SCC) were stained for the presence of hyaluronic acid and CD44.[263] Compared with normal epidermis, ISC and well-differentiated SCC samples showed an enhanced hyaluronic acid signal on carcinoma cells ($P < .001$), while CD44 expression resembled normal skin. Less-differentiated SCC samples had reduced and irregular expression of hyaluronic acid and CD44 on carcinoma cells. In BCC samples, hyaluronic acid was frequently present on cell nuclei; this was not noted in the other types of samples. Hyaluronic acid in the connective tissue stroma around tumors was more frequent in SCCs than BCC. Versican staining was positive around hair follicles and dermal blood vessels of normal skin. Peritumoral versican signal was present in a part of the BCCs but not in other tumors.

The immunohistochemical expression of hyaluronic acid synthase (HAS) and serum levels of hyaluronic acid correlation with the clinicopathological manifestations of endometrial carcinoma were determined.[264] Sera and cancer tissue was collected from 59 endometrial cancer patients and sera from 22 healthy postmenopausal women. Hyaluronic acid concentration was determined by inhibitory ELISA. The cancer tissues were immunostained by the avidin-biotin-peroxidase complex method using anti-HAS1, 2, and 3 and anti-CD44 antibody. The expression of HAS1 was related to the depth of myometrial invasion, histological grade, and lymph-vascular space involvement, but not HAS 2 and 3. CD44 expression was more frequent in HAS2- and HAS3-positive groups than in the HAS2- ($P = .0094$) or HAS3-negative ($P = .0134$) groups. The expression of HAS1 was not related to CD44 expression. Serum hyaluronic acid levels were higher in the endometrial cancer patients (418.4 $\pm$ 210.6 ng/mL) than in the healthy control group (99.5 $\pm$ 48.2 ng/mL; $P < .0001$). The levels increased with depth of myometrial invasion, histological grade, and lymph-vascular space involvement. Serum hyaluronic acid levels were higher in the HAS1-positive group than the HAS1-negative group ($P < .0001$); the expression of HAS2 and HAS3 were unrelated to serum hyaluronic acid levels.

The amount of hyaluronic acid present in human tumors was measured.[265] A total of 256 samples were collected after surgery from cancer patients between 1995 and 2002. The samples were fixed and stained and the grade of tumors evaluated. Samples were selected for the presence of benign and malignant histology. Histochemical localization of hyaluronic acid was accomplished by a biotinylated hyaluronic acid–affinity probe. Twenty samples were graded as well-differentiated tumors (astrocytoma, salivary gland, thyroid, infiltrating breast, stomach, urinary bladder, and colon tumors), all of which had intense hyaluronic acid staining in the tumor cells, intratumoral, and in the associated surrounding stroma. In the poorly differentiated tumor samples (astrocytomas, infiltrating breast, stomach, gall bladder, pancreas, caecum, prostate, ovary), cells showed almost no hyaluronic acid stain; the intratumoral and stromal areas showed moderate hyaluronic acid stain. Irrespective of their origin, the poorly differentiated tumor samples showed negative staining for hyaluronic acid in the tumor epithelial or stromal area. With regard to the surrounding tissue, there was intense hyaluronic acid staining in intratumoral and peritumoral areas of all the well-differentiated tumors compared with benign areas. Carcinomas from astrocytomas, gangliogliomas, thyroid, breast, and salivary samples displayed intense accumulation of hyaluronic acid in intratumoral areas. Highly aggressive poorly differentiated tumors of different origins demonstrated moderate to low levels of stromal hyaluronic acid in the immediate vicinity of the tumor cells. Epithelial cells of benign areas were mostly hyaluronic acid negative. In the 20 tumors studied, epithelial cell surfaces were positive for hyaluronic acid in the well-differentiated tumors compared with the poorly differentiated tumors.

Distributed for Comment Only -- Do Not Cite or Quote

Hyaluronic acid levels were measured by immunoradiometric techniques in 850 patients with invasive breast cancer.[266] The mean follow-up time was 55.1 months. Cytosolic hyaluronic acid levels in tumors ranged from 4 to 59 767 mg/mg protein; the median was 4960 mg/mg protein. Hyaluronic acid levels were higher in younger patients ($P = .0001$); the levels were also higher in premenopausal women compared with postmenopausal women ($P = .001$). Hyaluronic acid levels were higher in ductal or lobular histological type than other histological types (colloid, medullary, papillary) ($P = .001$). No association was found between hyaluronic acid intratumoral levels and relapse-free survival and overall survival. High hyaluronic acid intratumoral levels were associated with longer relapse-free survival ($P = .01$) in the subgroup of patients with ductal histological type tumors and those ($P = .01$) without any type of systemic adjuvant treatment.

Case Reports

The case of a 74-year-old man who was referred for worsening OA of the right knee was reported.[267] Previous treatments included arthroscopic debridement, physical therapy, and intra-articular steroid injection. He declined total knee replacement. Radiographs showed advanced OA with faint linear calcifications suggestive of chondrocalcinosis. Physical examination showed a mild varus deformity and anterior and medial joint tenderness. There was no joint effusion. Three intra-articular injections of hyaluronic acid (Hylan G-F 20, Synvisc) were administered weekly for 3 weeks. The first 2 injections were uncomplicated. One week after the third injection, the patient developed a large, painful right knee effusion. Synovial fluid analysis revealed 5300 white blood cells and 680 red blood cells/μL. Gram stain and cultures were negative. Enzyme-linked immunosorbent assay did not detect antibodies to *Borrelia borgdorferi*. Microscopic analysis revealed multiple intracellular rhomboid crystals typical of pseudogout. The patient underwent joint aspiration and conservative treatment, which improved his symptoms.

Adverse effects of intra-articular hyaluronic acid (Hylan GF-20) injections to a 46-year-old male patient with moderate medial compartment OA were reported.[268] He was otherwise healthy and had no history of allergy to food or drugs, including eggs or chicken. Three injections 1 week apart were performed. The third injection was performed without difficulty and was uneventful. Within 2 hours, the patient developed rapidly progressive painful swelling of the knee. A large effusion developed in less than 24 hours and was evaluated in the emergency room. He had a fever of 38.9°C (102°F) and complained of severe knee pain. Arthrocentesis yielded 100 cc of clear yellow fluid. The cell count was 9600 leukocytes with 27% neutrophils, 38% monocytes, 25% lymphocytes, and 16% eosinophils. There were no crystals. Gram stain revealed leukocytes but no bacteria. Cultures of synovial fluid and peripheral blood were negative at 72 hours. He was first treated with naproxen but returned to the emergency room 24 hours later. He then received an intra-articular injection of triamcinolone hexacetonide. The swelling and pain improved gradually over the next 5 days and was controlled with ibuprofen. The authors stated that the temporal pattern, marked synovial fluid eosinophilia, and response to corticosteroid injection were all consistent with an allergic reaction.

A 54-year-old white woman underwent treatment for facial lines and wrinkles with modified hyaluronic acid gel.[269] She was treated in April 1998 and November 1998 with only mild bruising and erythema at the injection sites, which resolved in 1 to 2 days. In June 1999, she again had treatments. Two weeks later, she developed acute, multiple, tender red nodules within the treatment areas. Physical examination showed multiple, discrete nodules measuring 0.5 to 1 cm in diameter in various stages of development. Some nodules exuded a coagulated, yellow, stringy material and appeared to be secondarily infected with frank pus. Other lesions were more indurated, almost fibrotic, with significant erythema but still with intact overlying skin. A culture detected no pathogenic bacteria. The patient was treated with minocycline and methylprednisolone. Warm saline compresses were also applied. The most purulent and fibrotic nodules were injected with triamcinolone acetonide for symptomatic relief. The symptoms rapidly cleared. However, 2 weeks later, the patient returned with recurrent inflammation. The same treatment was repeated with resolution of her symptoms.

A 53-year-old woman was described with an exudative reaction that increasingly turned granulomatous 2 days after the injection of hyualuronic acid (Hylaform).[270] The peak of the eczematous reaction was reached after 4 to 6 days, and it healed after a further 4 to 5 days.

Distributed for Comment Only -- Do Not Cite or Quote

Two patients with reactions to intra-articular injections for OA were reported.[271] The first was a 73-year-old woman with a long history of left knee pain. She had been treated with nonsteroidal anti-inflammatory drugs, steroid intra-articular injections, and physical therapy. A right Baker's cyst had been surgically removed 6 years prior. Physical examination showed crepitus with reduced flexion, a left popliteal cyst, and a slight knee effusion. Radiographs showed osteoarthritic changes and bilateral genu valgum. She underwent intra-articular injections of hyaluronic acid (Hylan GF-20) into the left knee. Before the first injection, the knee was aspirated, yielding 5 mL of clear synovial fluid. White and red blood cell counts were 90×10^6/L and 50×10^6/L, respectively. No crystal was identified, and routine cultures were negative. The first injection was uneventful. One week later, a second injection was given. Before the injection, 10 mL of synovial fluid was aspirated with a white blood cell count of 310×10^6/L, sterile and no crystals. Four hours after the second injection, she developed severe left knee pain with local inflammation signs and a fever of 38°C (100.4°F). The synovial fluid contained $80\,000 \times 10^6$/L white cells with 82% polymorphonuclear cells and remained sterile after 5 days. No crystal was detected. Treatment with piroxicam, paracetamol, and ice resulted in partial recovery within 7 days. At the third injection, a small effusion persisted, and 20 mL of fluid was aspirated. The white cell count had reduced to $15\,750 \times 10^6$/L with 72% granulocytes and remained sterile without detectable crystals. She was completely recovered from the reaction in 2 weeks. The second patient was a 59-year-old woman who had right knee OA for 7 years. There was no effusion and no erythema. Knee flexion was limited. Radiographs showed osteoarthritic changes without meniscocalcinosis. Pain did not diminish with NSAIDs or steroid intra-articular injection. Before the first hyaluronic acid (Hylan GF-20) injection, less than 1 mL of synovial fluid was aspirated and examined. White blood cell count was 90×10^6/L. After the first injection, the knee had a small effusion and swelling within 4 days and improved spontaneously. Before the second injection, the white blood cell count of the synovial fluid was 90×10^6/L with sterile cultures and without crystals. Two hours after the injection, she presented with severe knee pain, swelling, and effusion. Knee aspiration yielded 50 mL of inflammatory synovial fluid. The white cell count was $32\,600 \times 10^6$/L. The fluid was sterile in culture, and no crystals were found. The arthritis resolved within 7 days after treatment with paracetamol alone. The patient declined a third injection.

Ten cases were reported with clinical allergic reactions after wrinkle treatment with injectable hyaluronic acid.[40] The manifestations look like nettle rash reactions or, more often, like delayed and long-lasting inflammatory cutaneous reactions. The author suggests that even though the percentage of allergy to hyaluronic acid ($\pm 3\%$) is lower than injectable bovine collagen ($\pm 4\%$), allergy tests may be in order before treatment.

A case was reported of a 45-year-old woman who developed erythematous swelling of treated areas and mildly tender nodules at injection sites 3 days after soft-tissue augmentation of the nasolabial lines and oral commissures.[272] The same treatment 90 days prior was uneventful. The right side was worse than the left. Symptoms cleared after 10 to 15 days with treatment with hydrocortisone cream 2.5%. There were no sequelae at 90 days' follow-up.

A 54-year-old woman who had hyaluronic acid gel injected in her nasolabial folds was described.[273] The gel was produced by crosslinking part of the glucosaminoglycan molecule of hyaluronic acid under controlled conditions to yield a gel. The resulting polymer resides in the intercellular matrix of the skin for about 6 to 12 months. The woman developed redness and intermittent swelling of the nasolabial folds, followed by the development of severe palpable and painful erythematous nodular papulocystic lesions, which evolved into severe abscesses on both folds. The complications were resolved with drainage and surgical removal of the papulonodular material. A biopsy showed granulomas with severe foreign body reactions.

A patient was reported who presented with a possible granulomatous reaction to hyaluronic acid (Restylane, filler from bacterial sources) or a bovine collagen filler.[274] The authors state that adverse effects of classic foreign-body granuloma are rare and estimate more frequent reactions in patients who have been sensitized to these products, although they can also occur in nonsensitized patients. Skin tests on this patient showed that she was not sensitized to these 2 fillers.

A case of a 40-year-old man who was injected with hyaluronic acid (Restylane) along a prominent horizontal forehead furrow line and the vermillion border of his upper lip from the same syringe was

Distributed for Comment Only -- Do Not Cite or Quote

reported.[275] Five months later, he showed an elevated, lumpy red line in the forehead that developed over a 24-hour period. There was no pain or tenderness, and the inflammatory reaction gradually disappeared over the next 3 weeks. No reaction was evident on the lip, and the author suggested this was possibly due to the small amount of hyaluronic acid injected or the reaction was camouflaged by the natural redness and indistinct outline of the patient's vermillion border.

A case was reported of a 65-year-old woman who had a negative skin test result and still developed a hypersensitivity reaction to hyaluronic acid.[276] A skin test was performed on the left forearm with 0.1 mL intradermal injection of Restylane obtained from a 0.7-mL syringe of Restylane from the manufacturer. The syringe was sealed in an envelope. There was no hypersensitivity reaction noted at the site for 4 weeks after the test. The patient underwent treatment with Restylane over a period of almost 1 year at the nasolabial folds, lips, perioral rhytides; corners of the mouth; nasolabial folds and perioral rhytids; and perioral areas. Approximately 0.7 mL of Restylane was used for each treatment. Six weeks after her last treatment, the patient presented with slight edema of the nasolabial folds with no erythema. Massage did not relieve the condition, and she returned in 6 weeks with extensive erythema, edema, and induration in the injected regions. She was started on a 3-week prednisone taper starting at 40 mg. The patient improved over the next few weeks; then in mid-April 2004, she presented with a flare in the previously affected areas, coinciding with the tapering of the prednisone. The recommended biopsy was refused. Intralesional 1.0 mL triamcinolone acetonide (2 mg/kmL) was injected resulting in no change after 1 week. The patient was restarted on a longer course of prednisone at 40 mg, which was tapered over 8 weeks with slight results at follow-up. She received several monthly intralesional triamcinolone acetonide injections (5 mg/mL) to the inflamed areas when the erythema had faded and the previously raised thickened areas were almost flat.

Summary

Hyaluronic acid, sodium hyaluronate, and potassium hyaluronate function in cosmetics as skin conditioning agents and viscosity increasing agents. These compounds are formed by the bonding of N-acetyl-D-glucosamine with glucuronic acid in a disaccharide chain that can grow as long as 10 000 pairs in length. As the chain lengthens and coils into a spherical shape, the molecule mechanically holds water, which in turn allows small molecules to pass through and excludes or slows the passing of larger molecules. Adjacent chains can interact with each other to form a network. The viscosity of these molecules increases with concentration. Hyaluronic acid salts are soluble in saline but almost insoluble in organic solvents. Hyaluronic acid can act as an antioxidant but is degraded. It can be degraded by UV radiation.

Hyaluronic acid and its salts may be derived from rooster combs, bovine tracheas and vitreous, bacterial fermentation, and human umbilical cords. In cosmetics, hyaluronic acid (5 and 1800 kD) sources are bacterial fermentation and rooster combs. Impurities include proteins, DNA, and chondroitin sulfate when derived from animal sources.

Hyaluronic acid reportedly is used in 27 cosmetic product categories, at concentrations up to 1%. Sodium hyaluronate reportedly is used in 32 cosmetic product categories, with a maximum concentration of 2%. Potassium hyaluronate reportedly is used in 8 cosmetic product categories, although no use concentration data were available.

Noncosmetic uses include multiple medical uses, including treatments for tissue augmentation, dry eye, osteoarthritis, and wounds. Hyaluronic acid also has multiple surgical and drug delivery applications.

Hyaluronic acid is found naturally in avascular body compartments. In humans, it is most abundant in the skin but also found in synovial fluid, vitreous humor, tendon sheaths, and bursae. Hyaluronic acid is synthesized primarily by dermal fibroblasts and epidermal keratinocytes. Functions in the body include tissue hydration, lubrication, solute transport, cell migration and function, wound healing, and red blood cell aggregation and adhesion.

Hyaluronic acid has been detected to a maximum depth of 800 μm after dermal application to rats. In mice and humans, hyaluronic acid penetrated to the dermis. Hyaluronic acid can moderate the penetration of other chemicals such as diclofenac, causing a slower absorption of that drug and preferential accumulation in the epidermis.

Radioactive hyaluronic acid was found to transfer to the fetuses of pregnant rats injected on day 17 of pregnancy after 4 hours. Hyaluronic acid was found in the skin, plasma, blood, Harder's gland,

Distributed for Comment Only -- Do Not Cite or Quote

kidney, spleen, and livers. Similarly treated lactating rats were found to have radioactivity in their milk after 24 hours. In humans, hyaluronic acid has a half-life of 1 to 3 weeks in the cartilage, 2.5 to 5.5 minutes in the blood, and 12 hours in the skin. Radiolabeled hyaluronic acid injected into the anterior chamber of rabbits had a half-life of 14 hours, 50 hours for subcutaneous injection, and 30 hours for intramuscular injection. For sheep, hyaluronic acid has a half-life of 5.3 ± 1.1 minutes in the blood. When injected in the eye, hyaluronic acid has a half-life of 21 hours in cynomolgus monkeys. Hyaluronic acid has a half-life of 8 to 15 hours in rabbits when injected into the pleural space. Inflammation causes an increase in hyaluronic acid levels and half-life in rabbits.

In an acute toxicity study, no deaths were reported of mice orally administered >1200 mg/kg hyaluronic acid. Hyaluronic acid had no ill effects to guinea pigs, rats, or cats when injected into the middle ear. Rabbits had no sign of neurotoxicity from hyaluronic acid when injected into the spinous process.

Hyaluronic acid or sodium hyaluronate caused no ill effects to rats or rabbits when injected peritoneally. There was no short-term ototoxicity to guinea pigs, rats, or cats when hyaluronic acid was injected into the middle ear. There was no evidence of cytotoxicity from sodium hyaluronate to mouse bone marrow cells; however, hyaluronan-based hydrogels were cytotoxic to smooth muscle cells from the thoracic aorta of rats. The respiration of hyaluronic acid had no ill effects in dogs and sheep.

Owl monkeys and rhesus monkeys had no ill effects from repeated injection of hyaluronic acid into the eyes. Repeated injections of sodium hyaluronate into the eyes of owl monkeys increased the leukocyte count up to 200 cells/mm^3 after 48 hours. The severity of haze and flare in the eyes after the injections did not increase over time, and there was no immunogenic response. This experiment was continued for up to 9 years in 2 eyes with no adverse effects. When implanted into the bladder submucosa of rabbits and dogs, there was no inflammation, infection, irritation, foreign body response, tissue necrosis, or scarring for up to 24 months.

Rabbits had no sensitization response to multiple injections of hyaluronic acid from human umbilical cords or streptococcal fermentation. Repeating the experiment resulted in erythema from umbilical cord hyaluronic acid in 2 rabbits and 2 that received streptococcal hyaluronic acid. Tests for nonprecipitation, skin-sensitizing antibodies were negative during the 1-hour observation. Injected streptococcal hyaluronic acid samples containing 0.1% to 0.3% protein caused rabbits to form precipitating antibodies to the hyaluronic acid. No passive cutaneous anaphylaxis reactive antibodies were formed in rabbits for hyaluronic acid. Antibody response by rooster comb–derived hyaluronic acid in rats caused an enhanced secondary antibody response to birch pollen, egg albumen, and dog albumin. Neither commercial sodium hyaluronate preparations nor a crude rooster comb sodium hyaluronate preparation elicited a hyaluronic acid–specific antibody response in rabbits. Crosslinked hyaluronic acid caused acute cutaneous anaphylaxis and delayed-type hypersensitivity in guinea pigs up to 48 hours after injections.

Hyaluronic acid did not cause reproductive or developmental toxicity in studies using rats or white rabbits. Hyaluronic acid was not genotoxic in reverse mutagenicity tests on *S typhimurium* and *E coli*, in vivo micronucleus tests using mice, or in vitro and in vivo chromosomal aberration tests using Chinese hamster lung fibroblast cells.

Hyaluronic acid levels have been found to be increased in tissues surrounding some breast cancer; gastric cancer; poorly differentiated, serous histological type, advanced stage, and large primary tumor epithelial ovarian cancer; endometrial cancer; ganglioma; thyroid cancer; and salivary gland cancer. Hyaluronic acid levels have been found to be normal or reduced in association with breast cancer, early FIGO stage and mucinous histological-type epithelial ovarian cancer, and murine astrocytoma.

Mouse melanoma cell lines with high hyaluronic acid production had increased lung metastasis and lower survival than melanoma cell lines with low hyaluronic acid production. Medium from mesotheliaoma cell line culture, transferred to mesothelial cells in culture, caused increased hyaluronic acid production. Increased hyaluronic acid intensity in breast cancer patients was related to axillary lymph node positivity and poor survival. Mesothelioma cells genetically enhanced to produce hyaluronic acid grew 2- to 3-fold larger colonies in soft agar than non–hyaluronic acid–producing mesothelioma cells. Hyaluronic acid reduced LX-1 lung carcinoma tumor growth. Using rat carcinoma cells, hyaluronic acid synthase (HAS2) enhances tumor growth, whereas expression of hyaluronidase activity of Hyal1 delays tumor development.

Distributed for Comment Only -- Do Not Cite or Quote

Aneuploid human breast adnocarcinoma cells modified with antisense inhibition of HAS2 expression produced more HMW hyaluronic acid. When these cells were injected into mice, no primary tumors were established, and there was no metastasis to any organs, and the mice had longer survival than those injected with nonmodified cells.

Well-differentiated tumors (astrocytoma, salivary gland, thyroid, infiltrating breast, stomach, urinary bladder, and colon tumors) had intense hyaluronic acid staining in the tumor cells, intratumoral, and in the associated surrounding stroma. Poorly differentiated tumor samples (astrocytomas, infiltrating breast, stomach, gallbladder, pancreas, caecum, prostate, and ovary) with carcinoma or sarcoma had almost no hyaluronic acid when stained. Enhanced motility of human pancreatic carcinoma cells was dependent on the CD44–hyaluronic acid interaction where LMW hyaluronic acid induced angiogenesis, enhanced CD44 cleavage, and promoted the migration of the tumor cells in a CD44-dependent manner.

Stromal hyaluronic acid was not related to survival or recurrence-free survival from cutaneous melanoma. Compared with normal epidermis, in situ carcinomas and well-differentiated squamous cell carcinomas showed an enhanced hyaluronic acid signal on carcinoma cells, while CD44 expression resembled normal skin. Less-differentiated squamous cell carcinoma samples had reduced and irregular expression of hyaluronic acid and CD44 on carcinoma cells. In basal cell carcinoma samples, hyaluronic acid was frequently present on cell nuclei but not in the other types of samples.

Hyaluronidase applied to tumors or tumor cells injected into the footpads of mice reduced growth rates in human breast carcinoma. The in vivo application of hyaluronic acid increased the effectiveness of chemotherapeutic agents when applied to drug-resistant mammary carcinoma cells but had little effect on nonresistant cells in mice. Increasing the expression of hyaluronic acid in these MCF-7 cells increased the resistance to doxorubicin. Hyaluronic acid reduced the effectiveness of cisplatin in treating cisplatin-resistant head and neck squamous cell carcinoma. Hyaluronic acid in combination with paclitaxel increased survival of mice over paclitaxel alone; tumor metastases were reduced by a combination of paclitaxel and hyaluronic acid over paclitaxel alone.

There were no skin reactions to an immunogenicity test of sodium hyaluronate on humans (10 mg/mL). Leukocytosis or fever were not observed in healthy subjects and persons with decreased resistance to bacterial infections and impaired phagocytic activity when subcutaneously injected with 5 to 10 mg of hyaluronic acid. Phagocytic rate of polymorphonuclear leukocytes measured with IgG-coated latex particles was stimulated in all healthy subjects; neutrophils of all the immunocompromised subjects responded by increased rates of phagocytosis in both IgG- and serum-opsonized particles.

In multiple studies on the use of injected hyaluronic acid as a treatment of osteoarthritis, most treatments were successful. Adverse effects such as minor discomfort, bruising, headache, nausea, and increased white blood cell count were reported, all treatable with nonsteroidal anti-inflammatory medications and/or aspiration of the swollen joint. Some patients had severe pain, swelling, and very high white blood cell counts ($>10.0 \times 10^9$/L) in the aspirated fluid, usually after multiple treatments with hyaluronic acid. No treatment-related effects were reported with the use of hyaluronic acid as a component of a treatment for vesicoureteral reflux.

When tested for tissue augmentation, hyaluronic acid was found to be safe and nonreactive in most cases. Most cases of bruising, tenderness, discomfort, edema, and erythema lasted less than 3 days and resolved spontaneously. There were cases of redness, red spots, dark areas, and/or swelling. Some adverse effects required treatment. One study found that less than 2% of all treatments had adverse reactions, including erythema, acne papule formation, and ecchymotic changes. No antigenic or immunogenic responses were observed. In another study, 86% had swelling, 52% had redness after injections to the upper lip.

There were 144 reported adverse reactions reported from the use of hyaluronic acid for soft-tissue augmentation out of a possible 262 000 uses, 222 out of 144 000 in 1999. Reactions included localized hypersensitivity, swelling, erythema, edema, tenderness, inflammation, itching, discoloration, temporary lumpiness, and induration. Most resolved within 2 weeks. Of a total of 12 344 applications sold by another company and used on a total of 4320 patients between 1997 and 2001, 34 cases of hypersensitivity were reported.

Many case reports of adverse effects from hyaluronic acid have appeared, most related to its use to treat osteoarthritis or for tissue augmentation; all these cases were from injections and not topical use.

Distributed for Comment Only -- Do Not Cite or Quote

Discussion

While hyaluronic acid has multiple sources, including rooster combs, bovine sources, and bacterial fermentation, in cosmetics, the only sources of hyaluronic acid used are bacterial fermentation and rooster combs. Because there is an avian source for these cosmetic ingredients, the matter of avian flu was considered. Because the heat from the manufacturing process reliably kills the avian flu virus, no safety concern exists in this regard. While there are no specific infectious agent concerns, the CIR Expert Panel is mindful of the need to derive these ingredients only from disease-free animals. Bacterial sources should be free of pyrogens.

The Expert Panel recognized that these ingredients can enhance the penetration of other ingredients through the skin (eg, HC Yellow No. 4, Disperse Yellow 3). The Panel cautioned that care should be taken in formulating cosmetic products that may contain these ingredients in combination with any ingredients whose safety was based on their lack of dermal absorption data or when dermal absorption was a concern.

After reviewing inhalation toxicity data on dogs and sheep, the CIR Expert Panel determined that hyaluronic acid, sodium hyaluronate, and potassium hyaluronate can be used safely in sprays because the ingredient particle size is not respirable. The Panel reasoned that, for example, the particle size of anhydrous hair sprays (60-80 μm) and pump hair sprays (>80 μm) is large compared with the median aerodynamic diameter of 4.25 ± 1.5 μm for a respirable particulate mass.

The CIR Expert Panel considered that the amount of hyaluronic acid present naturally in human skin was relevant to considering the effect of exogenous hyaluronic acid. The amount of hyaluronic acid in the skin is approximately 0.6 mg/g skin. The average woman has a total surface area of 16 900 cm^2; approximately 15% of a 60-kg woman is skin which is approximately 9000 g. Dividing the weight of skin by the area of skin on a woman (9000 g/16 900 cm^2), the figure of 0.53 g skin/cm^2 is reached. The CIR Expert Panel estimated the amount of hyaluronic acid in skin by area to be 0.318 mg hyaluronic acid/cm^2 skin.

The CIR Expert Panel compared the amount of hyaluronic acid found in the skin to the maximum amount of hyaluronic acid applied to the skin by cosmetic products, as noted in this report, of 0.02 mg/cm^2 by a product with the maximum concentration of 2%, and found the contribution via application of such a cosmetic product to be negligible. Acute, short-term, and chronic toxicological studies indicated low toxicity.

The CIR Expert Panel recognized that hyaluronic acid has been linked to metastatic cancer and sought to resolve whether the relationship was causal. In that regard, one seminal study reported a reduced level of hyaluronic acid associated with an unfavorable prognosis of clinical stage 1 cutaneous melanoma. These results suggest that, in melanoma, hyaluronic acid does not play a role in the metastatic process. In another pivotal study, hyaluronic acid was studied using the hyaluronic acid receptor, CD44 (a cell surface glycoprotein that is involved in cell/cell and cell/matrix interactions) on epidermal keratinocyte tumors, specifically, basal cell carcinomas and squamous cell carcinomas. In basal cell carcinomas, CD44 expression was quite low. In squamous cell carcinomas, CD44 expression was variable. As the malignancy became less differentiated and, therefore, would be expected to have a higher risk for metastasis, the expression of hyaluronic acid decreased.

These key findings suggest that hyaluronic acid likely does not play a causal role in metastasis and that increased expression of hyaluronic acid genes may be a consequence of metastatic growth not the converse. These results, together with the levels of hyaluronic acid that would be applied to the skin, would further insure the safety of this ingredient in cosmetic products.

The CIR Expert Panel discussed the possible need for additional dermal irritation and sensitization, UV absorption, and/or photosensitization and photoirritation data. Taking into consideration the above-mentioned calculation on the amount of hyaluronic acid in the skin compared to the amount that might be contributed by the application of cosmetics, the Expert Panel decided that the amount of hyaluronic acid in cosmetics would be negligible and not a concern in these areas.

Even though adverse reactions to injected hyaluronic acid used in the treatment of osteoarthritis and tissue augmentation are reported, these data do not raise safety concerns regarding the use of hyaluronic acid in cosmetics. There were no reported reactions to topically applied hyaluronic acid, further supporting that hyaluronic acid at levels currently

Distributed for Comment Only -- Do Not Cite or Quote

used in cosmetics applied to the skin should not be of concern.

The CIR Expert Panel recognizes that there are data gaps regarding use and concentration of these ingredients. However, the overall information available on the types of products in which these ingredients are used and at what concentrations indicate a pattern of use, which was considered by the Expert Panel in assessing safety.

Conclusion

The CIR Expert Panel concluded that hyaluronic acid, sodium hyaluronate, and potassium hyaluronate are safe as cosmetic ingredients in the practices of use and concentrations as described in this safety assessment.

Financial Disclosure

The articles in this supplement were sponsored by the Cosmetic Ingredient Review. The Cosmetic Ingredient Review Program is financially supported by the Personal Care Products Council.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported. F. Alan Andersen, PhD, and Lillian C. Becker are employed by the Cosmetic Ingredient Review.

References

Unpublished sources cited in this report are available for review from the Director, Cosmetic Ingredient Review, 1101 17th St. Suite 412, Washington, DC 20036, USA.

1. Balazs EA, Laurent TC, Howe AF, Varga L. Irradiation of mucopolysaccharides with ultraviolet light and electrons. *Rad Res*. 1959;11:149.
2. Laurent TC. Biochemistry of hyaluronan. *Acta Otolaryngol*. 1987;Suppl 442:7-24.
3. Laurent TC, Laurent UBG, Fraser JRE. Functions of hyaluronan. *Ann Rheum Dis*. 1995;54:429-432.
4. Hascall VC, Laurent TC. Hyaluronan: structure and physical properties. 1997. Available at: http://www.glycoforum.gr.ip/science/hyaluronan/HA01/HA01E.html. Accessed February 21, 2005.
5. Sheehan JK, Gardner KH, Atkins ED. Hyaluronic acid: a double-helical structure in the presence of potassium at low pH and found also with the cations ammonium, rubdium and caesium. *J Molec Biol*. 1977;117:113-135.
6. Tan SW, Johns MR, Greenfield PF. Hyalruonic acid: a versatile biopolymer. *Aust J Biotech*. 1990;4:38-43.
7. Gottschalk TE, NcEwen GN Jr. *International Cosmetic Ingredient Dictionary and Handbook*. Washington, DC; CTFA; 2006.
8. Billek G, Billek D. Hyaluronic acid: the history of an active ingredient in cosmetics [in German]. *Parfuem Kosmet*. 1988;69:788-790; 792-795.
9. Sutherland IW. Novel and established applications of microbial polysaccharides. *Trends Biotechnol*. 1998; 16:41-46.
10. Laurent TC. *Structure of Hyaluronic Acid*. New York, NY: Academic Press; 1970.
11. Ogston AG. *The Biological Functions of the Glycosaminoglycans*. New York, NY: Academic Press; 1970.
12. Comper WD, Laurent TC. Physiological function of connective tissue polysaccharides. *Phyil Rev*. 1978;58:255.
13. Balazs EA, Band P. Hyaluronic acid: its structure and use. *Cosmetics & Toiletries*. 1984;99:65-72.
14. Morris ER, Reese DA, Welsh EJ. Conformation and dynamic interactions in hyaluronate solutions. *J Molec Biol*. 1980;138:383.
15. Cleland RL, Wang JL, Detweiler DM. Polyelectrolyte properties of sodium hyaluronate. 2: potentiometric titration of hyaluronic acid. *Macromolecules J*. 1982; 15:386-395.
16. Balazs EA, Laurent TC. Viscosity function of hyaluronic acid as a polyelectrolyte. *J Polym Sci*. 1951;6:665-667.
17. Cleland RL. Comparison of polyelectrolyte behaviour of hyaluronate with that of carboxymethyl cellulose. *Biopolymers*. 1968;6:1519-1529.
18. Scott JE, Cummings C, Brass A, Chen Y. Secondary and tertiary structures of hyaluronan in aqueous solution, investigated by rotary shadowing-electron microscopy and computer simulation. *Biochem J*. 1991;274: 699-705.
19. Scott JE. Secondary and tertiary structures of hyaluronan in aqueous solution: some biological consequences. 1998. Available at: http://www.glucoforum.gr.jp/science/hyaluronan/HA02/HA02E.html. Accessed February 21, 2005.
20. Lower E. Heralding hyaluronic acid. *Soap Perfum Cosmet*. 1998;71:41-42.
21. Denlinger JL, Balazs EA. Replacement of the liquid vitreous with sodium hyaluronate in monkeys. I: short-term evaluation. *Exp Eye Res*. 1980;31:80-99.
22. Milas M, Rinaudo M, Fouissac E. Molecular weight and rheotological measurements of sodium hyaluronate. *Cosmetics & Toiletries*. 1993;108:57-63.
23. Cleland RL, Stoomiller AC, Roden L, Laurent TC. Partial characterization of reaction products formed by the

Distributed for Comment Only -- Do Not Cite or Quote

degradation of hyaluronic acid with ascorbic acid. *Biochim Biophy Acta*. 1969;192:385.

24. Duranti F, Galti G, Bovani B, Calandra M, Rosati ML. Injectable hyaluronic acid gel for soft tissue augmentation: a clinical and histological study. *Dermatol Surg*. 1998;24:1317-1325.
25. Hyaluronic acid and sodium hyaluronate: sources, molecular weight and protein content. 2006. Unpublished data from CTFA; 2/23/06; 1p.
26. Kendall FE, Heidelberger M, Awson MH. Serologically inactive polysaccharide elaborated by mucoid strains of group A *Hemolytic streptococci*. *J Biol Chem*. 1937; 118:61-69.
27. Meyer D, Dubos R, Smyth EM. The hydorlysis of the polysaccharide acids of vitreous humor, umbilical cord and *Streptococcus* by aurolytic enzyme of pneumococcus. *J Biol Chem*. 1937;118:71-78.
28. Seastone CV. Virulence of group C hemolytic streptococci of animal origin. *J Exp Med*. 1939;70:361-378.
29. Kjems E, Lebech K. Isolation of hyaluronic acid from cultures of *Streptococci* in a chemically defined medium. *Acta Pathol Microbiol Scand B*. 1976;84:162-164.
30. Sugahara K, Schwarts NB, Dorfman A. Biosynthesis of hyaluronic acid by *Streptococcus*. *J Biol Chem*. 1979; 254:6252-6261.
31. Akasaka HSS, Setu M, Yanagi M, Fukushima S, Mitusui TJ. Industrial production of hyaluronic acid by Streptococcus zooepidemicus [in Japanese]. *Soc Cosmet Chem Jpn*. 1988;22:35-42.
32. Rapport MM, Linker A, Meyer K. The hydrolysis of hyaluronic acid by pneumococcal hyaluronidase. *J Biol Chem*. 1951;192:283-291.
33. MacLennan AP. The production of capsules, hyaluronic acid and hyaluronidases by group A and C *Streptococci*. *J Gen Microbiol*. 1956;14:134-142.
34. MacLennan AP. The isolation and characterization of a hyaluronidase produced by a capsulated strain of a group C *Streptococci*. *J Gen Microbiol*. 1956;14:143-152.
35. MacLennan AP. The production of capsules, hyaluronic acid and hyaluronidase by 25 strains of group C *Streptococci*. *J Gen Microbiol*. 1956;15:485-491.
36. Warren GH, Gray J. Isolation and purification of *Streptococal* hyaluronic acid. *Proc Soc Exp Biol Med*. 1959;102:125-127.
37. Warren GH, Gray J. Production of a polysaccharide by *Staphylococcus aureus III*: action of penicillin and polysaccharide on enzymic lysis. *Proc Soc Exp Biol Med* 1965;118:627-631.
38. Balazs EA. Hyaluronate-based compositions and cosmetic formulations containing same. US Patent No. 4,303,676. 1981.
39. Balazs EA, Leschiner A. Hyaluronan, its crosslinked derivative hylan and their medical applications. In: Inagaki H, Phillips GO, eds. *Cellulosics Utilization: Research and Rewards in Cellulosics* (*Proceedings of Nisshinbo International Conference on Cellulosics Utilization in the Near Future*). New York: Elsevier Applied Science; 1989:223-241.
40. Micheels P. Human anti-hyaluronic acid antibodies: is it possible? *Dermatol Surg*. 2001;27:185-191.
41. Piacquadio DJ, Larsen NE, Denlinger JL. Hylan b gel (Hylaform) as a soft tissue augmentation material. In: Klein, AW, ed. *Tissue Augmentation in Clinical Practice: Procedures and Techniques*. New York: Marcel Dekker; 1998:269-291.
42. Manna F, Dentini M, Desideri P, De Pitá O, Mortilla E, Maras B. Comparative chemical evaluation of two commercially available derivatives of hyaluronic acid (Hylaform from roster combs and Restylane from streptococcus) used for soft tissue augmentation. *J Eur Acad Dermatol Venerol*. 1999;13:183-192.
43. Goldberg VM, Coutts RD. Pseudoseptic reactions to hylan viscosupplementation: diagnosis and treatment. *Clin Orthoped*. 2003;4:130-137.
44. Laurent UBG, Tengblad A. Determination of hyaluronate in biological samples by a specific radioassay technique. *Anal Biochem*. 1980;109:386-394.
45. Girard N, Delpech A, Delpech B. Characterization of hyaluronic acid on tissue sections with hyaluronectin. *J Histochem Cytochem*. 1986;34:539-541.
46. Lindner JS, Huang SS. Low-angle laser light scattering (LALLS) of macromolecules. In: Barth, HG, Mays, JW, eds. *Modern Methods of Polymer Characterization*. New York: John Wiley & Sons; 1991:313-375.
47. Balazs EA, Freeman MI, Klöti R, Meyer-Schwickerath G, Regnault F, Sweeney DB. Hyaluronic acid and replacement of vitreous and aqueous humor. *Mod Probl Ophthalmol*. 1972;10:3-21.
48. Delpech B, Bertrand P, Maingonnat C. Immunoenzymoassay of the hyaluronic acid-hyaluronectin interaction: application to the detection of hyaluronic acid in serum of normal subjects and cancer patients. *Anal Biochem*. 1985;149:555-565.
49. Glasser DB, Edelhouser HF. Toxicity of surgical solutions. *Int Ophthalmol*. 1989;29:179-187.
50. Filion MC, Phillips NC. Pro-inflammatory activity of contaminating DNA in hyaluronic acid preparations. *J Pharmacy Pharmacol*. 2001;53:555-561.
51. World Health Organization (WHO). Epidemic and pandemic alert and response (EPR): avian influenza frequently asked questions. 2005. Available at: http://www.who.int/csr/disease/avian_influenza/avian_faqs/en/index.html. Accessed March 31, 2006.
52. Food and Drug Administration (FDA). Frequency of use of cosmetic ingredients. *FDA Database*. Washington, DC: FDA; 2005.
53. Cosmetic, Toiletry, and Fragrance Association (CTFA). Concentration of use hyaluronic acid, sodium hyaluronate and potassium hyaluronate. 2005. Unpublished data from CTFA; 11/15/2005; 6p.

Distributed for Comment Only -- Do Not Cite or Quote

54. Casado FJ, Nusimovich AD. LMW hyaluronic acid to induce epidermal regeneration. *Drug Cosm Ind*. 1991; 148:30-34, 74.
55. Ministry of Health, Labour and Welfare (MHLW). MHLW notification no. 331 of 2000, as amended on March 23, 2005. Tokyo, Japan: Ministry of Health Labour and Welfare, Evaluation and Licensing Division, Pharmaceutical and Food Safety Bureau; 2005.
56. European Economic Community (EEC). EEC Cosmetic Directive 76/768/EEC, as amended, Annexes I through VII. Brussells: EEC; 2001.
57. Jensen PA, Obrien D. *Industrial Hygiene*. John Wiley and Sons Inc: New York, NY; 1993.
58. Bower D. Unpublished information on hair spray particle sizes. Presented at: CIR Expert Panel meeting; September 9, 1999; Washington, D.C.
59. Håkansson L, Hällgren R, Venge P, Artursson G, Vedung S. Hyaluronic aid stimulates neutrophil function in vitro and in vivo: a review of experimental results and a presentation of a preliminary clincal trial. *Scand J Infect Dis*. 1980;Suppl 24:54-57.
60. Håkansson L, Hällgren R, Venge P. Effect of hyaluronic acid on phagocytosis of opsonized latex particles. *Scand J Immunol*. 1980;11(6):649-653.
61. Liu H, Mao J, Yao K, Yang G, Cui L, Cao Y. A study on a chitosan-gelatin-hyaluronic acid scaffold as artificial skin in vitro and its tissue engineering applications. *J Biomater Sci Polymer Ed*. 2004;15:25-40.
62. Ritt MJF, Hillebrand-Haverkort ME, ten Veen JH. Local treatment of facial lipdystrophy in patients receiving HIV protease inhibitor therapy. *Acta Chirurgiae Plasticae*. 2001;43:54-56.
63. Espallargues M, Pons JM. Efficacy and safety of viscosupplementation with hylan G-F 20 for the treatment of knee osteoarthritis. *Int J Tech Assess Health Care*. 2003;19:41-56.
64. Kelly MA, Moskowitz RW, Lieberman JR. Hyaluronan therapy: looking toward the future. *Amer J Orthopedics*. 2004;33:23-28.
65. Özgenel GY. Effects of hyaluronic acid on peripheral nerve scarring and regenration in rats. *Microsurgery*. 2003;23:575-581.
66. Polack FM, McNiece MT. The treatment of dry eyes with Na Hyaluronate (Healon): a preliminary report. *Cornea*. 1982;1:133.
67. Camber O, Lundgren P. Diffusion of some low molecular weight compounds in sodium hyaluronate. *Acta Pharm Suec*. 1985;22:315-320.
68. Lüke C, Dietlein T, Jacobi P, Konen W, Drieglstein GK. Intracorneal inclusion of high-molecular-weight sodium hyaluronate following detachment of Descemet's membrane during viscocanalostomy. *Cornea*. 2000;19:556-557.
69. Artola A, Alió JL, Bellot JL, Ruiz JM. Protective properties of viscoelastic substances (sodium hyaluronate and 2% hydroxymethylcellulose) against experimental free radical damage to the corneal endothelium. *Cornea*. 1993;12:109-114.
70. Percival SPB. Results of a clinical trial of sodium hyaluronate in lens implantation surgery. *Am Intraocular Implant Soc*. 1985;11:257-259.
71. Glasser DB, Matsuda M, Edelhouser HF. A comparison of the efficacy and toxicity of and intraocular pressure response to viscous solutions in the anterior chamber. *Arch Ophthalmol*. 1986;104:1819-1824.
72. Donnelly MJ, Hohen LT, Clark GM. Initial investigation of the efficacy and biosafety of sodium hyaluronate (Healon) as an aid to electrode array insertion. In: Clark GM, Cowan RS, eds. The International Cochlear Implant, Speech, and Hearing Symposium, 1994. *Ann Otol Rhinol Laryngol*. 1995;89:45-48.
73. Stenfors L-E, Berghem GD, Bloom GD, Helström S, Söderberg O. Exogenous hyaluronic acid (Healon) accelerates the healing of experimental myringotomies. *Auris-Nasus-Larynx (Tokyo)*. 1985;Suppl 1:217-218.
74. Laurent C, Söderberg O, Anniko M, Harwig S. Repair of chronic tympanic membrane perforations using applications of hyaluronan or rice papers prostheses. *J Oto-Rhino-Laryngology Rel Specialities*. 1991;53: 37-40.
75. Krauss MC. Recent advances in soft tissue augmentation. *Seminars Cutaneous Med Surg*. 1999;18:119-128.
76. Fujishiro M, Yahagi N, Kashimyra K, et al. Comparison of various submucosal injection solutions for maintaining mucosal elevation during endoscopic mucosal resection. *Endoscopy*. 2004;36:579-583.
77. Matsui Y, Inomata M, Izumi K, Sonoda K, Shiraishi N, Kitano S. Hyaluronic acid stimulates tumor-cell proliferation at wound sites. *Gastrointestinal Endoscopy*. 2004; 60:539-543.
78. Stevens MF, Hoppe M, Holthusen H, Lipfert P. Tetrodotoxin-induced conduction blockade is prolonged by hyalruonic acid with and without bupivacaine. *Acta Anaesthesiol*. 2004;48:128-134.
79. Cantor JO, Turino GM. Can exogenously administered hyaluronan improve respiratory function in patients with pulmonary emphysema? *Chest*. 2004;125:288-292.
80. McDermott ML, Edelhouser HF. Drug binding of opthalmic viscoelastic agents. *Arch Ophthalmol*. 1989; 107:261-263.
81. Park S, Kim JK, Suh H. Evaluation of antibiotic-loaded collagen-hyaluronic acid matrix as a skin substitute. *Biomaterials*. 2004;25:3689-3698.
82. Moroi Y, Fujita S, Fukagawa S, et al. Clinical evaluation of allogenic cultured dermal substitutes for intractable skin ulcers after tumor resection. *Eur J Dermatol*. 2004;14:172-176.
83. FDA. Synvisc premarket approval application: summary of safety and effectiveness data. 2000. Available at: http://www.fda.gov/edrh/pdf/p940015.html. Accessed January 5, 2005.

Distributed for Comment Only -- Do Not Cite or Quote

84. FDA. Summary of safety and effectiveness data: Nuflexxa. 2004. Available at: http://www.fda.gov/cdrh/mda/docs/p010029.html. Accessed January 5, 2005.
85. FDA. New device approval Restylane injectable gel: P020023. 2004. Available at: http://www.fda.gov/fdac/departs/2004/204upd.html. Accessed January 5, 2005.
86. Ramos-e-Silva M, Ribeiro de Castro MC. Hyaluronic acid in office practice. *Cosmetic Dermatol Update*. 2004;May/June:163-164.
87. Fraser JRE, Laurent TC, Laurent UBG. Hyaluronan: its nature, distribution, functions and turnover. *J Intern Med*. 1997;242:27-33.
88. United States Environmental Protection Agency (US EPA). Exposure Factors Handbook. 1997. Available at: http://cfpub.epa.gov/ncea/cfm/recordisplay.cfm?deid=12464&CFIC=1017888&CFTOKEN=55097998. Accessed June 12, 2006.
89. West DC, Hampson IN, Arnold F, Kumar S. Angiogenesis induced by degradation products of hyaluronic acid. *Science*. 1985;228:1324-1326.
90. Brecht M, Mayer U, Schlosser E, Prehm P. Increased hyaluronate synthesis is required for firoblast detachment and mitosis. *Biochem J*. 1986;239:445-450.
91. Kakehi K, Kinoshita M, Yasueda S. Hyaluronic acid: separation and biological implications. *J Chromatography*. 2003;797:347-355.
92. Spicer AP, McDonald JA. Eukaryotic hyaluronan syntheses. 1998. Available at: http://www.glycoforum.gr.jp/science/hyaluronan/HA07/HA07E.html. Accessed January 5, 2005.
93. Szirmai JA. Effect of steroid hormones on the glycosaminoglycans of target connective tissues. In: Balazs EA, Jeanloz RW, eds. *The Amino Sugars*. 1965;11b:129-154.
94. Pearce RH, Grimmer BJ. *The Nature of the Ground Substance*. New York, NY: Appleton; 1970.
95. Balazs EA, Gibbs DA. *The Rheological Properties and Biological Function of Hyaluronic Acid*. New York, NY: Academic Press; 1970.
96. Yates JR. *Mechanism of Water Uptake by Skin*. New York, NY: Wiley Interscience; 1971.
97. Laurent TC, Laurent UBG, Fraser JRE. Serum hyaluronan as a disease marker. *DUODECIM Ann Med*. 1996;28:241-253.
98. Balazs EA. *Sodium Hyaluronate and Viscosurgery*. New York, NY: John Wiley & Sons; 1983.
99. Balazs EA. Viscosurgery in the eye. *Ocular Inflammation Ther*. 1983;1:91-92.
100. Laurent TC, Pietruszkiewics A. The effect of hyaluronic acid on the sedimentation rate of other substances. *Biochem Biophys Acta*. 1961;49:258.
101. Balazs EA, Darzynkiewewics Z. *The effect of hyaluronic acid on fibroblast, mononuclear phagocytes and lymphocytes*. New York, NY: Academic Press; 1972.
102. Forrester JV, Wilkinson PC. Inhibition of leucocyte locomotion by hyaluronic acid. *J Cell Sci*. 1981;48:315.
103. Toole BP. Development role of hyaluronante. *Conect Tissue Res*. 1982;10:93.
104. Forrester JV, Lackie JM. Effect of hyaluronic acid on neutrophil adhesion. *J Cell Sci*. 1981;50:329-344.
105. Pisko EJ, Turner RA, Soderstrom LP, Panetti M, Foster SL, Treadway WJ. Inhibition of neutrophil phagocytosis and energy release by hyaluronic acid. *Clin Exp Rheumatol*. 1983;1:41.
106. Dahlgren C, Björkstén B. Effect of hyaluronic acid on polymorphonuclear leucocyte cell surface properties. *Scand J Haemotol*. 1982;28:376-380.
107. Greco RM, Iocono JA, Ehrlich HP. Hyaluronic acid stimulates human fibroblast proliferation within a collagen matrix. *J Cell Physiol*. 1998;177:465-473.
108. Prince CW. Roles of hyaluronan in bone resorption. *BMC Musculoskeletal Disorders*. 2004;5:12-14.
109. Shepard S, Becker H, Hartmann JX. Using hyaluronic acid to create a fetal-like environment in vitro. *Annals Plastic Surg*. 1996;36:65-69.
110. Iocono JA, Ehrlich HP, Keefer KA, Krummel TM. Hyaluronan induces scarless repair in mouse limb organ culture. *J Ped Surg*. 1998;33:564-567.
111. Weigel PH, Fuller GM, LeBoeuf RD. A model for the role of hyaluronic acid and fibrin in the early events during the inflammatory response and wound healing. *J Theor Biol*. 1986;119:219-234.
112. Tammi R, Tammi M. Hyaluronan in the epidermis. 1998. Available at: http://www.glcoforum/gr.jp/science/hyaluronan/HA04/HA04E.html. Accessed January 5, 2005.
113. Toole BP, Gross J. The extracellular matrix of the regenerating new limb: synthesis and removal of hyaluronate prior to differentiation. *Develop Biol*. 1971;25:57.
114. Toole BP, Underhill CB. Regulation of morphogenesis by the pericellular matrix. In: Yamada KM, ed. *Cell Interactions and Development*. New York, NY: John Wiley & Sons; 1983: 203.
115. Bychkov SM, Kuz'mina SA. Role of glycosaminoglycans and proteoglycans in erythrocyte aggregation and adhesion. *Bull Eksp Biol Med*. 1997;83:284-288.
116. Knudson CB, Toole BP. Changes in the pericellular matrix during differentiation of limb bud mesoderm. *Develop Biol*. 1985;112:308-318.
117. Salustri A, Fulop C. The role of hyaluronan during ovulation and fertilization. 1998. Available at: http://www.glycofourm.gr.jp/science/hyaluronan/HA03/HA03E.html. Accessed January 5, 2005.
118. Sliwa L. Hyaluronic acid and chemoattractant substance from follicular fluid: in vitro effect of human sperm migration. *Adv Reprod*. 1999;3:113-115.
119. Turley EA, Noble PW, Bourguignon LY. Signaling properties of hyaluronan receptors. *J Biol Chem*. 2002;277:4581-4584.

Distributed for Comment Only -- Do Not Cite or Quote

120. Toole BP. *Glycosaminoglycans in Morphogenesis*. New York, NY: Plenum Publ; 1981.
121. Toole BP, Goldberg RL, Chi-Rosso G, Underhill CB, Orkin RW. *Hyaluronate Cell Interaction*. New York, NY: Alan R Liss Inc; 1984.
122. Lark MW, Culp LA. Selective solubilization of hyaluronic acid from fibroblast substratum adhesive sites. *J Biol Chem*. 1982;257:14073-14080.
123. Abatangelo G, Cortivo R, Martelli M, Vecchia P. Dell detachment mediated by hyaluronic acid. *Exp Cell Res*. 1982;137:73-78.
124. Bernanke DH, Markwald RR. Effects of two glycosaminoglycans on seeding of cardiac cushion tissue cells into a collagen-lattice culture system. *Anat Rec*. 1984;210:25-31.
125. Erickson CA, Turley EA. Substrate formed by combinations of extracellualr matrix components alter neural crest cell motility in vitro. *J Cell Sci*. 1983;61:299-323.
126. Turley EA, Bowman P, Kytryk MA. Effects of hyaluronate and hyaluronate binding proteins on cell motile and contact behaviour. *J Cell Sci*. 1985;78:133-146.
127. Feinberg RN, Beebe DC. Hyaluronate in vasculogenesis. *Science*. 1983;10:1177-1179.
128. Font J, Aubery M. Inaccessibility of certain *Ricinus* lectin binding sites due to the increase in hyaluronic acid during chick embryo development. *Differentiation*. 1983;25:23-26.
129. Hamasima N. Effects of hyaluronic acid on the periimplantational development of mouse embros in vitro. *Develop Growth Differ*. 1982;24:353-357.
130. Toole BP, Trelstad RL. Hyaluronate production and removal during corneal development in the chick. *Develop Biol*. 1971;26:28-35.
131. Schoenwolf GC, Fischer M. Analysis of the effects of Streptomyces hyaluronidase on formation of the neural tube. *J Embryol Exp Morph*. 1983;73:1-15.
132. Silberstein GB, Daniel CW. Glycosaminoglycans in the basal lamina and extracellular matrix of serially aged mouse mammary ducts. *Mechanisms Ageing Develop*. 1984;24:151-162.
133. Sunderland CA, Bulmer JN, Luscombe M, Redman CWG, Stirrat GM. Immunohistological and biochemical evidence for a role of hyaluronic acid in the growth and development of the placenta. *J Reprod Immunol*. 1985;8:197-212.
134. Lamberg SI, Yuspa SH, Hascall VC. Synthesis of hyaluornic acid is decreased and synthesis of proteoglycans is increased when cultured mouse epidermal cells differentiate. *J Invest Derm*. 1986;86:659-667.
135. Forrester JV, Balazs EA. Inhibition of phagocytosis by high molecular weight hyaluronate. *Immunol*. 1980; 40:435-446.
136. Ahlgren T, Jarstrand C. Hyaluronic acid enhances phagocytosis of human monocytes in vitro. *J Clin Immunol*. 1984;4:246-249.
137. Cohn ZA, Parks E. The regulation of pinocytosis in mouse macrophages. II: factors inducing vesicle formation. *J Exp Med*. 1967;125:213-232.
138. Delmage JM, Powars DR, Jaynes PK, Allerton SE. The selective supression of immunogenicity by hyaluronic acid. *Ann Clin Lab Sci*. 1986;16:303-310.
139. Sheehan KM, DeLott LB, West RA, Bonnema JD, DeHeer DH. Hyaluronic acid of high molecular weight inhibits proliferation and induces cell death in U937 macrophage cells. *Life Sci*. 2004;75:3087-3102.
140. Forteza RM, Conner GE. Hyaluronan and airway mucosal defense. 2002. Available at: http://www.hlycoforum.gr.jp/science/hyaluronan/HA25/HA25E.html. Accessed January 5, 2005.
141. Clarris BJ, Fraser JRE, Rodda SJ. Effect of cell-bound hyaluronic acid on infectivity of Newcastle disease virus for human synovial cell in vitro. *Ann Rheum Dis*. 1974;33:240-242.
142. Trudel J, Massia SP. Assessment of the cytotoxicity of photocrosslinked dextran and hyaluronan-based hydrogels to vascular smooth muscle cells. *Biomaterials*. 2002;23:3299-3307.
143. Trommer H, Warewig S, Bottcher R, et al. The effects of hyaluronan and its fragments on lipid models exposed to UV irradiation. *Int J Pharm*. 2003;254: 223-224.
144. Sattar A, Rooney S, Kumar D, Pye DC, West I, Ledger P. Application of angiogenic oligosaccharides of hyaluronan increases blood vessel numbers in rat skin. *J Invest Derm*. 1994;103:576-579.
145. Brown TJ, Alcorn D, Fraser JRE. Absorption of hyaluronan applied to the surface of intact skin. *J Invest Derm*. 1999;113:740-746.
146. Iwata Y, Akima K, Sato I, Hasegawa M. Studies of the metabolic fate of sodium hyaluronate (SL-1010) (IV): transfer into the fetus, and excretion into milk and bile after intravenous administration of SL-1010 in rats [in Japanese]. *Yakuri To Chiryo*. 1991;19:379-389.
147. Laurent UBG, Fraser JRE, Laurent TC. An experimental technique to study the turnover of concentrated hyaluronan in the anterior chamber of the rabbit. *Exp Eye Res*. 1988;46:49-58.
148. Lebel L, Fraser JRE, Kimpton WS, Gabrielsson J, Berdin B, Laurent TC. A pharmacokinetic model of intravenously adminstered hyaluronan in sheep. *Pharm Res*. 1989;6:677-682.
149. Laurent UBG, Fraser JRE. Disappearance of concentrated hyaluronan from the anterior chamber of monkey eyes. *Exp Eye Res*. 1990;51:65-69.

Distributed for Comment Only -- Do Not Cite or Quote

150. Allen SR, Fraser FRE, Laurent UBG, Reed RK, Laurent TC. Turnover of hyaluronan in the rabbit pleural space. *J Appl Physiol*. 1992;73:1457-1460.
151. Edelstam GAB, Laurent UBG, Lundkvist ÖE, Fraser JRE, Laurent TC. Concentration and turnover of intraperitoneal hyaluronan during inflammation. *Inflammation*. 1992;16:459-469.
152. Scuri M, Abraham WM. Hyaluronan blocks human neutrophil elastase (HNE)-induced airway responses in sheep. *Pulmonary Pharm Therapeutics*. 2003; 16:335-340.
153. Surendrakumar K, Martyn GP, Hodgers ECM, Jansen M, Blair JA. Sustained release of insulin from sodium hyaluronate based dry powder formulations after pulmonary delivery to beagle dogs. *J Controlled Release*. 2003;91:385-394.
154. Bousquet M-T, Ågerup B. Restylane lip implantation: European experience. *Oper Techniq Ocul Orbital Recon Surg*. 1999;2:172-176.
155. FDA. Summary of safety and effectiveness data: Deflux. 2001. http://www.fda.gov/cdrh/mda/docs/p000029.html. Accessed January 5, 2005.
156. Humphrey JH. Antigenic properties of hyaluronic acid. *Biochem J*. 1943;37:460-463.
157. Quinn RW, Singh KP. Antigenicity of hyaluronic acid. *Proc Soc Exp Biol Med*. 1957;95:290-294.
158. Hammerman D, Sandson J. Immunologic cross-reactions between streptococcal hyaluronic acid and proteoglycans of human connective tissue. In: Balazs EA, ed. *Chemistry and Molecular Biology of the Intercellular Matrix*. London: Academic Press; 1970;3:1537-1549.
159. Richter AW. Non-immunogenicty of purified hyaluronic acid preparation tested by passive cutaneous anaphaxis. *Int Arch Allergy Appl Immunol*. 1974;47: 211-217.
160. Uhlin A, Richter W, Björkstén B. Enhanced humoral immune response in rats by hyaluronic acid. *Int J Immy-nopharmacol*. 1982;4:529-532.
161. Goomer RS, Leslie K, Maris T, Amiel D. Native hyaluronan produces less hypersensitivity than cross-linked hyaluronan. *Clin Orthop Related Res*. 2005;434: 239-245.
162. Anniko M, Hellström S, Laurent C. Reversible changes in inner ear functions following hyaluronan application in the middle ear. *Acta Otolaryngol (Stoch)*. 1987;Suppl 442:72-75.
163. Bjurström B, Slepeck N, Angelborg C. A histopathological study of the inner ear after the adminstration of hyaluronan into the middle ear of the guinea pig. *Acta Otolaryngol (Stoch)*. 1987;Suppl 442:62-65.
164. Engström B, Bjurström S, Jansson B, Engström H, Angelborg C. An ultrastructural and functional study of the inner ear after adminstration of hyaluronan into the middle ear of the guinea pig. *Acta Otolaryngol (Stoch)*. 1987;442:66-71.
165. Lim Y-J, Sim W-S, Kim Y-C, Lee S-C, Choi Y-L. The neurotoxicity of epidural hyaluronic acid in rabbits: a light and electron microscopic examination. *Anesth Analg*. 2003;97:1716-1720.
166. Furuhashi T, Takei A, Nakayoshi H. Reproduction studies of sodium hyaluronate (SPH) (4) perinatal and postnatal test in rats [in Japanese]. *NRI Life Sci*. 1985;29:139-153.
167. Wada K, Hashimoto Y, Mizutani M, Tanaka C. Reproductive and developmental toxicity studies of sodium hyaluronate (SL-1010) (III) [in Japanese]. *Jpn Pharmocol Ther*. 1991;19:111-119.
168. Tateda C, Nagaoka S, Nagai T, Nakamura T. Reproductive and developmental toxicity study (IV) on sodium hyaluronate (SH) (4): study on subcutaneous administration to rabbits during organogenesis period [in Japanese]. *Jpn Pharmocol Ther*. 1992;20:51-58.
169. Matsuura T, Nakajima H, Maeda H, et al. Teratological study of high molecular weight sodium hyaluronate (NRD101) in rabbits [in Japanese]. *Yakuri To Chiryo*. 1994;22:205-213.
170. Sugiyama C, Yagame O. Mutagenicity studies of sodium hyaluronate (SL-1010). I: reverse mutation test on bacteria [in Japanese]. *Jpn Pharmocol Ther*. 1991;19:S177-S181.
171. Aruga F, Miwa Y, Fuzimura T, Ohata S. Micronucleus test of sodium hyaluronate (SH) using mice [in Japanese]. *Jpn Pharmocol Ther*. 1992;20:775-777.
172. Onishi M, Nagata T, Saigou K, Sameshima H, Nagata R. Mutagenicity studies of sodium hyaluronate (SH) [in Japanese]. *Yakuri To Chiryo*. 1992;20:767-774.
173. Aruga F, Nagasawa Y, Miwa Y, Tanaka R, Sugiyama H, Ota S. Mutagenicity test of high molecular weight sodium hyaluronate (NRD101) [in Japanese]. *Yakuri To Chiryo*. 1994;22:S627-S636.
174. McBride WH, Bard JBL. Hyaluronidase-sensitive halos around adherent cells: their role in blocking lymphocyte-mediated cytolysis. *J Exp Med*. 1979;149: 507-515.
175. Asplund T, Versnel MA, Laurent TC, Heldin P. Human mesothelioma cells produce factors that stimulate the production of hyaluronan by mesothelial cells and fibroblasts. *Cancer Res*. 1993;53:388-392.
176. Zhang L, Underhill CB, Chen L. Hyaluronan on the surface of tumor cells is correlated with metastatic behavior. *Cancer Res*. 1995;55:428-433.
177. Li Y, Heldin P. Hyaluronan production increases the malignant properties of mesothelioma cells. *Br J Cancer*. 2001;85:600-607.
178. Enegd B, King JAJ, Stylli S, Paradiso L, Kaye AH, Novak U. Overexpression of hyaluronan synthase-2 reduces the turmorigenic potential of glioma cells

Distributed for Comment Only -- Do Not Cite or Quote

lacking hyaluronidase activity. *Neurosurgery*. 1992;50: 1311-1318.

179. Ghatak S, Misra S, Toole BP. Hyaluronan oligosaccharides inhibit anchorage-independent growth of tumor cells by suppressing the phosphoinositide 3-kinase/Akt cell survival pathway. *J Biol Chem*. 2002;277: 38013-38020.
180. Jacobson A, Rahmanian M, Rubin K, Heldin P. Expression of hyaluronan synthase 2 or hyaluronidase 1 differentially affect the growth rate of transplantable colon carcinoma cell tumors. *Int J Cancer*. 2002; 102:212-219.
181. Udabage L, Brownlee GR, Waltham M, et al. Antisense-mediated suppression of hyaluronan synthase 2 inhibits the turmorigenesis and progression of breast cancer. *Cancer Res*. 2005;65:6139-6150.
182. Sugahara K, Hirata T, Hayasaka H, Stern R, Murai T, Miyasaka M. Tumor cells enhance their own CD44 cleavage and motility by generating hyaluronan fragments. *J Biol Chem*. 2006;281:5861-5868.
183. Shuster S, Frost GI, Csóka AB, Formby B, Stern R. Hyaluronidase reduces human breast cancer zenografts in SCID mice. *Int J Cancer*. 2002;102:192-197.
184. Misra S, Ghatak S, Zoltan-Jones A, Toole BP. Regulation of multi-drug resisitance in cancer cells by hyaluronan. *J Biol Chem*. 2003;278:25285-25288.
185. Wang SJ, Bourguignon LYW. Hyaluronan-CD44 promotes phospholipase C-mediated Ca2+ signaling and cisplatin resistance in head and neck cancer. *Arch Otolaryngol Head Neck Surg*. 2006;132:19-24.
186. Yin D, Ge Z, Yang W, Liu C, Yuan Y. Inhibition of tumor metastasis in vivo by combination of paclitaxel and hyaluronic acid. *Cancer Letters*. 2006;243:71-79.
187. Laugier J-P, Shuster S, Rosdy M, Csóka AB, Stern R, Maibach HI. Topical hyaluronidase decreases hyaluronic acid and CD44 in human skin and in reconstituted human epidermis: evidence that hyaluronidase can permeate the stratum corneum. *Br J Dermatol*. 2000;142:226-233.
188. Fraser JRE, Appelgren L-E, Laurent TC. Tissue uptake of circulating hyaluronic acid: a whole body autoradiographic study. *Cell Tissue Res*. 1983;233:285-293.
189. Engström-Laurent A, Laurent UBG, Lilja K, Laurent TC. Concentration of sodium hyalruonate in serum. *Scand J Clin Lab Invest*. 1985;45:497-504.
190. Fraser JRE, Kimpton WG, Laurent TC, Cahill RNP, Vakakis N. Uptake and degradation of hyaluronan in lymphatic tissue. *Biochem J*. 1988;256:153-158.
191. Fraser JRE, Laurent TC, Pertoft H, Baxter E. Plasma clearance, tissue distribution and metabolism of hyaluronic acid injected intravenously in the rabbit. *Biochem J*. 1981;200:415-424.
192. Fraser JRE, Laurent TC, Engström-Laurent A. Elimination of hyaluronic acid from the bloodstream in the human. *Clin Exp Pharmacol Physiol*. 1984; 11:17-25.
193. Smedsrød B, Pertoft H, Eridsson S, Fraser JRE, Laurent TC. Studies in vitro on the uptake and degradation of sodium hyaluronate in rat liver endothelial cells. *Biochem J*. 1984;223:617-626.
194. Fraser JRE, Alcorn D, Laurent TC, Robinson AD, Ryan GB. Uptake of circulating hyaluronic acid by the rat liver: cellular localization in situ. *Cell Tissue Res*. 1985;242:505-510.
195. Rodén L, Campbell P, Fraser JRE, Laurent TC, Pertoft H, Thompson JN. Enzymic pathways of hyaluronan catabolism. *Ciba Found Symp*. 1989;143:60-86.
196. Laurent TC, Fraser JRE. Hyaluronan. *FASEB J*. 1992; 6:2397-2404.
197. Deguine V, Menasche M, Ferrari P, Fraisse L, Pouliquen Y, Rober L. Free radical depolymerization of hyaluronan by Maillard reaction products: roe in liquefaction of aging vitreous. *Int J Biol Macromol*. 1998;22:17-22.
198. Ågren UM, Tammi RH, Tammi MI. Reactive oxygen species contribute to epidermal hyaluronan catobolism in human skin organ culture. *Free Radic Biol Med*. 1997;23:996-1001.
199. Engström-Laurent A, Lööf L, Nyberg A, Schröder T. Increased serum levels of hyaluronate in liver disease. *Hepatology*. 1985;5:638-642.
200. Fraser JRE, Engström-Laurent A, Nyberg A, Laurent TC. Removal of hyaluronic acid from circulation in rheumatoid disease and primary biliary cirrhosis. *J Lab Clin Med*. 1986;107:79-85.
201. Gibson PR, Fraser JRE, Brown TJ, et al. Hemodynamic and liver function predictors of serum hyaluronan in alcoholic liver disease. *Hepatology*. 1992;15: 1054-1059.
202. Engström-Laurent A, Hällgren R. Circulating hyaluronate in rheumatoid arthritis: relationship to inflammatory activity and the effect of corticosteroid therapy. *Ann Rheum Dis*. 1985;44:83-88.
203. Homicz MR, Watson D. Review of infectable material for soft tissue augmentation. *Facial Plastic Surg*. 2004;20:21-29.
204. Draelos ZD. The use of biological additives in cosmetic products: part 1. *Cosmetic Derm*. 1994;7:16-17.
205. Brown MB, Marriot C, Martin GP. The effect of hyaluronan on the in vitro deposition of diclofenac within the skin. *Int J Tissue Reac*. 1995;17:133-140.
206. Brown MB, Ingham S, Moore A, et al. *A Preliminary Study of the Effect of Hyaluronan in Drug Delivery*. London, England: Royal Society of Medicine Press; 1995.
207. Lin W, Maibach HI. *Percutaneous Absorption of Diclofenac in Hyaluronic Acid Gel: In Vitro Study in Human Skin*. London, England: Royal Society of Medicine Press; 1996.

Distributed for Comment Only -- Do Not Cite or Quote

208. Brown MB, Hanpanitcharoen M, Martin GP. An in vitro investigation into the effect of glycosaminoglycans on the skin partitioning and deposition of NSAIDs. *Int J Pharm*. 2001;225:113-121.
209. Brown MB, Forbes B, Martin GP. *The Use of Hyaluronan in Tropical Delivery*. Cambridge, England: Woodhead Publishers; 2002.
210. Amr SK. The effect of homogenised skin on the activity of licosamide antibiotics. Paper presented at: Proceedings of Millennium Congress of Pharmaceutical Science; April 16-20, 2000; San Francisco, CA.
211. Brown MB, Moore A. *The Effects of a Novel Formulation of Cyclosporin on Antibody and Cell-Mediated Immune Reactions in the Pleural Cavity of Rats*. London, England: Royal Society of Medicine Press; 1996.
212. Mazir T, Martin GP, Brown MB. Dermal delivery cyclosporin A entrapped liposomal gells: preformulation Franz Cell diffusion studies using silastic membranes. *STP Pharm*. 2001;3:1429.
213. Jarvis B, Figgitt DP. Topical 3% dicolfenac in 2.5% hyaluronic acid gel: a review of its use in patients with actinic keratoses. *Amer J Clin Dermatol*. 2003;4:203-213.
214. De La Motte CA, Hascall VC, Calabro A, Yen-Leiberman B, Strong SA. Mononuclear leukocytes preferentially bind via CD44 to hyaluronan on human intestinal mucosal smooth muscle cells after virus infection or treatment with poly(I.C.). *J Biol Chem*. 1999;274:30747-30755.
215. De La Motte CA, Hascall VC, Drazba J, Bandyopadhyay SK, Strong SA. Mononuclear leukocytes bind to specific hyaluronan structures on colon mucosal smooth muscle cells treated with polyinosinic acid: polycytidylic acid. *Amer J Path*. 2003;163: 121-133.
216. Maytin EV, Chung HH, Seetharaman VM. Hyalronan participates in the epidermal response to disruption of the permeability barrier in vivo. *Amer J Path*. 2004;165:1331-1341.
217. Engström-Laurent A, Hallgren R. Circulating hyaluronic acid levels vary with physical activity in healthy subjects and in rheumatoid arthritis patients: relationship to synovitis mass and morning stiffness. *Arthritis Rheum*. 1987;30:1333-1338.
218. Lundin A, Engström-Laurent A, Hallgren R, Michaelsson G. Circulating hyaluronate in psoriasis. *Br J Dermatol*. 1985;112:663-671.
219. Berg S, Brodin B, Hesselvik F, Laurent TC, Maller R. Elevated levels of plasma hyaluronan in septicaemia. *Scand J Clin Lab Invest*. 1988;48:727-732.
220. Håkansson L, Hällgren R, Venge P. Regulation of granulocyte function by hyaluronic acid: in vitro and in vivo effects on phagocytosis, locomotion, and metabolism. *J Clin Invest*. 1980;66:298-305.
221. Tammi R, Paukkonen K, Wang C, Horsmanheimo M, Tammi M. Hyaluronan and CD44 in psoriatic skin, intense staining for hyaluronan on dermal capillary loops and reduced expression of CD44 and hyaluronan in keratinocyte-leukocyte interfaces. *Arch Dermatol Res*. 1994;286:21-29.
222. Richter AW, Ryde EM, Zetterström EO. Non-immunogenicity of a purified sodium hyaluronate preparation in man. *Int Arch Allergy Appl Immunol*. 1979;59:45-48.
223. Altman RD, Åkermark Bearleiu AD, Schnitzer T. Efficacy and safety of a single intra-articular injection of non-animal stabilized hyaluronic acid (NASHA) in patients with osteoarthritis of the knee. *Osteoarthritis Cartilage*. 2004;12:642-649.
224. Neustadt DH. Long-term efficacy and safety of intra-articular sodium hyaluronate (Hyalgan) in patients with osteoarthritis of the knee. *Clin Exp Rheumatol*. 2003;21:307-311.
225. Chen AL, Desai P, Adler E, De Cesare PE. Granulomatous inflammation after hylan G-F 20 viscosupplementation of the knee: a report of six cases. *J Bone Joint Surg*. 2002;84A:1142-1147.
226. Olenius M. The first clinical study using a new biodegradable implant for the treatment of lips, wrinkles, and folds. *Aesth Plast Surg*. 1998;22:97-101.
227. Lowe NJ, Maxwell CA, Lowe P, Duick MG, Shah K. Hyaluronic acid skin fillers: adverse reactions and skin testing. *J Am Acad Dermatol*. 2001;45:930-933.
228. Friedman PM, Mafong EA, Dauvar ANB, Geronems RG. Safety data of injectable nonanimal stabilized hyaluronic acid gel for soft tissue augmentation. *Dermatol Surg*. 2002;28:491-494.
229. André P. Evaluation of the safety of a non-animal stabilized hyaluronic acid (NASHA - Q - Medical, Sweden) in European countries: a retrospective study from 1997 to 2001. *J Eur Acad Dermatol Venerol*. 2004; 18:422-425.
230. Toole BP. Mini review: hyaluronan promotes the malignant phenotype. *Glycobiology*. 2002;12:37R-42R.
231. Auvinen P, Tammi R, Parkkinen J, et al. Hyaluronan in periturmoral stroma and malignant cells associates with breast cancer spreading and predicts survival. *Amer J Path*. 2000;156:529-536.
232. Gately CL, Muul LM, Greenwood MA, et al. In vitro studies on the cell-mediated immune response to human brain tumors. II: leucocyte-induced coats of glycosaminoglycan increase the resistance of glioma cells to cellular immune attack. *J Immunol*. 1984;133: 3387-3395.
233. Setälä LP, Tammi MI, Tammi RH, et al. Hyaluronan expression in gastric cancer cells is associated with the local and nodal spread and reduced survival rate. *Br J Cancer*. 1998;79:1133-1138.
234. Anttila MA, Tammi RH, Tammi MI, Syrjänen KJ, Saarikoski SV, Kosma V-M. High levels of stromal hyaluronan predict poor disease outcome

Distributed for Comment Only -- Do Not Cite or Quote

in epithelial ovarian cancer. *Cancer Res*. 2000;60: 150-155.

235. Stenvinkel P, Heimbürger O, Wang T, Lindholm B, Bergström J, Elinder C-G. High serum hyaluronan indicates poor survival in renal replacement therapy. *Amer J Kidney Dis*. 1999;34:1083-1088.
236. Zeng C, Toole BP, Kinney SD, Kuo J-W, Stamenkovic I. Inhibition of tumor growth in vivo by hyaluronan oligomers. *Int J Cancer*. 1998;77:396-401.
237. Toole BP. Hyaluronan: from extracellular glue to pericellular cue. *Nature Rev*. 2004;4:528-539.
238. Bracey AW, Wu AHB, Aceves J, Chow T, Carlile S, Hoots WK. Platelet dysfunction associated with Wilm's tumor and hyaluronic acid. *Am J Hematol*. 1987;24: 247-257.
239. Heldin P, Laurent TC, Heldin C-H. Effect of growth factors on hyaluronan synthesis in cultured human fibroblasts. *Biochem J*. 1989;258:919-922.
240. Fraizer GE, Bowen-Pope DF, Vogel AM. Production of platelet-derived growth factor by cultured Wilms' tumor cells and fetal kidney cells. *J Cell Physiol*. 1987;133:169-174.
241. Deutsch HF. Some properties of a human serum hyaluronic acid. *J Biol Chem*. 1957;224:767-774.
242. Tomasi TB, Van B, Roberson W, Naeye R, Reichlin M. Serum hyperviscosity and metabolic acidosis due to circulating hyaluronic acid. *J Clin Invest*. 1966;45: 1080-1081.
243. Morse BS, Nussbaum M. The detection of hyaluronic acid in the serum and urine of a patient with nephroblastoma. *Am J Med*. 1967;42:966-1002.
244. Allerton SE, Beierle JW, Powars DR, Bavetta LA. Abnormal extracellular components in Wilm's tumor. *Cancer Res*. 1970;30:679-683.
245. Wu AHB, Parker OS, Ford L. Hyperviscosity caused by hyaluronic acid in serum in a case of Wilms' tumor. *Clin Chem*. 1984;30:914-916.
246. Longaker MT, Adzick NS, Sadigh D, et al. Hyaluronic acid-stimulating activity in the pathophysiology of Wilms' tumors. *J Nat Cancer Inst*. 1990;82:195-199.
247. Kumar S, West DC, Ponting JM, Gattameaeni HR. Sera of children with renal tumours contain low-molecular-mass hyaluronic acid. *Int J Cancer*. 1989; 44:445-448.
248. Kumar S, West DC, Ponting JM. Hyaluronic acid and childhood renal tumours. *J Nat Cancer Inst*. 1990;82:973-974.
249. Meyer K, Chaffe E. Hyaluronic acid in the pleural fluid associated with a malignant tumor involving the pleura and peritoneum. *J Biol Chem*. 1940;133:83-91.
250. Roboz J, Greaves J, Lilides D, Chahinian AP, Holland JF. Hyaluronic acid content of effusions as a diagnostic aid for malignant mesothelioma. *J Cancer Res*. 1985;45:1850-1854.
251. Frebourg T, Lerebours G, Delpech B, et al. Serum hyaluronate in malignant pleural mesothelioma. *Cancer J*. 1987;59:2104-2107.
252. Dahl IMS, Solheim ØP, Erikstein B, Müller E. A longitudinal study of the hyaluronan level in the serum of patients with malignant mesothelioma under treatment. *Cancer*. 1989;64:68-73.
253. Chevrier A, Girard B, Delpech B, Gilbert D. Inhibition of active E rosette forming T lymphocytes by hyaluronic acid: evidence of a receptor for hyaluronic acid on a lymphocyte subpopulation. *Biomedicine*. 1982;36:100-103.
254. Stern R. Hyaluronan metabolism: a major paradox in cancer biology. *Pathologie Biologie*. 2005;53:372-382.
255. Cooper EH, Forbes MA. Serum hyaluronic acid levels in cancer. *Br J Cancer*. 1988;58:668-669.
256. Dahl IMS, Laurent TC. Concentration of hyaluronan in the serum of untreated cancer patients with special reference to patients with mesothelioma. *Cancer*. 1988;62:326-330.
257. Schüller J, Fogl U, Bosse CH, Schernthaner G. Serum hyaluronic acid levels in cancer patients with different tumor types. Abstract presented at: Sixth International Conference on Differentiation of Normal Neoplastic Cells; July 20-August 2 1990; Vancouver, Canada.
258. Delpech B, Chevallier B, Reinhardt N, et al. Serum hyaluronan (hyaluronic acid) in breast cancer patients. *Int J Cancer*. 1990;46:388-390.
259. Ponting J, Howell A, Pye D, Kumar S. Prognostic relevance of serum hyaluronan levels in patients with breast cancer. *Int J Cancer*. 1992;52:873-876.
260. Abildgaard N, Lanng Nielsen JL, Heickendorff L. Connective tissue components in serum in multiple myeloma: analyses of propeptides of type I and type III procollagens, type I collagen telopeptide and hyaluronan. *Am J Hematol*. 1994;46:173-178.
261. Pauli BU, Knudson W. Tumor invasion: a consequence of destructive and compositional matrix alterations. *Human Path*. 1988;19:628-639.
262. Hedman M, Arnberg H, Wernlund J, Riska H, Brodin O. Tissue polypeptide antigen (TPA), hyaluronan and CA 125 as serum markers in malignant mesothelioma. *Anticancer Res*. 2003;23:531-536.
263. Karvinen S, Kosma V-M, Tammi MI, Tammi R. Hyaluronan, CD44 and versican in epidermal keratinocyte tumours. *Br J Dermatol*. 2003;148:86-94.
264. Yabushita H, Kishida T, Fusano K, et al. Role of hyaluronan and hyaluronan synthase in endometrial cancer. *Oncology Reports*. 2005;131:101-105.
265. Boregowda RK, Appaiah HN, Siddaiah M, et al. Expression of hyaluronan in human tumor progression. *J Carcinog*. 2006;5:2.
266. Carte MD, González LO, Luz Lamelas M, et al. Expression and clinical signification of cytosolic hyaluronan

Distributed for Comment Only -- Do Not Cite or Quote

levels in invasive breast cancer. *Breast Cancer Res Treatment*. 2006;97:329-337.
267. Ali Y, Weinstein M, Jokl P. Acute pseudogout following intra-articular injection of high molecular weight hyaluronic acid. *Amer J Med*. 1999;107:641-642.
268. Allen E, Krohn K. Adverse reactions to hylan G-F 20. *J Rheumotol*. 2000;27:6.
269. Lupton JR, Alster TS. Cutaneous hypersensitivity reaction to injectable hyaluronic acid gel. *Dermatol Surg*. 2000;26:135-137.
270. Raulin C, Greve B, Hartschuh W, Soegding K. Exudative granulomatous reaction to hyaluronic acid (Hylaform). *Contact Dermatitis*. 2000;43:178-179.
271. Bernardear C, Bucki B, Lioté F. Acute arthritis after intra-articular hyaluronate injection: onset of effusions without crystal. *Ann Rheum Dis*. 2001;60:518-520.
272. Kavouni A, Stanec JJ. Human antihyaluronic acid antibodies. *Dermatol Surg*. 2002;28:359-360.
273. Hönig JF, Brink U, Korabiowska M. Severe granulomatous allergic tissue reaction after hyaluronic acid injection in the treatment of facial lines and its surgical correction. *J Craniofacial Surg*. 2003;14:197-200.
274. Lombardi T, Samson J, Plantier F, Husson C, Küffer R. Orofacial granulomas after injection of cosmetic fillers: histopathologic and clinical study of 11 cases. *J Oral Pathol Med*. 2004;33:115-120.
275. Jordan DR. Delayed inflammatory reaction to hyaluronic acid (Restylane). *Ophthal Plastic Reconstr Surg*. 2005;21:401-402.
276. Patel VJ, Bruck MC, Katz BE. Hypersensitivity reaction to hyaluronic acid with negative skin testing. *Plastic Reconstructive Surg*. 2006;117:92e-94e.
277. Brown MB, Jones SA. Hyaluronic acid: a unique topical vehicle for the localized delivery of drugs to the skin. *J Eur Acad Dermatol Venerol*. 2005;19:308-318.
278. Rosato A, Banzato A, De Luca G, et al. HYTADI-p20: a new paclitaxel-hyaluronic acid hydrosoluble bioconjugate for treatment of superficial bladder cancer. *Urol Oncol Sem Orig Invest*. 2006;24:207-215.
279. Goa KL, Benfield P. Hyaluronic acid: a review of its pharmacology and use as a surgical aid in ophthalmology, and its therapeutic potential in joint disease. *Drugs*. 1994;47:536-566.
280. Laurent TC. "The tree": hyaluronan research in the 20th century. 2002. Available at: http://www.glcoforum.gr.jp/science/hyaluronan/HA23/HA23E.html. Accessed January 5, 2005.
281. Ota R, Hashimoto Y, Matsumoto A, Mizutani M, Tanaka C. Reproductive and developmental toxicity studies of sodium hyaluronate (SL01010) (IV): perinatal and postnatal study in rats [in Japanese]. *Yakuri To Chiryo*. 1991;19:121-135.
282. Tanaka C, Sasa H, Hirama S, et al. Reproductive and developmental toxicity studies of sodium hyaluronate (SL-1010) (I): adminstration test in rats before pregnancy and in early stage of pregnancy [in Japanese]. *Jpn Pharmocol Ther*. 1991;19:81-92.
283. Tanaka C, Sasa H, Hirama S, et al. Reproductive and developmental toxicity studies of sodium hyaluronate (SL-1010) (II): teratogencity study in rats (study on administration during the period of fetal organogenesis in rats) [in Japanese]. *Jpn Pharmocol Ther*. 1991;19:93-110.
284. Ono C, Iwama A, Nakajima Y, Kitsuya A, Nakumura T. Reproductive and developmental toxicity studies on sodium hyaluronate (SH) (I): study on subcutaneous administration to rats during the period of organogenesis [in Japanese]. *Yakuri To Chiryo*. 1992;20:11-26.
285. Ono C, Fufiwara Y, Koura S. Reproductive and developmental toxicity study on sodium hyaluronate (SH) (2): study on subcutaneous administration to rats prior to and in the early stages of pregnancy [in Japanese]. *Yakuri To Chiryo*. 1992;20:27-35.
286. Ono C, Ishitobi H, Kozuka K, Konagai S, Nakamura T. Reproductive and developmental toxicity study (III): on sodium hyaluronate (SH) [in Japanese]. *Yakuri To Chiryo*. 1992;20:37-50.
287. Matsuura T, Nakajima H, Maeda H, et al. Teratological study of high molecular weight sodium hyaluronate (NRD101) in rats [in Japanese]. *Yakuri To Chiryo*. 1994;22:185-203.
288. Matsuura T, Nakajima H, Aeda H, et al. Perinatal and postnatal study of high molecular weight sodium hyaluronate (NRD101) in rats [in Japanese]. *Yakuri To Chiryo*. 1994;22:215-233.
289. Hattori M, Inoue S, Ogura H, et al. Reproductive and developmental toxicity study of a 1% solution sodium hyaluronate (SI-4402). 1: fertility study in rats by subcutaneous administration [in Japanese]. *Oyo Yakuri*. 1995;A50:93-103.
290. Kumada J, Nishiwaki K, Iriyama K, et al. Reproductive and developmental toxicity study of a 1% solution of sodium hyaluronate (SI-4402) [in Japanese]. *Oyo Yakuri*. 1995;50:105-122.
291. Carrabba M, Paresce E, Angelini M, Re KA, Torchiana EEM, Perbellini A. The safety and efficacy of different dose schedules of hyaluronic acid in the treatment of painful osteoarthritis of the knee with joint effusion. *Eur J Rheumatol Inflamm*. 1995;15:25-31.
292. Bragantini AM, Cassini M, DeBastiani B, Perebellini A. Controlled single-blind trial of intraarticularly-injected hyaluronic acid (Hyalgan) in osteoarthritis of the knee. *Clin Trial J*. 1987;24:333-341.
293. Grecomoro G, Martorana U, DiMarco C. Intra-articular treatment with sodium hyaluronate in gonarthrosis: a controlled clincal trial versus placebo. *Pharmacotherapeutica*. 1987;5:137-141.
294. Dixon ASF, Jacoby RK, Berry H, Hamiton EBD. Clinical trial of intraarticular injection of sodium

Distributed for Comment Only -- Do Not Cite or Quote

hyaluronate in patients with osteoarthritis of the knee. *Curr Med Res Opin*. 1988;11:205-213.

295. Dougados M, Mguyen M, Listrat V, Amor B. High molecular weight sodium hyaluronate (hyalectin) in osteoarthritis of the knee: a 1 year placebo-controlled trial. *Osteoarthritis Cartilage*. 1993;1:97-103.
296. Henderson EB, Smith EC, Pegley F, Blake DR. Intra-articular injections of 750 kDa hyaluronan in the treatment of osteoarthritis: a randomised single centre double-blind trial of 91 patients demonstrating lack of efficacy. *Ann Rheum Dis*. 1994;53:529-534.
297. Corrado EM, Peluso GF, Gigliotti S, et al. The effects of intra-articular administration of hyaluronic acid on osteoarthritis of the knee: a clinical study with immunological and biochemical evaluations. *Eur J Rheumatol Inflamm*. 1995;15:47-56.
298. Listrat V, Ayral X, Patarnello F, et al. Arthroscopic evaluation of potential structure modifying activity of hyaluronan (Hyalgan) in osteoarthritis of the knee. *Osteoarthritis Cartilage*. 1997;5:153-160.
299. Altman RD, Moskowitz R. Intraarticular sodium hyaluronate (Hyalgan) in the treatment of patients with osteoarthritis of the knee: a randomized clinical trial. *J Rheumatol*. 1998;25:2203-2212.
300. Huskisson EC, Donnelly S. Hyaluronic acid in the treatment of osteoarthritis of the knee. *Rheumatology*. 1999;38:602-607.
301. Wobig M, Dickhut A, Maier R, Vetter G. Viscosupplementation with hylan G-F 20: a 26-week controlled trial of efficacy and safety in the osteoarthritic knee. *Clin Ther*. 1998;20:410-423.
302. Puhl W, Bernau A, Greiling H, et al. Intra-articular sodium hyaluronate in osteoarthritis of the knee: a multicenter, double-blind study. *Osteoarthritis Cartilage*. 1993;1:233-241.
303. Dahlberg L, Lohmander LS, Ryd L. Intraarticular injections of hyaluronan in patients with cartilage abnormalities and knee pain. *Arthritis Rheum*. 1994;37:521-528.
304. Lohmander LS, Dalén N, Englund G, et al. Intra-articular hyaluronan injections in the treatment of osteoarthritis of the knee: a randomised, double blind, placebo controlled multicentre trial. *Ann Rheum Dis*. 1996;55:424-431.
305. Wu J-J, Shih L-Y, Hsu H-C, Chen T-H. The double-blind test of sodium hyaluronate (ARTZ) on osteoarthritic knees. *Chin Med J (Taipei)*. 1997; 59:99-106.

For reprints and permissions queries, please visit SAGE's Web site at http://www.sagepub.com/journalsPermissions.nav

Draft 2022 CIR Priorities

Concentration of use by FDA Product Category – Hyaluronates Not Previously Reviewed by CIR*

Sodium Acetylated Hyaluronate
Hydrolyzed Hyaluronic Acid
Hydrolyzed Sodium Hyaluronate
Hydrolyzed Calcium Hyaluronate
Zinc Hydrolyzed Hyaluronate

Ingredient	**Product Category**	**Maximum Concentration of Use**
Sodium Acetylated Hyaluronate	Foundations	0.002%
Sodium Acetylated Hyaluronate	Face and neck products Not spray	 0.1%
Sodium Acetylated Hyaluronate	Moisturizing products Not spray	 0.0085-0.1%
Sodium Acetylated Hyaluronate	Other skin care preparations	0.1%
Hydrolyzed Hyaluronic Acid	Other eye makeup preparations	0.01%
Hydrolyzed Hyaluronic Acid	Shampoos (noncoloring)	0.002%
Hydrolyzed Hyaluronic Acid	Tonics, dressings, and other hair grooming aids	0.02%
Hydrolyzed Hyaluronic Acid	Hair dyes and colors	0.002%
Hydrolyzed Hyaluronic Acid	Skin cleansing (cold creams, cleansing lotions, liquids, and pads)	0.01%
Hydrolyzed Hyaluronic Acid	Face and neck products Not spray	 0.01%
Hydrolyzed Hyaluronic Acid	Moisturizing products Not spray	 0.1-0.2%
Hydrolyzed Hyaluronic Acid	Night products Not spray	 0.15%
Hydrolyzed Hyaluronic Acid	Other skin care preparations	0.2%
Hydrolyzed Sodium Hyaluronate	Face and neck products Not spray	 0.15%
Hydrolyzed Sodium Hyaluronate	Moisturizing products Not spray	 0.0015%

*Ingredients included in the title of the table but not found in the table were included in the concentration of use survey, but no uses were reported.

Information collected in 2021
Table prepared: July 6, 2021

Concentration of Use by FDA Product Category – Ingredients Previously Reviewed by CIR

Hyaluronic Acid
Sodium Hyaluronate
Potassium Hyaluronate

Ingredient	Product Category	Maximum Concentration of Use
Hyaluronic Acid	Other bath preparations	0.0089%
Hyaluronic Acid	Eye lotions	0.001%
Hyaluronic Acid	Hair conditioners	0.0036%
Hyaluronic Acid	Shampoos (noncoloring)	0.0036%
Hyaluronic Acid	Hair dyes and colors	0.002%
Hyaluronic Acid	Foundations	0.000002-0.1%
Hyaluronic Acid	Lipstick	0.003-0.05%
Hyaluronic Acid	Makeup bases	0.1%
Hyaluronic Acid	Other shaving preparations	0.008%
Hyaluronic Acid	Face and neck products Not spray	 0.003-0.3%
Hyaluronic Acid	Body and hand products Not spray	 0.05%
Hyaluronic Acid	Moisturizing products Not spray	 0.08-0.2%
Hyaluronic Acid	Night products Not spray	 0.15%
Hyaluronic Acid	Paste masks and mud packs	0.83%
Sodium Hyaluronate	Other baby products	0.005%
Sodium Hyaluronate	Eye shadows	0.097-0.96%
Sodium Hyaluronate	Eye lotions	0.1%
Sodium Hyaluronate	Eye makeup removers	0.12%
Sodium Hyaluronate	Mascaras	0.0001-0.1%
Sodium Hyaluronate	Other eye makeup preparations	0.001-0.1%
Sodium Hyaluronate	Shampoos (noncoloring)	0.01%
Sodium Hyaluronate	Tonics, dressings, and other hair grooming aids	2%
Sodium Hyaluronate	Other hair preparations (noncoloring)	0.005%
Sodium Hyaluronate	Blushers	0.05%
Sodium Hyaluronate	Face powders	0.001-0.099%
Sodium Hyaluronate	Foundations	0.015-0.2%
Sodium Hyaluronate	Lipstick	0.24-0.39%
Sodium Hyaluronate	Rouges	0.001%
Sodium Hyaluronate	Other makeup preparations	0.1-0.025%
Sodium Hyaluronate	Other manicuring preparations	0.025%
Sodium Hyaluronate	Bath soaps and detergents	0.01%
Sodium Hyaluronate	Deodorants Not spray	 0.013%

Sodium Hyaluronate	Aftershave lotions	0.1%
Sodium Hyaluronate	Other shaving preparations Razor lube strip	 0.01%
Sodium Hyaluronate	Skin cleansing (cold creams, cleansing lotions, liquids, and pads)	0.0001-0.1%
Sodium Hyaluronate	Face and neck products Not spray	 0.005-7.5%
Sodium Hyaluronate	Body and hand products Not spray	 0.00002-0.86%
Sodium Hyaluronate	Moisturizing products Not spray	 0.001-0.4%
Sodium Hyaluronate	Night products Not spray	 0.00001-0.3%
Sodium Hyaluronate	Paste masks and mud packs	0.024%
Sodium Hyaluronate	Other skin care preparations Spray	0.02-0.1% 0.01%

*Ingredients included in the title of the table but not found in the table were included in the concentration of use survey, but no uses were reported

Information collected in 2021
Table prepared: January 10, 2022

Memorandum

TO: Bart Heldreth, Ph.D.
Executive Director - Cosmetic Ingredient Review

FROM: Carol Eisenmann, Ph.D.
Personal Care Products Council

DATE: November 9, 2022

SUBJECT: Hyaluronic Acid and Sodium Hyaluronate

Active Concepts. 2020. Dermal and ocular irritation tests AC HYA Solution 1% (contains 1% Hyaluronic Acid).

Active Concepts. 2018. Dermal and ocular irritation tests AC Hyalurosome (contains 1% Hyaluronic Acid).

Active Concepts. 2018. *In chemico* skin sensitization AC Hyalurosome (contains 1% Hyaluronic Acid).

Active Concepts. 2018. OECD TG 422D: In vitro skin sensitization AC Hyalurosome (contains 1% Hyaluronic Acid).

Active Concepts. 2022. Dermal and ocular irritation tests AC NanoVesicular System P3 (contains 3% Hyaluronic Acid).

Active Concepts. 2022. Dermal and ocular irritation tests AC Moisture-Plex Advance (contains 0.5% Sodium Hyaluronate).

Distributed for Comment Only -- Do Not Cite or Quote

Dermal and Ocular Irritation Tests

info@activeconceptsllc.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

Tradename: AC HYA Solution 1% Contains 1% Hyaluronic Acid mixture tested at 100%

Code: 17001

CAS #: 7732-18-5 & 9004-61-9

Test Request Form #: 7504

Lot #: 72198P

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Danaher*
Principle Investigator: *Jennifer Goodman*

Test Performed:
In Vitro EpiDerm™ Dermal Irritation Test (EPI-200-SIT)
EpiOcular™ Eye Irritation Test (OCL-200-EIT)

SUMMARY

In vitro dermal and ocular irritation studies were conducted to evaluate whether **AC HYA Solution 1%** would induce dermal or ocular irritation in the EpiDerm™ and EpiOcular™ model assays.

The product was tested according to the manufacture's protocol. The test article solution was found to be **non-irritating**. Reconstructed human epidermis and cornea epithelial model were incubated in growth media overnight to allow for tissue equilibration after shipping from MatTek Corporation, Ashland, MA. Test substances were applied to the tissue inserts and incubated for 60 minutes for liquid and solid substances in the EpiDerm™ assay and 30 minutes for liquid substances and 90 minutes for solid substances in the EpiOcular™ assay at 37°C, 5% CO_2, and 95% relative humidity (RH). Tissue inserts were thoroughly washed and transferred to fresh plates with growth media. After post substance dosing incubation is complete, the cell viability test begins. Cell viability is measured by dehydrogenase conversion of MTT [(*3-4,5-dimethyl thiazole 2-yl*)], present in the cell mitochondria, into blue formazan salt that is measured after extraction from the tissue. The irritation potential of the test chemical is dictated by the reduction in tissue viability of exposed tissues compared to the negative control.

Under the conditions of this assay, the test article was considered to be **non-irritant**. The negative and positive controls performed as anticipated.

This information is presented in good faith but is not warranted as to accuracy of results. Also, freedom from patent infringement is not implied.
This information is offered solely for your investigation, verification, and consideration.

 Version#2/05-19-20/Form#53

Distributed for Comment Only -- Do Not Cite or Quote

Dermal and Ocular Irritation Tests

info@activeconceptsllc.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

I. Introduction

A. Purpose

In vitro dermal and ocular irritation studies were conducted to evaluate whether a test article would induce dermal or ocular irritation in the EpiDerm™ and EpiOcular™ model assays. MatTek Corporation's reconstructed human epidermal and human ocular models are becoming a standard in determining the irritancy potential of test substances. They are able to discriminate between irritants and non-irritants. The EpiDerm™ assay has accuracy for the prediction of UN GHS R38 skin irritating and no-label (non-skin irritating) test substances. The EpiOcular™ assay can differentiate chemicals that have been classified as R36 or R41 from the EU classifications based on Dangerous Substances Directive (DSD) or between the UN GHS Cat 1 and Cat 2 classifications.

II. Materials

A. Incubation Conditions: 37°C at 5% CO_2 and 95% relative humidity

B. Equipment: Forma humidified incubator, ESCO biosafety laminar flow hood, Synergy HT Microplate reader; Pipettes

C. Media/Buffers: DMEM based medium; DPBS; sterile deionized H_2O

D. Preparation: Pre-incubate (37°C) tissue inserts in assay medium; Place assay medium and MTT diluent at 4°C, MTT concentrate at -20°C, and record lot numbers of kit components

E. Tissue Culture Plates: Falcon flat bottom 96-well, 24-well, 12-well, and 6-well tissue culture plates

F. Reagents: MTT (1.0mg/mL); Extraction Solution (Isopropanol); SDS (5%); Methyl Acetate

G. Other: Nylon Mesh Circles (EPI-MESH); Cotton tip swabs; 1mL tuberculin syringes; Ted Pella micro-spatula; 220mL specimen containers; sterile disposable pipette tips; Parafilm

III. Test Assay

A. Test System

The reconstructed human epidermal model, EpiDerm™, and cornea epithelial model, EpiOcular™, consist of normal human-derived epidermal keratinocytes which have been cultured to form a multilayer, highly differentiated model of the human epidermis and cornea epithelium. These models consist of organized basal, spinous, and granular layers, and the EpiDerm™ systems also contains a multilayer stratum corneum containing intercellular lamellar lipid layers that the EpiOcular™ system is lacking. Both the EpiDerm™ and EpiOcular™ tissues are cultured on specially prepared cell culture inserts.

B. Negative Control

Sterile DPBS and sterile deionized water are used as negative controls for the EpiDerm™ and EpiOcular™ assays, respectfully.

C. Positive Control

Known dermal and eye irritants, 5% SDS solution and Methyl Acetate, were used as positive controls for the EpiDerm™ and EpiOcular™ assays, respectfully.

This information is presented in good faith but is not warranted as to accuracy of results. Also, freedom from patent infringement is not implied.
This information is offered solely for your investigation, verification, and consideration.

Dermal and Ocular Irritation Tests

info@activeconceptsllc.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

D. Data Interpretation Procedure

a. EpiDerm™

An irritant is predicted if the mean relative tissue viability of the 3 tissues exposed to the test substance is reduced by 50% of the mean viability of the negative controls and a non-irritant's viability is > 50%.

b. EpiOcular™

An irritant is predicted if the mean relative tissue viability of the 2 tissues exposed to the test substance is reduced by 60% of the mean viability of the negative controls and a non-irritant's viability is > 40%.

IV. Method

A. Tissue Conditioning

Upon MatTek kit arrival at Active Concepts, LLC the tissue inserts are removed from their shipping medium and transferred into fresh media and tissue culture plates and incubated at 37°C at 5% CO_2 and 95% relative humidity for 60 minutes. After those 60 minutes the inserts are transferred into fresh media and tissue culture plates and incubated at 37°C at 5% CO_2 and 95% relative humidity for an additional 18 to 21 hours.

B. Test Substance Exposure

a. EpiDerm™

30µL (liquid) or 25mg (solid) of the undiluted test substance is applied to 3 tissue inserts and allowed to incubate for 60 minutes in a humidified incubator (37°C, 5% CO_2, 95% RH).

b. EpiOcular™

Each tissue is dosed with 20µL DPBS prior to test substance dosing. 50µL (liquid) or 50mg (solid) of the undiluted test substance is applied to 2 tissue inserts and allowed to incubate for 90 minutes in a humidified incubator (37°C, 5% CO_2, 95% RH).

C. Tissue Washing and Post Incubation

a. EpiDerm™

All tissue inserts are washed with DPBS, dried with cotton tipped swab, and transferred to fresh media and culture plates. After 24 hours the inserts are again transferred into fresh media and culture plates for an additional 18 to 20 hours.

b. EpiOcular™

Tissue inserts are washed with DPBS and immediately transferred into 5mL of assay medium for 12 to 14 minutes. After this soak the inserts are transferred into fresh media and tissue culture plates for 120 minutes for liquid substances and 18 hours for solid substances.

D. MTT Assay

Tissue inserts are transferred into 300µL MTT media in pre-filled plates and incubated for 3 hours at 37°C, 5% CO_2, and 95% RH. Inserts are then removed from the MTT medium and placed in 2mL of the extraction solution. The plate is sealed and incubated at room temperature in the dark for 24 hours. After extraction is complete the tissue inserts are pierced with forceps and 2 x 200µL aliquots of the blue formazan solution is transferred into a 96 well plate for Optical Density reading. The spectrophotometer reads the 96-well plate using a wavelength of 570 nm.

V. Acceptance Criterion

A. Negative Control

The results of this assay are acceptable if the mean negative control Optical Density (OD_{570}) is ≥ 1.0 and ≤ 2.5 (EpiDerm™) or ≥ 1.0 and ≤ 2.3 (EpiOcular™).

This information is presented in good faith but is not warranted as to accuracy of results. Also, freedom from patent infringement is not implied.
This information is offered solely for your investigation, verification, and consideration.

Distributed for Comment Only -- Do Not Cite or Quote

info@activeconceptsllc.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

B. Positive Control

a. EpiDerm™

The assay meets the acceptance criterion if the mean viability of positive control tissues expressed as a % of the negative control is ≤ 20%.

b. EpiOcular™

The assay meets the acceptance criterion if the mean viability of positive control tissues is < 60% of control viability.

C. Standard Deviation

Since each irritancy potential is predicted from the mean viability of 3 tissues for EpiDerm™ and 2 tissues for EpiOcular™, the variability of the replicates should be < 18% for EpiDerm™ and < 20% EpiOcular™.

VI. Results

A. Tissue Characteristics

The tissue inserts included in the MatTek EpiDerm™ and EpiOcular™ assay kits were in good condition, intact, and viable.

B. Tissue Viability Assay

The results are summarized in Figure 1. In no case was the tissue viability ≤ 50% for EpiDerm™ or ≤ 60% for EpiOcular™ in the presence of the test substance. The negative control mean exhibited acceptable relative tissue viability while the positive control exhibited substantial loss of tissue viability and cell death.

C. Test Validity

The data obtained from this study met criteria for a valid assay.

VII. Conclusion

Under the conditions of this assay, the test article substance was considered to be **non-irritating**. The negative and positive controls performed as anticipated.

This information is presented in good faith but is not warranted as to accuracy of results. Also, freedom from patent infringement is not implied.
This information is offered solely for your investigation, verification, and consideration.

Dermal and Ocular Irritation Tests

info@activeconceptsllc.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

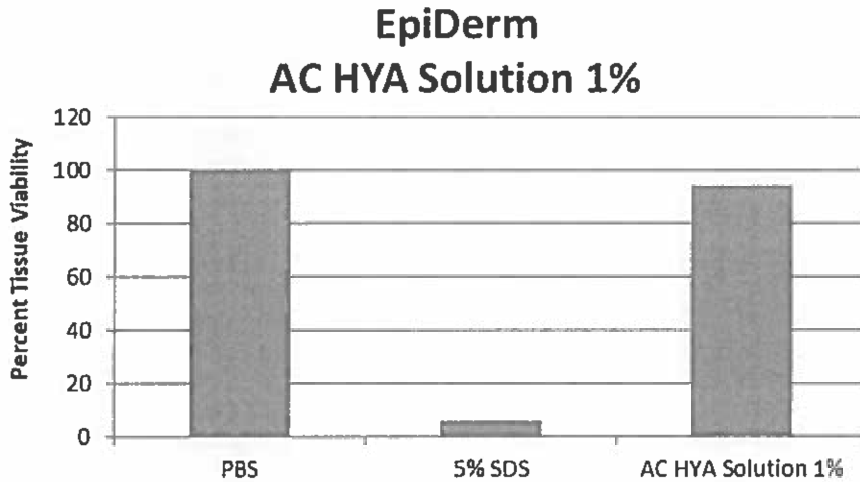


Figure 1: EpiDerm tissue viability

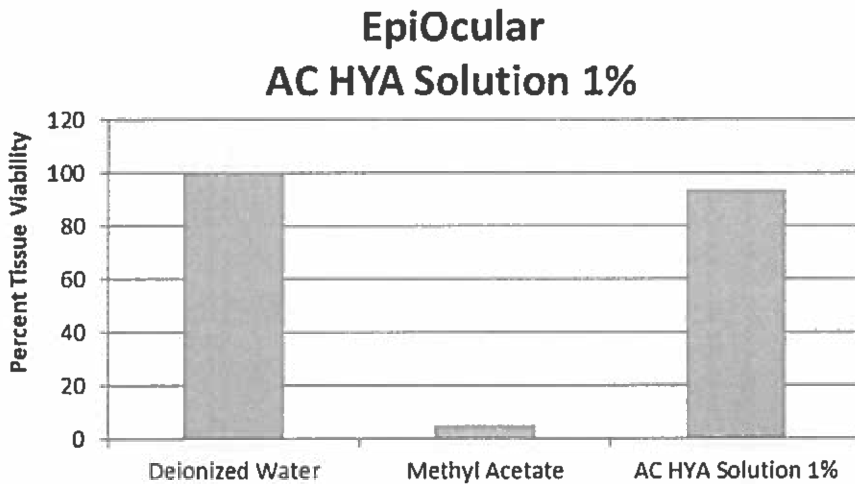


Figure 2: EpiOcular tissue viability

This information is presented in good faith but is not warranted as to accuracy of results. Also, freedom from patent infringement is not implied.
This information is offered solely for your investigation, verification, and consideration.

Dermal and Ocular Irritation Tests

info@activeconceptsllc.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

Sample: AC Hyalurosome Contains 1% Hyaluronic Acid

Code: 26001 mixture tested at 100%

CAS #: 9006-65-9 & 8002-43-5 & 68554-70-1 & 9004-61-9

Test Request Form/Submission #: 730

Lot #: 31243

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Danaher*
Principle Investigator: *Jennifer Goodman*

Test Performed:
In Vitro EpiDerm™ Dermal Irritation Test (EPI-200-SIT)
EpiOcular™ Eye Irritation Test (OCL-200-EIT)

SUMMARY

In vitro dermal and ocular irritation studies were conducted to evaluate whether **AC Hyalurosome** would induce dermal or ocular irritation in the EpiDerm™ and EpiOcular™ model assays.

The product was tested according to the manufacture's protocol. The test article solution was found to be **non-irritating**. Reconstructed human epidermis and cornea epithelial model were incubated in growth media overnight to allow for tissue equilibration after shipping from MatTek Corporation, Ashland, MA. Test substances were applied to the tissue inserts and incubated for 60 minutes for liquid and solid substances in the EpiDerm™ assay and 30 minutes for liquid substances and 90 minutes for solid substances in the EpiOcular™ assay at 37°C, 5% CO_2, and 95% relative humidity (RH). Tissue inserts were thoroughly washed and transferred to fresh plates with growth media. After post substance dosing incubation is complete, the cell viability test begins. Cell viability is measured by dehydrogenase conversion of MTT [(*3-4,5-dimethyl thiazole 2-yl*)], present in the cell mitochondria, into blue formazan salt that is measured after extraction from the tissue. The irritation potential of the test chemical is dictated by the reduction in tissue viability of exposed tissues compared to the negative control.

Under the conditions of this assay, the test article was considered to be **non-irritant**. The negative and positive controls performed as anticipated.

This information is presented in good faith but is not warranted as to accuracy of results. Also, freedom from patent infringement is not implied.
This information is offered solely for your investigation, verification, and consideration.

Distributed for Comment Only -- Do Not Cite or Quote

Dermal and Ocular Irritation Tests

info@activeconceptsllc.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

I. Introduction

A. Purpose

In vitro dermal and ocular irritation studies were conducted to evaluate whether a test article would induce dermal or ocular irritation in the EpiDerm™ and EpiOcular™ model assays. MatTek Corporation's reconstructed human epidermal and human ocular models are becoming a standard in determining the irritancy potential of test substances. They are able to discriminate between irritants and non-irritants. The EpiDerm™ assay has accuracy for the prediction of UN GHS R38 skin irritating and no-label (non-skin irritating) test substances. The EpiOcular™ assay can differentiate chemicals that have been classified as R36 or R41 from the EU classifications based on Dangerous Substances Directive (DSD) or between the UN GHS Cat 1 and Cat 2 classifications.

II. Materials

A. Incubation Conditions: 37°C at 5% CO_2 and 95% relative humidity

B. Equipment: Forma humidified incubator, ESCO biosafety laminar flow hood, Synergy HT Microplate reader; Pipettes

C. Media/Buffers: DMEM based medium; DPBS; sterile deionized H_2O

D. Preparation: Pre-incubate (37°C) tissue inserts in assay medium; Place assay medium and MTT diluent at 4°C, MTT concentrate at -20°C, and record lot numbers of kit components

E. Tissue Culture Plates: Falcon flat bottom 96-well, 24-well, 12-well, and 6-well tissue culture plates

F. Reagents: MTT (1.0mg/mL); Extraction Solution (Isopropanol); SDS (5%); Methyl Acetate

G. Other: Nylon Mesh Circles (EPI-MESH); Cotton tip swabs; 1mL tuberculin syringes; Ted Pella micro-spatula; 220mL specimen containers; sterile disposable pipette tips; Parafilm

III. Test Assay

A. Test System

The reconstructed human epidermal model, EpiDerm™, and cornea epithelial model, EpiOcular™, consist of normal human-derived epidermal keratinocytes which have been cultured to form a multilayer, highly differentiated model of the human epidermis and cornea epithelium. These models consist of organized basal, spinous, and granular layers, and the EpiDerm™ systems also contains a multilayer stratum corneum containing intercellular lamellar lipid layers that the EpiOcular™ system is lacking. Both the EpiDerm™ and EpiOcular™ tissues are cultured on specially prepared cell culture inserts.

B. Negative Control

Sterile DPBS and sterile deionized water are used as negative controls for the EpiDerm™ and EpiOcular™ assays, respectfully.

C. Positive Control

Known dermal and eye irritants, 5% SDS solution and Methyl Acetate, were used as positive controls for the EpiDerm™ and EpiOcular™ assays, respectfully.

This information is presented in good faith but is not warranted as to accuracy of results. Also, freedom from patent infringement is not implied.
This information is offered solely for your investigation, verification, and consideration.

Distributed for Comment Only -- Do Not Cite or Quote

Dermal and Ocular Irritation Tests

info@activeconceptsllc.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

D. Data Interpretation Procedure

a. EpiDerm™

An irritant is predicted if the mean relative tissue viability of the 3 tissues exposed to the test substance is reduced by 50% of the mean viability of the negative controls and a non-irritant's viability is > 50%.

b. EpiOcular™

An irritant is predicted if the mean relative tissue viability of the 2 tissues exposed to the test substance is reduced by 60% of the mean viability of the negative controls and a non-irritant's viability is > 40%.

IV. Method

A. Tissue Conditioning

Upon MatTek kit arrival at Active Concepts, LLC the tissue inserts are removed from their shipping medium and transferred into fresh media and tissue culture plates and incubated at 37°C at 5% CO_2 and 95% relative humidity for 60 minutes. After those 60 minutes the inserts are transferred into fresh media and tissue culture plates and incubated at 37°C at 5% CO_2 and 95% relative humidity for an additional 18 to 21 hours.

B. Test Substance Exposure

a. EpiDerm™

30µL (liquid) or 25mg (solid) of the undiluted test substance is applied to 3 tissue inserts and allowed to incubate for 60 minutes in a humidified incubator (37°C, 5% CO_2, 95% RH).

b. EpiOcular™

Each tissue is dosed with 20µL DPBS prior to test substance dosing. 50µL (liquid) or 50mg (solid) of the undiluted test substance is applied to 2 tissue inserts and allowed to incubate for 90 minutes in a humidified incubator (37°C, 5% CO_2, 95% RH).

C. Tissue Washing and Post Incubation

a. EpiDerm™

All tissue inserts are washed with DPBS, dried with cotton tipped swab, and transferred to fresh media and culture plates. After 24 hours the inserts are again transferred into fresh media and culture plates for an additional 18 to 20 hours.

b. EpiOcular™

Tissue inserts are washed with DPBS and immediately transferred into 5mL of assay medium for 12 to 14 minutes. After this soak the inserts are transferred into fresh media and tissue culture plates for 120 minutes for liquid substances and 18 hours for solid substances.

D. MTT Assay

Tissue inserts are transferred into 300µL MTT media in pre-filled plates and incubated for 3 hours at 37°C, 5% CO_2, and 95% RH. Inserts are then removed from the MTT medium and placed in 2mL of the extraction solution. The plate is sealed and incubated at room temperature in the dark for 24 hours. After extraction is complete the tissue inserts are pierced with forceps and 2 x 200µL aliquots of the blue formazan solution is transferred into a 96 well plate for Optical Density reading. The spectrophotometer reads the 96-well plate using a wavelength of 570 nm.

V. Acceptance Criterion

A. Negative Control

The results of this assay are acceptable if the mean negative control Optical Density (OD_{570}) is ≥ 1.0 and ≤ 2.5 (EpiDerm™) or ≥ 1.0 and ≤ 2.3 (EpiOcular™).

This information is presented in good faith but is not warranted as to accuracy of results. Also, freedom from patent infringement is not implied.
This information is offered solely for your investigation, verification, and consideration.

Dermal and Ocular Irritation Tests

info@activeconceptsllc.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

B. Positive Control

a. EpiDerm™

The assay meets the acceptance criterion if the mean viability of positive control tissues expressed as a % of the negative control is ≤ 20%.

b. EpiOcular™

The assay meets the acceptance criterion if the mean viability of positive control tissues is < 60% of control viability.

C. Standard Deviation

Since each irritancy potential is predicted from the mean viability of 3 tissues for EpiDerm™ and 2 tissues for EpiOcular™, the variability of the replicates should be < 18% for EpiDerm™ and < 20% EpiOcular™.

VI. Results

A. Tissue Characteristics

The tissue inserts included in the MatTek EpiDerm™ and EpiOcular™ assay kits were in good condition, intact, and viable.

B. Tissue Viability Assay

The results are summarized in Figure 1. In no case was the tissue viability ≤ 50% for EpiDerm™ or ≤ 60% for EpiOcular™ in the presence of the test substance. The negative control mean exhibited acceptable relative tissue viability while the positive control exhibited substantial loss of tissue viability and cell death.

C. Test Validity

The data obtained from this study met criteria for a valid assay.

VII. Conclusion

Under the conditions of this assay, the test article substance was considered to be **non-irritating**. The negative and positive controls performed as anticipated.

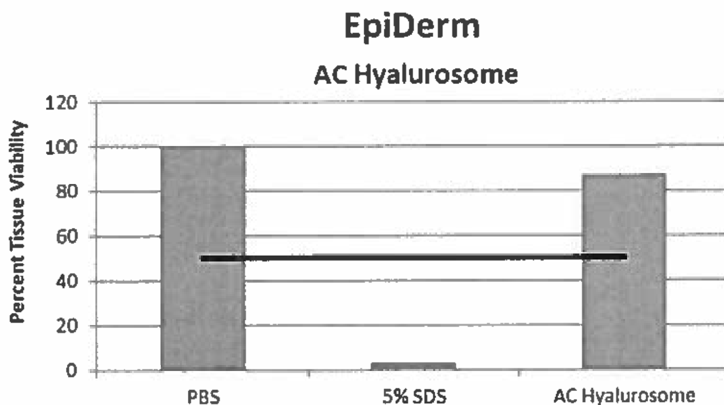


Figure 1: EpiDerm tissue viability

This information is presented in good faith but is not warranted as to accuracy of results. Also, freedom from patent infringement is not implied.
This information is offered solely for your investigation, verification, and consideration.

Dermal and Ocular Irritation Tests

info@activeconceptsllc.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

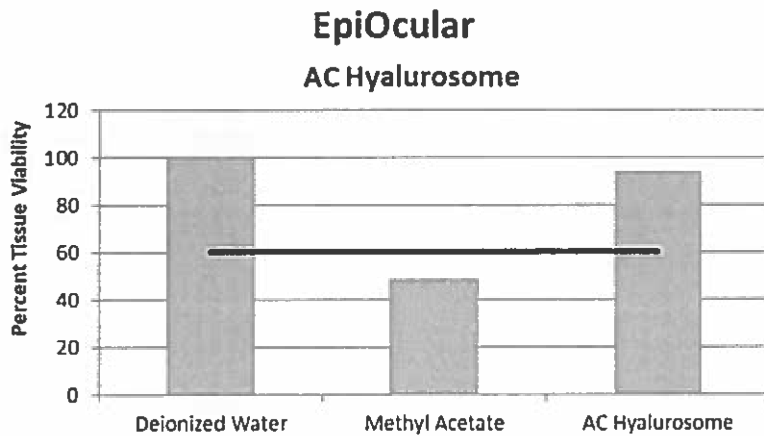


Figure 2: EpiOcular tissue viability

This information is presented in good faith but is not warranted as to accuracy of results. Also, freedom from patent infringement is not implied.
This information is offered solely for your investigation, verification, and consideration.

Distributed for Comment Only -- Do Not Cite or Quote

OECD TG 442C: *In Chemico* Skin Sensitization

info@activeconceptsllc.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

Tradename: AC Hyalurosome contains 1% Hyaluronic Acid

Code: 26001

CAS #: 9006-65-9 & 8002-43-5 & 68554-70-1 & 9004-61-9

Test Request Form #: 5160

Lot #: 58447P

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Danaher*
Principle Investigator: *Jennifer Goodman*

Test Performed:
OECD TG 442C: *In Chemico* Skin Sensitization Direct Peptide Reactivity Assay (DPRA)

Introduction

A skin sensitizer is a substance that will lead to an allergic response following skin contact[1]. Haptenation is the covalent binding of a hapten, or low-molecular weight substance or chemical, to proteins in the skin. This is considered the prominent mechanism which defines a chemical as a sensitizer. Haptenation is described as a "molecular initiating event" in the OECD Adverse Outcome Pathway (AOP) for skin sensitization which summarizes the key events known to be involved in chemically-induced allergic contact dermatitis[2]. The direct peptide reactivity assay (DPRA) is designed to mimic the covalent binding of electrophilic chemicals to nucleophilic centers in skin proteins by quantifying the reactivity of chemicals towards the model synthetic peptides containing cysteine and lysine. The DPRA is able to distinguish sensitizers from non-sensitizer with 82% accuracy (sensitivity of 76%; specificity of 92%)[3].

This assay was conducted to determine skin sensitization hazard of **AC Hyalurosome** in accordance with European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) and OECD Test Guideline 442C.

Assay Principle

The DPRA is an *in chemico* method which addresses peptide reactivity by measuring depletion of synthetic heptapeptides containing either cysteine or lysine following 24 hours incubation with the test substance. The peptide is a custom material containing phenylalanine to aid in detection. Depletion of the peptide in the reaction mixture is measured by HPLC with gradient elution and UV detection at 220 nm. Cysteine and lysine peptide percent depletion values are then calculated and used in a prediction model which allows assigning the test chemical to one of four reactivity classes used to support the discrimination between sensitizers and non-sensitizers.

1. United Nations Economic Commission (UNECE) (2013) Global Harmonized System of Classification and Labelling of Chemicals (GHS) 5th Revised Edition
2. OECD (2012). The Adverse Outcome Pathway for Skin Sensitization Initiated by Covalent Binding to Proteins. Part 1: Scientific Evidence. Series on Testing and Assessment No. 168
3. EC EURL ECVAM (2012) Direct peptide reactivity assay (DPRA) validation study report: pp 1 -74

Information contained in this technical literature is believed to be accurate and is offered in good faith for the benefit of the customer. The company, however, cannot assume any liability or risk involved in the use of its chemical products since the conditions of use are beyond our control. Statements concerning the possible use of our products are not intended as recommendations to use our products in the infringement of any patent. We make no warranty of any kind, expressed or implied, other than that the material conforms to the applicable standard specification.

Distributed for Comment Only -- Do Not Cite or Quote

info@activeconceptsllc.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

Materials

A. **Equipment:**	HPLC-UV (Waters Breeze - Waters 2998 Photodiode Array Detector); Pipettes; Analytical balance
B. **HPLC/Guard Columns:**	Agilent Zorbax SB-C18 2.1mm x 100mm x 3.5μm; Phenomenex Security Guard C18 4mm x 2mm
C. **Chemicals:**	Trifluoroacetic acid; Ammonium acetate; Ammonium hydroxide; Acetonitrile; Cysteine peptide (Ac-RFAACAA-COOH); Lysine peptide (Ac-RFAAKAA-COOH); Cinnamic aldehyde
D. **Reagents/Buffers:**	Sodium phosphate buffer (100mM); Ammonium acetate buffer (100mM)
E. **Other:**	Sterile disposable pipette tips

Methods

Solution Preparation:

- 0.667mM Cysteine Peptide in 100mM Phosphate Buffer (pH 7.5)
- 0.667mM Lysine Peptide in 100mM Ammonium Acetate Buffer (pH 10.2)
- 100mM Cinnamic Aldehyde in Acetonitrile
- 100mM* **AC Hyalurosome** in Acetonitrile

*For mixtures and multi-constituent substances of known composition such as **PRODUCT**, a single purity should be determined by the sum of the proportion of its constituents (excluding water), and a single apparent molecular weight determined by considering the individual molecular weights of each component in the mixture (excluding water) and their individual proportions. The resulting purity and apparent molecular weight can then be used to calculate the weight of test chemical necessary to prepare a 100 mM solution.

Reference Controls:

- Reference Control A: For calibration curve accuracy
- Reference Control B: For peptide stability over analysis time of experiment
- Reference Control C: For verification that the solvent does not impact percent peptide depletion

Sample, Reference Control, and Co-Elution Control Preparation:

- Once these solutions have been made they should be incubated at room temperature, protected from light, for 24±2 hours before running HPLC analysis.
- Each chemical should be analyzed in triplicate.

1:10 Ratio, Cysteine Peptide 0.5mM Peptide, 5mM Test Chemical	1:50 Ratio, Lysine Peptide 0.5mM Peptide, 25mM Test Chemical
• 750μL Cysteine Peptide Solution (or 100mM Phosphate Buffer, pH 7.5, for Co-Elution Controls) • 200μL Acetonitrile • 50μL Test Chemical Solution (or Acetonitrile for Reference Controls)	• 750μL Lysine Peptide Solution (or 100mM Ammonium Acetate Buffer, pH 10.2, for Co-Elution Controls) • 250μL Test Chemical Solution (or Acetonitrile for Reference Controls)

Information contained in this technical literature is believed to be accurate and is offered in good faith for the benefit of the customer. The company, however, cannot assume any liability or risk involved in the use of its chemical products since the conditions of use are beyond our control. Statements concerning the possible use of our products are not intended as recommendations to use our products in the infringement of any patent. We make no warranty of any kind, expressed or implied, other than that the material conforms to the applicable standard specification.

Distributed for Comment Only -- Do Not Cite or Quote

info@activeconceptsllc.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

Calibration Curve:

- Standards are prepared in a solution of 20% Acetonitrile:Buffer
  - For the Cysteine peptide using the phosphate buffer, pH 7.5
  - For the Lysine peptide using the ammonium acetate buffer, pH 10.2

	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5	Standard 6	Standard 7
mM Peptide	0.534	0.267	0.1335	0.0667	0.0334	0.0167	0.000

HPLC Analysis:

- HPLC-UV system should be equilibrated at 30°C with 50% Mobile Phase A (0.1% (v/v) trifluoroacetic acid in water) and 50% Mobile Phase B (0.085% (v/v) trifluoroacetic acid in acetonitrile) for 2 hours
- Absorbance is measured at 220nm
- Flow Conditions:

Time	Flow	%A	%B
0 minutes	0.35 mL/min	90	10
10 minutes	0.35 mL/min	75	25
11 minutes	0.35 mL/min	10	90
13 minutes	0.35 mL/min	10	90
13.5 minutes	0.35 mL/min	90	10
20 minutes	End Run		

Data and Reporting

Acceptance Criteria:

1. The following criteria must be met for a run to be considered valid:
   a. Standard calibration curve should have an $r^2 > 0.99$.
   b. Mean percent peptide depletion values of three replicates for the positive control cinnamic aldehyde should be between 60.8% and 100% for the cysteine peptide and between 40.2% and 69% for the lysine peptide and the maximum standard deviation should be <14.9 for the percent cysteine depletion and <11.6 for the percent lysine depletion.
   c. Mean peptide concentration of reference controls A should be 0.50±0.05mM and the coefficient of variable of the peptide peak areas for reference B and C in acetonitrile should be <15.0%.

2. The following criteria must be met for a test chemical's results to be considered valid:
   a. Maximum standard deviation should be <14.9 for percent cysteine depletion and <11.6 for percent lysine depletion.
   b. Mean peptide concentration of the three reference control C should be 0.50±0.05mM.

Information contained in this technical literature is believed to be accurate and is offered in good faith for the benefit of the customer. The company, however, cannot assume any liability or risk involved in the use of its chemical products since the conditions of use are beyond our control. Statements concerning the possible use of our products are not intended as recommendations to use our products in the infringement of any patent. We make no warranty of any kind, expressed or implied, other than that the material conforms to the applicable standard specification.

OECD TG 442C: *In Chemico* Skin Sensitization

info@activeconceptsllc.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

Prediction Model:

Cysteine 1:10/Lysine 1:50 Prediction Model		
Mean of Cysteine and Lysine % Depletion	**Reactivity Class**	**Prediction**
0% < Mean % Depletion < 6.38%	Minimal Reactivity	Non-sensitizer
6.38% < Mean % Depletion < 22.62%	Low Reactivity	Sensitizer
22.62% < Mean % Depletion < 42.47%	Moderate Reactivity	Sensitizer
42.47% < Mean % Depletion < 100%	High Reactivity	Sensitizer

If co-elution occurs with the lysine peptide, than the cysteine 1:10 prediction model can be used:

Cysteine 1:10 Prediction Model		
Mean of Cysteine and Lysine % Depletion	**Reactivity Class**	**Prediction**
0% < Cys % Depletion < 13.89%	Minimal Reactivity	Non-sensitizer
13.89% < Cys % Depletion < 23.09%	Low Reactivity	Sensitizer
23.09% < Cys % Depletion < 98.24%	Moderate Reactivity	Sensitizer
98.24% < Cys % Depletion < 100%	High Reactivity	Sensitizer

Therefore the measured values of % depletion in the three separated runs for each peptide depletion assay include:

Cysteine 1:10/Lysine 1:50 Prediction Model		
Mean of Cysteine and Lysine % Depletion	**Reactivity Class**	**Prediction**
3.12	Minimal Reactivity	Non-sensitizer
3.11	Minimal Reactivity	Non-sensitizer
3.09	Minimal Reactivity	Non-sensitizer

Cysteine 1:10 Prediction Model		
Mean of Cysteine and Lysine % Depletion	**Reactivity Class**	**Prediction**
3.11	Minimal Reactivity	Non-sensitizer
3.15	Minimal Reactivity	Non-sensitizer
3.08	Minimal Reactivity	Non-sensitizer

Results and Discussion

The data obtained from this study met criteria for a valid assay and the controls performed as anticipated.

Percent peptide depletion is determined by the following equation:

$$Percent\ Peptide\ Depletion = \left[1 - \left(\frac{Peptide\ Peak\ Area\ in\ Replicate\ Injection}{Mean\ Peptide\ Peak\ Area\ in\ Reference\ Controls\ C}\right)\right] \times 100$$

Based on HPLC-UV analysis of **AC Hyalurosome (26001)** we can determine this product is not classified as a sensitizer and is not predicted to cause allergic contact dermatitis. The Mean Percent Depletion of Cysteine and Lysine was 3.11% causing minimal reactivity in the assay giving us the prediction of a non-sensitizer.

Information contained in this technical literature is believed to be accurate and is offered in good faith for the benefit of the customer. The company, however, cannot assume any liability or risk involved in the use of its chemical products since the conditions of use are beyond our control. Statements concerning the possible use of our products are not intended as recommendations to use our products in the infringement of any patent. We make no warranty of any kind, expressed or implied, other than that the material conforms to the applicable standard specification.

OECD TG 442D: *In Vitro* Skin Sensitization

info@activeconceptsllc.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

Tradename: AC Hyalurosome contains 1% Hyaluronic Acid

Code: 26001

CAS #: 9006-65-9 & 8002-43-5 & 68554-70-1 & 9004-61-9

Test Request Form #: 5161

Lot #: 58447P

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Danaher*
Principle Investigator: *Jennifer Goodman*

Test Performed:
OECD TG 442D: In Vitro Skin Sensitization *ARE-Nrf2 Luciferase Test Method*

Introduction

Skin sensitization refers to an allergic response following skin contact with the tested chemical, as defined by the United Nations Globally Harmonized System of Classification and Labelling of Chemicals[1]. Substances are classified as skin sensitizers if there is evidence in humans that the substance can lead to sensitization by skin contact or positive results from appropriate tests, both *in vivo* and *in vitro*. Utilization of the KeratinoSens™ cell line allows for valid *in vitro* testing for skin sensitization.

This assay was conducted to determine skin sensitization potential of **AC Hyalurosome** in accordance with the UN GHS.

Assay Principle

The ARE-Nrf2 luciferase test method addresses the induction of genes that are regulated by antioxidant response elements (ARE) by skin sensitizers. The Keap1-Nrf2-ARE pathways have been shown to be major regulator of cytoprotective responses to oxidative stress or electrophilic compounds. These pathways are also known to be involved in the cellular processes in skin sensitization. Small electrophilic substances such as skin sensitizers can act on the sensor protein Keap1 (Kelch-like ECH-associated protein 1), by covalent modification of its cysteine residue, resulting in its dissociation from the transcription factor Nrf2 (nuclear factor-erythroid 2-related factor 2). The dissociated Nrf2 can then activate ARE-dependent genes such as those coding for phase II detoxifying enzymes.

The skin sensitization assay utilizes the KeratinoSens™ method which uses an immortalized adherent human keratinocyte cell line (HaCaT cell line) that has been transfected with a selectable plasmid to quantify luciferase gene induction as a measure of activation of Keap1-Nrf2-antioxidant/electrophile response element (ARE). This test method has been validated by independent peer review by the EURL-ECVAM. The addition of a luciferin containing reagent to the cells will react with the luciferase produced in the cell resulting in luminescence which can be quantified with a luminometer.

1. United Nations (UN) (2013). Globally Harmonized System of Classification and Labelling of Chemicals (GHS). Fifth revised edition, UN New York and Geneva, 2013

Information contained in this technical literature is believed to be accurate and is offered in good faith for the benefit of the customer. The company, however, cannot assume any liability or risk involved in the use of its chemical products since the conditions of use are beyond our control. Statements concerning the possible use of our products are not intended as recommendations to use our products in the infringement of any patent. We make no warranty of any kind, expressed or implied, other than that the material conforms to the applicable standard specification

OECD TG 442D: *In Vitro* Skin Sensitization

info@activeconceptsllc.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

Materials

A. **Incubation Conditions:**	37°C at 5% CO_2 and 95% relative humidity (RH)
B. **Equipment:**	Humidified incubator; Biosafety laminar flow hood; Microplate Reader; Pipettes
C. **Cell Line:**	KeratinoSens™ by Givaudan Schweiz AG
D. **Media/Buffers:**	Dulbecco's Modified Eagle Medium (DMEM); Fetal Bovine Serum (FBS); Phosphate Buffered Saline (PBS); Geneticin
E. **Culture Plate:**	Flat bottom 96-well tissue culture treated plates
F. **Reagents:**	Dimethyl Sulfoxide (DMSO); Cinnamic Aldehyde; ONE-Glo Reagent; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); sodium lauryl sulfate (SLS)
G. **Other:**	Sterile disposable pipette tips; wash bottles

Methods

KeratinoSens™ were into seeded four 96-well tissue culture plates and allowed to grow to 80 – 90% confluency in DMEM containing 10% FBS and 500µg/mL G418 geneticin. Twelve test concentrations of **AC Hyalurosome** were prepared in DMSO with a concentration range from 0.98 - 2000 µM. These 12 concentrations were assayed in triplicate in 2 independently performed experiments. The positive control was cinnamic aldehyde for which a series of 5 concentrations prepared in DMSO had final test concentrations of 4 – 64 µM. The negative control was a 1% test concentration of DMSO.

24 hour post KeratinoSens™ seeding, the culture media was removed and replaced with fresh media containing 10% FBS without G418 geneticin. 50 µL of the above described test concentrations was added to the appropriate wells. The treated plates were then incubated for 48 hours at 37°C in the presence of 5% CO_2 and 95% relative humidity. After treatment incubation was complete the media was removed and the wells were washed with PBS 3 times.

One of the four plates was used for a cytotoxicity endpoint, where MTT was added to the wells and incubated for 4 hours at 37°C in the presence of 5% CO_2. SLS was then added to the wells and incubated overnight at room temperature. A spectrometer measured the absorbance at 570 nm. The absorbance values (optical density) were then used to determine the viability of each well by comparing the optical density of each test material treated well to that of the solvent control wells to determine the IC_{50} and IC_{30} values.

The remaining 3 plates were used in the luciferase induction endpoint of the assay. 100 µL of Promega's ONE-Glo Reagent was added to 100 µL of fresh media containing 10% FBS without geneticin. Cells were incubated for 5 minutes to induce cell lysis and release luciferin into the media. Plates were read with a luminometer and $EC_{1.5}$ and maximum response (I_{max}) values were obtained.

Information contained in this technical literature is believed to be accurate and is offered in good faith for the benefit of the customer. The company, however, cannot assume any liability or risk involved in the use of its chemical products since the conditions of use are beyond our control. Statements concerning the possible use of our products are not intended as recommendations to use our products in the infringement of any patent. We make no warranty of any kind, expressed or implied, other than that the material conforms to the applicable standard specification.

Distributed for Comment Only -- Do Not Cite or Quote

info@activeconceptsllc.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

Data and Reporting

Acceptance Criteria:

1. Gene induction obtained with the positive control, cinnamic aldehyde, should be statistically significant above the threshold of 1.5 in at least one of the tested concentrations (from 4 to 64 µM).
2. The EC1.5 value should be within two standard deviations of the historical mean and the average induction in the three replicates for cinnamic aldehyde at 64 µM should be between 2 and 8.
3. The average coefficient of variability of the luminescence reading for the negative (solvent) control DMSO should be below 20% in each experiment.

A KeratinoSens™ prediction is considered positive if the following conditions are met:

1. The Imax is higher than 1.5-fold and statistically significantly higher as compared to the solvent (negative) control
2. The cellular viability is higher than 70% at the lowest concentration with a gene induction above 1.5 fold (i.e., at the EC1.5 determining concentration)
3. The $EC_{1.5}$ value is less than 1000 µM (or < 200 µg/ml for test chemicals with no defined MW)
4. There is an apparent overall dose-response for luciferase induction

Results

Compound	Classification	$EC_{1.5}$ (µM)	IC_{50}	I_{max}
Cinnamic aldehyde	Sensitizer	19	289.19 µM	31.1
DMSO	Non-Sensitizer	No Induction	243.24 µM	0.15
AC Hyalurosome	Non-Sensitizer	No Induction	> 1000 µM	0.33

Table 1: Overview of KeratinoSens™ Assay Results (I_{max} equals the average induction values Fg.1)

Information contained in this technical literature is believed to be accurate and is offered in good faith for the benefit of the customer. The company, however, cannot assume any liability or risk involved in the use of its chemical products since the conditions of use are beyond our control. Statements concerning the possible use of our products are not intended as recommendations to use our products in the infringement of any patent. We make no warranty of any kind, expressed or implied, other than that the material conforms to the applicable standard specification.

Distributed for Comment Only -- Do Not Cite or Quote

info@activeconceptsllc.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

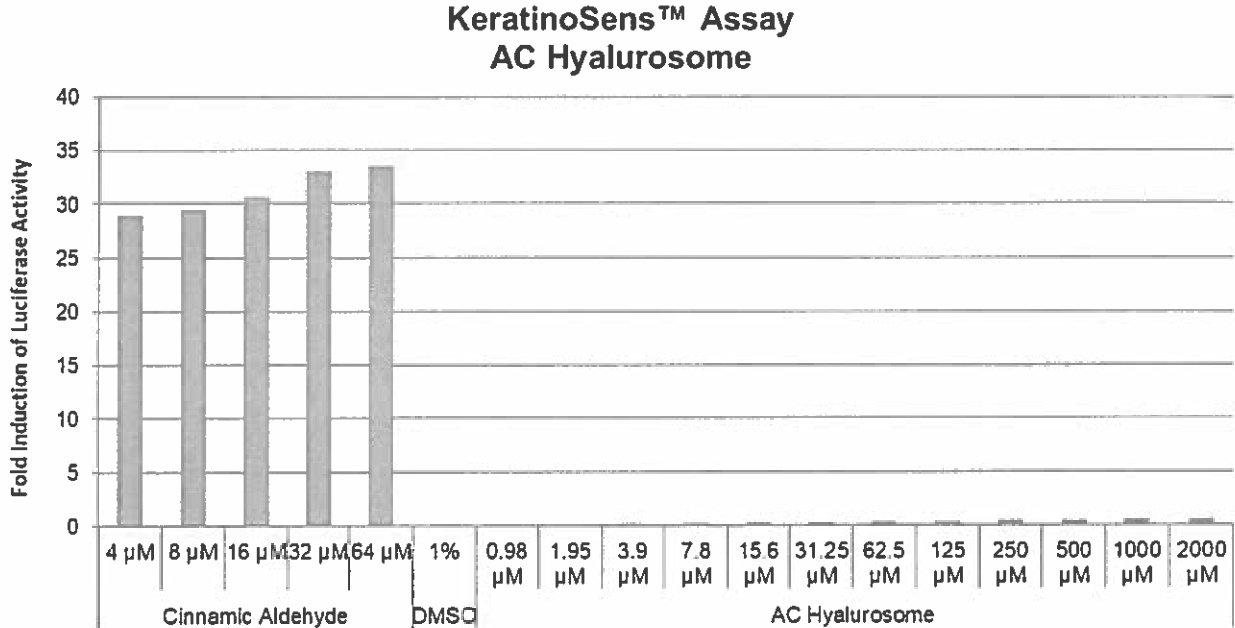


Figure 1: Fold Induction of Luciferase

Discussion

As shown in the results, **AC Hyalurosome (26001)** was not predicted to be a skin sensitizer based on the KeratinoSens™ ARE-Nrf2 Luciferase Test Method as there was not a significant increase in luciferase expression. It can be concluded that **AC Hyalurosome** can be safely used in cosmetics and personal care products at typical use levels.

Information contained in this technical literature is believed to be accurate and is offered in good faith for the benefit of the customer. The company, however, cannot assume any liability or risk involved in the use of its chemical products since the conditions of use are beyond our control. Statements concerning the possible use of our products are not intended as recommendations to use our products in the infringement of any patent. We make no warranty of any kind, expressed or implied, other than that the material conforms to the applicable standard specification.

Distributed for Comment Only -- Do Not Cite or Quote

Dermal and Ocular Irritation Tests

info@activeconceptsllc.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

Tradename: AC NanoVesicular System P3 Contains 3% Hyaluronic Acid

Code: 60051 mixture tested at 100%

CAS #: 8002-43-5 & 84604-16-0 & 9007-28-7 & 9004-61-9 & 59-02-9 & 92113-31-0

Test Request Form #: 9650

Lot #: 9126300

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Danaher*
Principle Investigator: *Daniel Shill*

Test Performed:
In Vitro EpiDerm™ Dermal Irritation Test (EPI-200-SIT)
EpiOcular™ Eye Irritation Test (OCL-200-EIT)

SUMMARY

In vitro dermal and ocular irritation studies were conducted to evaluate whether **AC NanoVesicular System P3** would induce dermal or ocular irritation in the EpiDerm™ and EpiOcular™ model assays.

The product was tested according to the manufacture's protocol. The test article solution was found to be **non-irritating**. Reconstructed human epidermis and cornea epithelial model were incubated in growth media overnight to allow for tissue equilibration after shipping from MatTek Corporation, Ashland, MA. Test substances were applied to the tissue inserts and incubated for 60 minutes for liquid and solid substances in the EpiDerm™ assay and 30 minutes for liquid substances and 90 minutes for solid substances in the EpiOcular™ assay at 37°C, 5% CO_2, and 95% relative humidity (RH). Tissue inserts were thoroughly washed and transferred to fresh plates with growth media. After post substance dosing incubation is complete, the cell viability test begins. Cell viability is measured by dehydrogenase conversion of MTT [(*3-4,5-dimethyl thiazole 2-yl*)], present in the cell mitochondria, into blue formazan salt that is measured after extraction from the tissue. The irritation potential of the test chemical is dictated by the reduction in tissue viability of exposed tissues compared to the negative control.

Under the conditions of this assay, the test article was considered to be **non-irritant**. The negative and positive controls performed as anticipated.

Information contained in this technical literature is believed to be accurate and is offered in good faith for the benefit of the customer. The company, however, cannot assume any liability or risk involved in the use of its chemical products since the conditions of use are beyond our control. Statements concerning the possible use of our products are not intended as recommendations to use our products in the infringement of any patent. We make no warranty of any kind, expressed or implied, other than that the material conforms to the applicable standard specification.

Dermal and Ocular Irritation Tests

info@activeconceptsllc.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

I. Introduction

A. Purpose

In vitro dermal and ocular irritation studies were conducted to evaluate whether a test article would induce dermal or ocular irritation in the EpiDerm™ and EpiOcular™ model assays. MatTek Corporation's reconstructed human epidermal and human ocular models are becoming a standard in determining the irritancy potential of test substances. They are able to discriminate between irritants and non-irritants. The EpiDerm™ assay has accuracy for the prediction of UN GHS R38 skin irritating and no-label (non-skin irritating) test substances. The EpiOcular™ assay can differentiate chemicals that have been classified as R36 or R41 from the EU classifications based on Dangerous Substances Directive (DSD) or between the UN GHS Cat 1 and Cat 2 classifications.

II. Materials

A. Incubation Conditions:	37°C at 5% CO_2 and 95% relative humidity
B. Equipment:	Forma humidified incubator, ESCO biosafety laminar flow hood, Synergy HT Microplate reader; Pipettes
C. Media/Buffers:	DMEM based medium; DPBS; sterile deionized H_2O
D. Preparation:	Pre-incubate (37°C) tissue inserts in assay medium; Place assay medium and MTT diluent at 4°C, MTT concentrate at -20°C, and record lot numbers of kit components
E. Tissue Culture Plates:	Falcon flat bottom 96-well, 24-well, 12-well, and 6-well tissue culture plates
F. Reagents:	MTT (1.0mg/mL); Extraction Solution (Isopropanol); SDS (5%); Methyl Acetate
G. Other:	Nylon Mesh Circles (EPI-MESH); Cotton tip swabs; 1mL tuberculin syringes; Ted Pella micro-spatula; 220mL specimen containers; sterile disposable pipette tips; Parafilm

III. Test Assay

A. Test System

The reconstructed human epidermal model, EpiDerm™, and cornea epithelial model, EpiOcular™, consist of normal human-derived epidermal keratinocytes which have been cultured to form a multilayer, highly differentiated model of the human epidermis and cornea epithelium. These models consist of organized basal, spinous, and granular layers, and the EpiDerm™ systems also contains a multilayer stratum corneum containing intercellular lamellar lipid layers that the EpiOcular™ system is lacking. Both the EpiDerm™ and EpiOcular™ tissues are cultured on specially prepared cell culture inserts.

B. Negative Control

Sterile DPBS and sterile deionized water are used as negative controls for the EpiDerm™ and EpiOcular™ assays, respectfully.

C. Positive Control

Known dermal and eye irritants, 5% SDS solution and Methyl Acetate, were used as positive controls for the EpiDerm™ and EpiOcular™ assays, respectfully.

Information contained in this technical literature is believed to be accurate and is offered in good faith for the benefit of the customer. The company, however, cannot assume any liability or risk involved in the use of its chemical products since the conditions of use are beyond our control. Statements concerning the possible use of our products are not intended as recommendations to use our products in the infringement of any patent. We make no warranty of any kind, expressed or implied, other than that the material conforms to the applicable standard specification.

Dermal and Ocular Irritation Tests

info@activeconceptsllc.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

D. Data Interpretation Procedure

a. EpiDerm™

An irritant is predicted if the mean relative tissue viability of the 3 tissues exposed to the test substance is reduced by 50% of the mean viability of the negative controls and a non-irritant's viability is > 50%.

b. EpiOcular™

An irritant is predicted if the mean relative tissue viability of the 2 tissues exposed to the test substance is reduced by 60% of the mean viability of the negative controls and a non-irritant's viability is > 40%.

IV. Method

A. Tissue Conditioning

Upon MatTek kit arrival at Active Concepts, LLC the tissue inserts are removed from their shipping medium and transferred into fresh media and tissue culture plates and incubated at 37°C at 5% CO_2 and 95% relative humidity for 60 minutes. After those 60 minutes the inserts are transferred into fresh media and tissue culture plates and incubated at 37°C at 5% CO_2 and 95% relative humidity for an additional 18 to 21 hours.

B. Test Substance Exposure

a. EpiDerm™

30µL (liquid) or 25mg (solid) of the undiluted test substance is applied to 3 tissue inserts and allowed to incubate for 60 minutes in a humidified incubator (37°C, 5% CO_2, 95% RH).

b. EpiOcular™

Each tissue is dosed with 20µL DPBS prior to test substance dosing. 50µL (liquid) or 50mg (solid) of the undiluted test substance is applied to 2 tissue inserts and allowed to incubate for 90 minutes in a humidified incubator (37°C, 5% CO_2, 95% RH).

C. Tissue Washing and Post Incubation

a. EpiDerm™

All tissue inserts are washed with DPBS, dried with cotton tipped swab, and transferred to fresh media and culture plates. After 24 hours the inserts are again transferred into fresh media and culture plates for an additional 18 to 20 hours.

b. EpiOcular™

Tissue inserts are washed with DPBS and immediately transferred into 5mL of assay medium for 12 to 14 minutes. After this soak the inserts are transferred into fresh media and tissue culture plates for 120 minutes for liquid substances and 18 hours for solid substances.

D. MTT Assay

Tissue inserts are transferred into 300µL MTT media in pre-filled plates and incubated for 3 hours at 37°C, 5% CO_2, and 95% RH. Inserts are then removed from the MTT medium and placed in 2mL of the extraction solution. The plate is sealed and incubated at room temperature in the dark for 24 hours. After extraction is complete the tissue inserts are pierced with forceps and 2 x 200µL aliquots of the blue formazan solution is transferred into a 96 well plate for Optical Density reading. The spectrophotometer reads the 96-well plate using a wavelength of 570 nm.

V. Acceptance Criterion

A. Negative Control

The results of this assay are acceptable if the mean negative control Optical Density (OD_{570}) is ≥ 1.0 and ≤ 2.5 (EpiDerm™) or ≥ 1.0 and ≤ 2.3 (EpiOcular™).

Information contained in this technical literature is believed to be accurate and is offered in good faith for the benefit of the customer. The company, however, cannot assume any liability or risk involved in the use of its chemical products since the conditions of use are beyond our control. Statements concerning the possible use of our products are not intended as recommendations to use our products in the infringement of any patent. We make no warranty of any kind, expressed or implied, other than that the material conforms to the applicable standard specification.

Dermal and Ocular Irritation Tests

info@activeconceptsllc.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

B. Positive Control

a. EpiDerm™

The assay meets the acceptance criterion if the mean viability of positive control tissues expressed as a % of the negative control is ≤ 20%.

b. EpiOcular™

The assay meets the acceptance criterion if the mean viability of positive control tissues is < 60% of control viability.

C. Standard Deviation

Since each irritancy potential is predicted from the mean viability of 3 tissues for EpiDerm™ and 2 tissues for EpiOcular™, the variability of the replicates should be < 18% for EpiDerm™ and < 20% EpiOcular™.

VI. Results

A. Tissue Characteristics

The tissue inserts included in the MatTek EpiDerm™ and EpiOcular™ assay kits were in good condition, intact, and viable.

B. Tissue Viability Assay

The results are summarized in Figure 1. In no case was the tissue viability ≤ 50% for EpiDerm™ or ≤ 60% for EpiOcular™ in the presence of the test substance. The negative control mean exhibited acceptable relative tissue viability while the positive control exhibited substantial loss of tissue viability and cell death.

C. Test Validity

The data obtained from this study met criteria for a valid assay.

VII. Conclusion

Under the conditions of this assay, the test article substance was considered to be **non-irritating**. The negative and positive controls performed as anticipated.

Information contained in this technical literature is believed to be accurate and is offered in good faith for the benefit of the customer. The company, however, cannot assume any liability or risk involved in the use of its chemical products since the conditions of use are beyond our control. Statements concerning the possible use of our products are not intended as recommendations to use our products in the infringement of any patent. We make no warranty of any kind, expressed or implied, other than that the material conforms to the applicable standard specification.

Dermal and Ocular Irritation Tests

info@activeconceptsllc.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

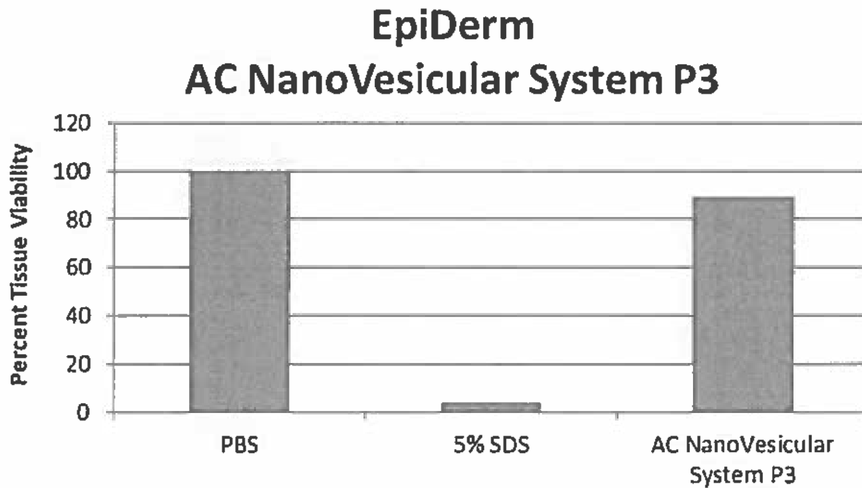


Figure 1: EpiDerm tissue viability

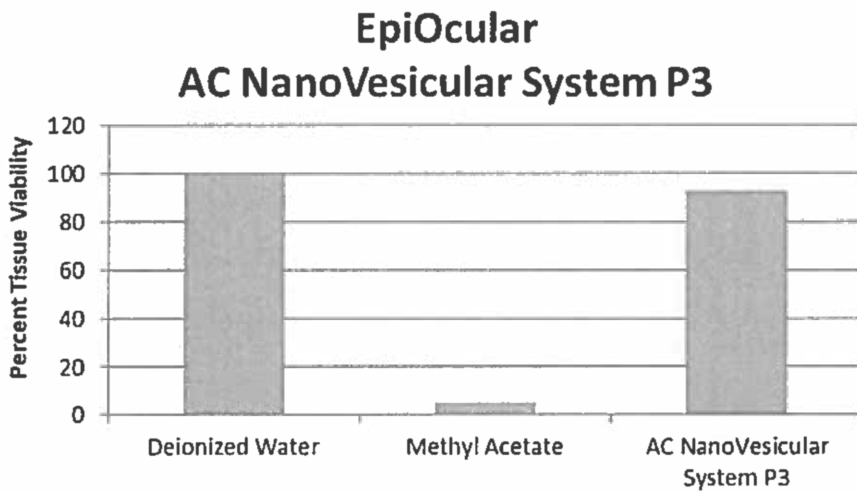


Figure 2: EpiOcular tissue viability

Information contained in this technical literature is believed to be accurate and is offered in good faith for the benefit of the customer. The company, however, cannot assume any liability or risk involved in the use of its chemical products since the conditions of use are beyond our control. Statements concerning the possible use of our products are not intended as recommendations to use our products in the infringement of any patent. We make no warranty of any kind, expressed or implied, other than that the material conforms to the applicable standard specification.

Dermal and Ocular Irritation Tests

info@activeconceptsllc.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

Tradename: AC Moisture-Plex Advanced contains 0.5% Sodium Hyaluronate

Code: 16503 mixture tested at 100%

CAS #: 56-81-5 & 7732-18-5 & 28874-51-3 & 57-13-6 & 99-20-7 & 125275-25-4 & 9067-32-7

Test Request Form #: 9651

Lot #: 8900200

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Danaher*
Principle Investigator: *Daniel Shill*

Test Performed:
In Vitro EpiDerm™ Dermal Irritation Test (EPI-200-SIT)
EpiOcular™ Eye Irritation Test (OCL-200-EIT)

SUMMARY

In vitro dermal and ocular irritation studies were conducted to evaluate whether **AC Moisture-Plex Advanced** would induce dermal or ocular irritation in the EpiDerm™ and EpiOcular™ model assays.

The product was tested according to the manufacture's protocol. The test article solution was found to be **non-irritating**. Reconstructed human epidermis and cornea epithelial model were incubated in growth media overnight to allow for tissue equilibration after shipping from MatTek Corporation, Ashland, MA. Test substances were applied to the tissue inserts and incubated for 60 minutes for liquid and solid substances in the EpiDerm™ assay and 30 minutes for liquid substances and 90 minutes for solid substances in the EpiOcular™ assay at 37°C, 5% CO_2, and 95% relative humidity (RH). Tissue inserts were thoroughly washed and transferred to fresh plates with growth media. After post substance dosing incubation is complete, the cell viability test begins. Cell viability is measured by dehydrogenase conversion of MTT [(*3-4,5-dimethyl thiazole 2-yl*)], present in the cell mitochondria, into blue formazan salt that is measured after extraction from the tissue. The irritation potential of the test chemical is dictated by the reduction in tissue viability of exposed tissues compared to the negative control.

Under the conditions of this assay, the test article was considered to be **non-irritant**. The negative and positive controls performed as anticipated.

Information contained in this technical literature is believed to be accurate and is offered in good faith for the benefit of the customer. The company, however, cannot assume any liability or risk involved in the use of its chemical products since the conditions of use are beyond our control. Statements concerning the possible use of our products are not intended as recommendations to use our products in the infringement of any patent. We make no warranty of any kind, expressed or implied, other than that the material conforms to the applicable standard specification.

Distributed for Comment Only -- Do Not Cite or Quote

Dermal and Ocular Irritation Tests

info@activeconceptsllc.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

I. Introduction

A. Purpose

In vitro dermal and ocular irritation studies were conducted to evaluate whether a test article would induce dermal or ocular irritation in the EpiDerm™ and EpiOcular™ model assays. MatTek Corporation's reconstructed human epidermal and human ocular models are becoming a standard in determining the irritancy potential of test substances. They are able to discriminate between irritants and non-irritants. The EpiDerm™ assay has accuracy for the prediction of UN GHS R38 skin irritating and no-label (non-skin irritating) test substances. The EpiOcular™ assay can differentiate chemicals that have been classified as R36 or R41 from the EU classifications based on Dangerous Substances Directive (DSD) or between the UN GHS Cat 1 and Cat 2 classifications.

II. Materials

A. Incubation Conditions:	37°C at 5% CO_2 and 95% relative humidity
B. Equipment:	Forma humidified incubator, ESCO biosafety laminar flow hood, Synergy HT Microplate reader; Pipettes
C. Media/Buffers:	DMEM based medium; DPBS; sterile deionized H_2O
D. Preparation:	Pre-incubate (37°C) tissue inserts in assay medium; Place assay medium and MTT diluent at 4°C, MTT concentrate at -20°C, and record lot numbers of kit components
E. Tissue Culture Plates:	Falcon flat bottom 96-well, 24-well, 12-well, and 6-well tissue culture plates
F. Reagents:	MTT (1.0mg/mL); Extraction Solution (Isopropanol); SDS (5%); Methyl Acetate
G. Other:	Nylon Mesh Circles (EPI-MESH); Cotton tip swabs; 1mL tuberculin syringes; Ted Pella micro-spatula; 220mL specimen containers; sterile disposable pipette tips; Parafilm

III. Test Assay

A. Test System

The reconstructed human epidermal model, EpiDerm™, and cornea epithelial model, EpiOcular™, consist of normal human-derived epidermal keratinocytes which have been cultured to form a multilayer, highly differentiated model of the human epidermis and cornea epithelium. These models consist of organized basal, spinous, and granular layers, and the EpiDerm™ systems also contains a multilayer stratum corneum containing intercellular lamellar lipid layers that the EpiOcular™ system is lacking. Both the EpiDerm™ and EpiOcular™ tissues are cultured on specially prepared cell culture inserts.

B. Negative Control

Sterile DPBS and sterile deionized water are used as negative controls for the EpiDerm™ and EpiOcular™ assays, respectfully.

C. Positive Control

Known dermal and eye irritants, 5% SDS solution and Methyl Acetate, were used as positive controls for the EpiDerm™ and EpiOcular™ assays, respectfully.

Information contained in this technical literature is believed to be accurate and is offered in good faith for the benefit of the customer. The company, however, cannot assume any liability or risk involved in the use of its chemical products since the conditions of use are beyond our control. Statements concerning the possible use of our products are not intended as recommendations to use our products in the infringement of any patent. We make no warranty of any kind, expressed or implied, other than that the material conforms to the applicable standard specification.

Distributed for Comment Only -- Do Not Cite or Quote

info@activeconceptsllc.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

D. Data Interpretation Procedure

a. EpiDerm™

An irritant is predicted if the mean relative tissue viability of the 3 tissues exposed to the test substance is reduced by 50% of the mean viability of the negative controls and a non-irritant's viability is > 50%.

b. EpiOcular™

An irritant is predicted if the mean relative tissue viability of the 2 tissues exposed to the test substance is reduced by 60% of the mean viability of the negative controls and a non-irritant's viability is > 40%.

IV. Method

A. Tissue Conditioning

Upon MatTek kit arrival at Active Concepts, LLC the tissue inserts are removed from their shipping medium and transferred into fresh media and tissue culture plates and incubated at 37°C at 5% CO_2 and 95% relative humidity for 60 minutes. After those 60 minutes the inserts are transferred into fresh media and tissue culture plates and incubated at 37°C at 5% CO_2 and 95% relative humidity for an additional 18 to 21 hours.

B. Test Substance Exposure

a. EpiDerm™

30µL (liquid) or 25mg (solid) of the undiluted test substance is applied to 3 tissue inserts and allowed to incubate for 60 minutes in a humidified incubator (37°C, 5% CO_2, 95% RH).

b. EpiOcular™

Each tissue is dosed with 20µL DPBS prior to test substance dosing. 50µL (liquid) or 50mg (solid) of the undiluted test substance is applied to 2 tissue inserts and allowed to incubate for 90 minutes in a humidified incubator (37°C, 5% CO_2, 95% RH).

C. Tissue Washing and Post Incubation

a. EpiDerm™

All tissue inserts are washed with DPBS, dried with cotton tipped swab, and transferred to fresh media and culture plates. After 24 hours the inserts are again transferred into fresh media and culture plates for an additional 18 to 20 hours.

b. EpiOcular™

Tissue inserts are washed with DPBS and immediately transferred into 5mL of assay medium for 12 to 14 minutes. After this soak the inserts are transferred into fresh media and tissue culture plates for 120 minutes for liquid substances and 18 hours for solid substances.

D. MTT Assay

Tissue inserts are transferred into 300µL MTT media in pre-filled plates and incubated for 3 hours at 37°C, 5% CO_2, and 95% RH. Inserts are then removed from the MTT medium and placed in 2mL of the extraction solution. The plate is sealed and incubated at room temperature in the dark for 24 hours. After extraction is complete the tissue inserts are pierced with forceps and 2 x 200µL aliquots of the blue formazan solution is transferred into a 96 well plate for Optical Density reading. The spectrophotometer reads the 96-well plate using a wavelength of 570 nm.

V. Acceptance Criterion

A. Negative Control

The results of this assay are acceptable if the mean negative control Optical Density (OD_{570}) is ≥ 1.0 and ≤ 2.5 (EpiDerm™) or ≥ 1.0 and ≤ 2.3 (EpiOcular™).

Information contained in this technical literature is believed to be accurate and is offered in good faith for the benefit of the customer. The company, however, cannot assume any liability or risk involved in the use of its chemical products since the conditions of use are beyond our control. Statements concerning the possible use of our products are not intended as recommendations to use our products in the infringement of any patent. We make no warranty of any kind, expressed or implied, other than that the material conforms to the applicable standard specification.

Distributed for Comment Only -- Do Not Cite or Quote

info@activeconceptsllc.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

B. Positive Control

a. EpiDerm™

The assay meets the acceptance criterion if the mean viability of positive control tissues expressed as a % of the negative control is ≤ 20%.

b. EpiOcular™

The assay meets the acceptance criterion if the mean viability of positive control tissues is < 60% of control viability.

C. Standard Deviation

Since each irritancy potential is predicted from the mean viability of 3 tissues for EpiDerm™ and 2 tissues for EpiOcular™, the variability of the replicates should be < 18% for EpiDerm™ and < 20% EpiOcular™.

VI. Results

A. Tissue Characteristics

The tissue inserts included in the MatTek EpiDerm™ and EpiOcular™ assay kits were in good condition, intact, and viable.

B. Tissue Viability Assay

The results are summarized in Figure 1. In no case was the tissue viability ≤ 50% for EpiDerm™ or ≤ 60% for EpiOcular™ in the presence of the test substance. The negative control mean exhibited acceptable relative tissue viability while the positive control exhibited substantial loss of tissue viability and cell death.

C. Test Validity

The data obtained from this study met criteria for a valid assay.

VII. Conclusion

Under the conditions of this assay, the test article substance was considered to be **non-irritating**. The negative and positive controls performed as anticipated.

Information contained in this technical literature is believed to be accurate and is offered in good faith for the benefit of the customer. The company, however, cannot assume any liability or risk involved in the use of its chemical products since the conditions of use are beyond our control. Statements concerning the possible use of our products are not intended as recommendations to use our products in the infringement of any patent. We make no warranty of any kind, expressed or implied, other than that the material conforms to the applicable standard specification.

Dermal and Ocular Irritation Tests

info@activeconceptsllc.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

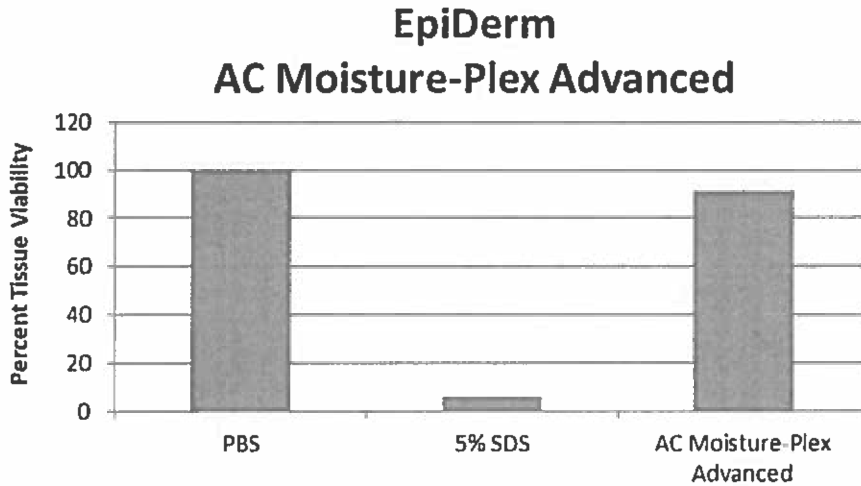


Figure 1: EpiDerm tissue viability

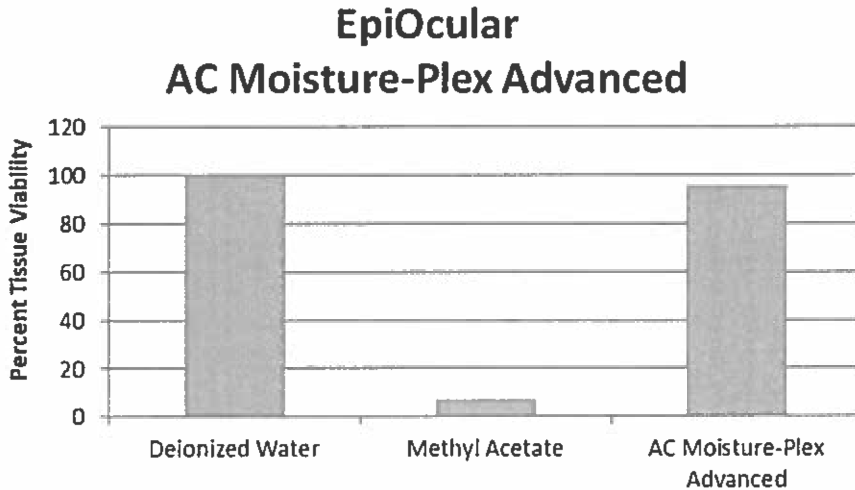


Figure 2: EpiOcular tissue viability

Information contained in this technical literature is believed to be accurate and is offered in good faith for the benefit of the customer. The company, however, cannot assume any liability or risk involved in the use of its chemical products since the conditions of use are beyond our control. Statements concerning the possible use of our products are not intended as recommendations to use our products in the infringement of any patent. We make no warranty of any kind, expressed or implied, other than that the material conforms to the applicable standard specification.

Distributed for Comment Only -- Do Not Cite or Quote

Memorandum

TO: Bart Heldreth, Ph.D.
Executive Director - Cosmetic Ingredient Review

FROM: Carol Eisenmann, Ph.D.
Personal Care Products Council

DATE: November 21, 2022

SUBJECT: Hyaluronic Acid, Sodium Acetylated Hyaluronate and Sodium Hyaluronate

Anonymous. 2020. Human repeat insult patch test (formula contains 0.2% Hyaluronic Acid).

Anonymous. 2020. Human repeated insult patch test with challenge (formula contains 0.2% Sodium Acetylated Hyaluronate).

Anonymous. 2019. Repeated insult patch test (formula contains 1.5% Sodium Hyaluronate).

HUMAN REPEAT INSULT PATCH TEST

formula contains 0.2% Hyaluronic Acid

CONDUCTED FOR:

DATE OF ISSUE:

February 28, 2020

TABLE OF CONTENTS

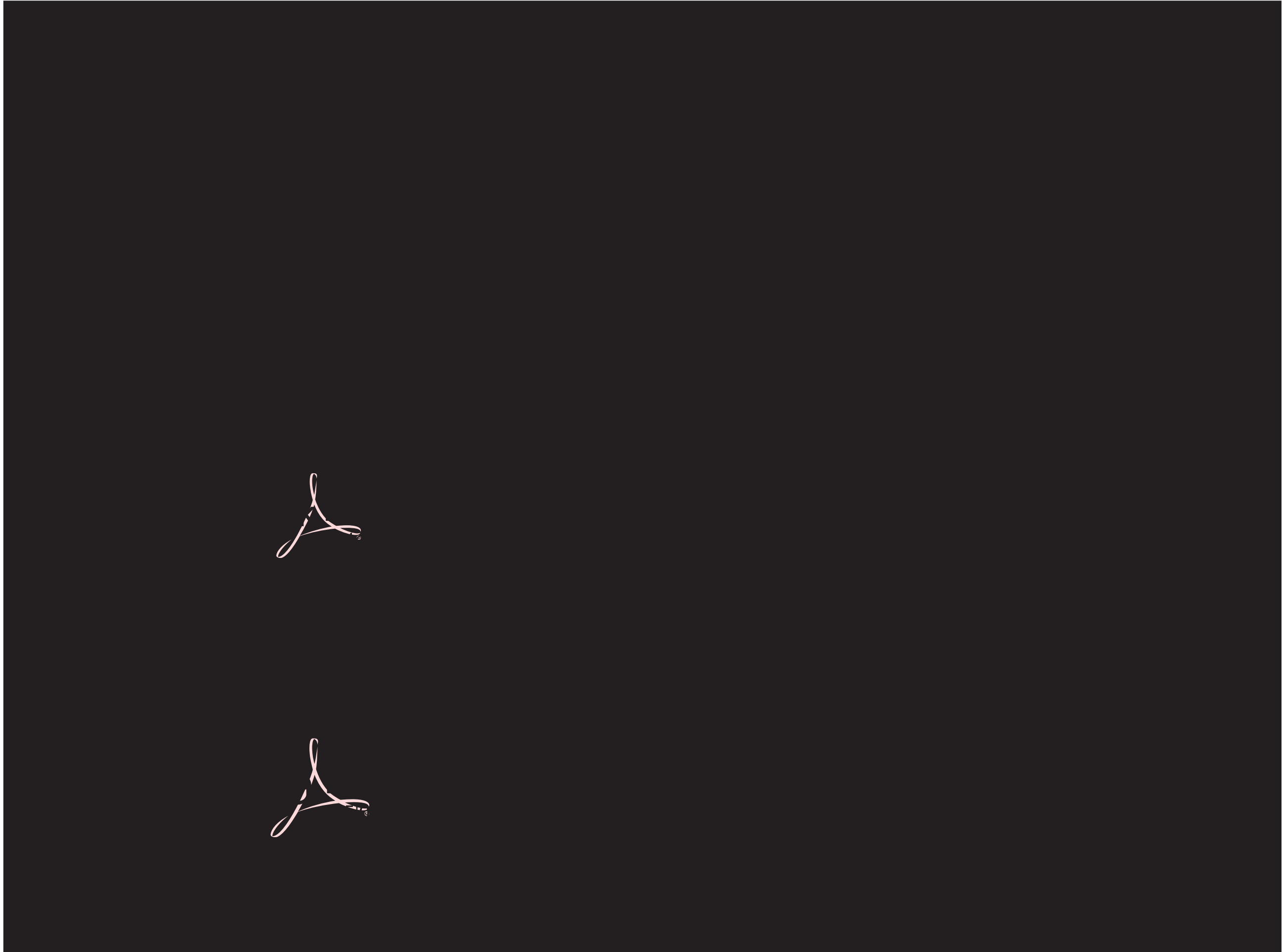
SIGNATURES 1
STATEMENT OF QUALITY CONTROL 1
TITLE OF STUDY 2
SPONSOR 2
STUDY MATERIAL 2
DATE STUDY INITIATED 2
DATE STUDY COMPLETED 2
DATE OF ISSUE 2
INVESTIGATIVE PERSONNEL 2
CLINICAL SITE 2
SUMMARY 3
1.0 OBJECTIVE 4
2.0 RATIONALE 4
3.0 STUDY DESIGN 4
3.1 STUDY POPULATION 4
3.1.1 Inclusion Criteria 4
3.1.2 Exclusion Criteria 4
3.1.3 Informed Consent 5
3.2 DESCRIPTION OF STUDY 5
3.2.1 Outline of Study Procedures 5
3.2.2 Definitions Use for Grading Responses 6
3.2.3 Evaluation of Responses 6
4.0 NATURE OF STUDY MATERIAL 6
4.1 STUDY MATERIAL SPECIFICATIONS 6
4.2 STORAGE, HANDLING, AND DOCUMENTATION OF STUDY MATERIAL 6
4.3 APPLICATION OF STUDY MATERIAL 7
4.4 DESCRIPTION OF PATCH CONDITIONS 7
5.0 INTERPRETATION 7
6.0 DOCUMENTATION AND RETENTION OF DATA 7
7.0 RESULTS & DISCUSSION 8
8.0 CONCLUSION 8
9.0 REFERENCES 8

APPENDICES

I SUMMARY TABLES

II DATA LISTINGS

III INFORMED CONSENT DOCUMENT



STATEMENT OF QUALITY CONTROL

The Quality Control Unit of the Dermatological Safety Department conducted a 100% review of all study-related documents. The protocol was reviewed prior to the start of the study, and the medical screening forms and informed consent documents were reviewed in-process of the study. The regulatory binder and study data were reviewed post-study to ensure accuracy. The study report was reviewed and accurately reflects the data for this study.

[1] ICH Topic E6 "Note for guidance on Good Clinical Practices (CPMP/ICH/135/95)" – ICH Harmonised Tripartite Guideline for Good Clinical Practices having reached Step 5 of the ICH Process at the ICH Steering Committee meeting on 1 May 1996.

TITLE OF STUDY

Human Repeat Insult Patch Test

SPONSOR

STUDY MATERIAL

DATE STUDY INITIATED

December 30, 2019

DATE STUDY COMPLETED

February 7, 2020

DATE OF ISSUE

February 28, 2020

INVESTIGATIVE PERSONNEL

SUMMARY

One (1) product, [redacted] was evaluated as supplied to determine its ability to sensitize the skin of volunteer subjects with normal skin using an occlusive 8 mm Finn Chamber human repeat insult patch test. One hundred fifteen (115) subjects completed the study.

Under the conditions employed in this study, [redacted] was non-sensitizing and non-irritating.

1.0 OBJECTIVE

The objective of the study was to determine the ability of the study material to cause sensitization by repeated topical applications to the skin of humans under controlled patch test conditions.

2.0 RATIONALE

Substances that come into contact with human skin need to be evaluated for their propensity to irritate and/or sensitize. Once an appropriate pre-clinical safety evaluation has been performed, a reproducible, standardized, quantitative patch evaluation procedure must be used to demonstrate that a particular material can be applied safely to human skin without significant risk of adverse reactions. The method herein employed is generally accepted for such a purpose.

Repeated insult patch evaluation is a modified predictive patch study that can detect weak sensitizers that require multiple applications to induce a cell-mediated (Type IV) immune response sufficient to cause an allergic reaction. Irritant reactions may also be detected using this evaluation method, although this is not the primary purpose of this procedure. Results are interpreted according to interpretive criteria based upon published works, as well as the clinical experience of Inc. These interpretive criteria are periodically reviewed and amended as new information becomes available.

3.0 STUDY DESIGN

3.1 STUDY POPULATION

A sufficient number of subjects were to be enrolled to provide 100 completed subjects. In the absence of any sensitization reactions in this sample size (100 evaluable subjects), a 95% upper confidence bound on the population rate of sensitization would be 3.5%.

3.1.1 Inclusion Criteria

Individuals eligible for inclusion in the study were those who:

1. Were healthy males or females, 18 to 75 years of age, in good general health;
2. Were free of any systemic or dermatologic disorder which, in the opinion of the investigative personnel, would have interfered with the study results or increased the risk of adverse events (AEs);
3. Were of any skin type or race providing the skin pigmentation allowed discernment of erythema;
4. Had completed a medical screening procedure; and
5. Had read, understood, and signed an informed consent (IC) agreement.

3.1.2 Exclusion Criteria

Individuals excluded from participation in the study were those who:

1. Had any visible skin disease at the study site which, in the opinion of the investigative personnel, would have interfered with the evaluation;
2. Were receiving systemic or topical drugs or medication which, in the opinion of the investigative personnel, would have interfered with the study results;

3. Had psoriasis and/or active atopic dermatitis/eczema;
4. Were females who were pregnant, planning to become pregnant during the study, or breast-feeding; and/or
5. Had a known sensitivity to cosmetics, skin care products, or topical drugs as related to the material being evaluated.

3.1.3 Informed Consent

A properly executed IC document was obtained from each subject prior to entering the study. The signed IC document is maintained in the study file. In addition, the subject was provided with a copy of the IC document (see Appendix III).

3.2 DESCRIPTION OF STUDY

3.2.1 Outline of Study Procedures

Subjects participated in the study over a 6-week period involving 3 phases: (1) Induction, (2) Rest, and (3) Challenge. Prior to study entry, the subjects were screened to assure that they met the inclusion/exclusion criteria. Informed consent was obtained. Each subject was provided with a schedule of the study activities. All subjects were told to avoid wetting the patches and were asked not to engage in activities that caused excessive perspiration. They were instructed to notify the staff if they experienced any discomfort beyond mild itching or observed any adverse changes at the patch sites, while on the study or within 2 weeks of completing the study.

The Induction Phase consisted of a series of 9 applications of the study material and subsequent evaluations of the application sites. Patches were applied on Mondays, Wednesdays, and Fridays for 3 consecutive weeks. Subjects returned to the facility at 48-hour intervals to have the patches removed, the sites evaluated, and identical patches applied to the same sites. Patches applied on a Friday remained in place for 72 hours until Monday. The sites were evaluated on the following Monday, ie, 72 hours after patch application.[2]

Following the 9th evaluation, the subjects were dismissed for a Rest Period of approximately 10-15 days.

The Challenge Phase was initiated during the sixth week of the study. Identical patch was applied to site previously unexposed to the study material. The patches were removed 48 hours after application. The sites were graded immediately and 24 hours following patch removal (ie, 48 and 72 hours after patch application). Rechallenge was to be performed if there was evidence of possible sensitization.

To be considered a completed case, a subject must have had 9 applications and subsequent readings during Induction and a single application and 2 readings at Challenge. Only completed cases were used to assess sensitization.

[2] A Monday or Friday holiday could have resulted in evaluation at 96 hours after patch application.

3.2.2 Definitions Use for Grading Responses

The following symbols were used to express the response observed at the time of examination.

SYMBOL		REACTION
-	=	No reaction
?	=	Minimal or doubtful response, slightly different from surrounding normal skin
+	=	Definite erythema, no edema
++	=	Definite erythema, definite edema
+++	=	Definite erythema, definite edema and vesiculation

SPECIAL NOTATIONS

E	=	Marked/severe erythema
S	=	Spreading of reaction beyond patch site (ie, reaction where material did not contact skin)
p	=	Papular response > 50%
pv	=	Papulovesicular response > 50%
D	=	Damage to epidermis: oozing, crusting and/or superficial erosions
I	=	Itching
X	=	Subject absent
PD	=	Patch dislodged
NA	=	Not applied
NP	=	Not patched (due to reaction achieved)
N9G	=	No ninth grading

3.2.3 Evaluation of Responses

All responses were graded by a trained dermatologic evaluator meeting certification requirements to standardize the assignment of response grades.

4.0 NATURE OF STUDY MATERIAL

4.1 Study Material Specifications

Formula No.	:
Batch No.	:
Amount Applied	: 20mg
Special Instructions	: Switched to semi-occlusive patch if reactions ≥ ++ occurred.

4.2 Storage, Handling, and Documentation of Study Material

Receipt of the material used in this study was documented in a general logbook, which serves as a permanent record of the receipt, storage, and disposition of all study materials received by On the basis of information provided by the Sponsor, the study material was considered reasonably safe for evaluation on human subjects. A sample of the study material was reserved and will be stored for a period of 6 months. All study material is kept in a locked product storage room accessible to

clinical staff members only. At the conclusion of the clinical study, the remaining study material was discarded or returned to the Sponsor and the disposition documented in the logbook.

4.3 APPLICATION OF STUDY MATERIAL

Study material was applied to the patch as instructed. The patch was applied to the infrascapular area of the back, either to the right or left of the midline, or to the upper arm.

4.4 DESCRIPTION OF PATCH CONDITIONS

Material evaluated under occlusive patch conditions is applied to an 8 mm Finn Chamber affixed to Scanpor tape. Liquid study material is soaked into a small filter disk placed within the Finn Chamber. Patches are secured with hypoallergenic non-occlusive tape (Micropore, 3M Company), as needed.

Material evaluated under semi-occlusive patch conditions is applied to a 2 cm x 2 cm Webril™ pad. The pad is affixed to the skin with hypoallergenic tape (Micropore).

5.0 INTERPRETATION

Sensitization is characterized by an acute allergic contact dermatitis. Typical sensitization reactions begin with an immunologic response in the dermis resulting in erythema, edema formation, and secondary epidermal damage (vesiculation), sometimes extending beyond the patch site and often accompanied by itching. Sensitization reactions tend to be delayed. The reaction typically becomes evident between 24 and 48 hours, peaks at 48-72 hours and subsequently subsides. The reaction is often greater at 72 hours than at 48 hours. The severity of the reaction is generally greater during the Challenge Phase of a Repeated Insult Patch Test (RIPT) than that seen during Induction.

Irritant reactions are characterized as a non-immunologic, localized, superficial, exudative, inflammatory response of the skin due to an externally applied material. The typical initial reaction does not develop much edema or vesiculation but results in scaling, drying, cracking, oozing, crusting, and erosions. The reaction is usually sharply delineated, not spreading beyond the patch site. Irritant reactions are typically evident by 24 hours and diminish over the next 48-72 hours. Removal of the offending agent results in gradual improvement of the epidermal damage. The reaction seen at 72 hours is, therefore, less severe than that seen at 48 hours. Finally, the severity of the reaction experienced in the Challenge Phase is generally similar to that seen during Induction.

If the results of the study indicate the likelihood of sensitization, the recommended practice is to rechallenge the subjects who have demonstrated sensitization-like reactions to confirm that these reactions are, indeed, associated with the product. Our preferred Rechallenge procedure involves the application of the product to naive sites, under both occlusive and semi-occlusive patch conditions. Use of the semi-occlusive patch condition helps to differentiate irritant and sensitization reactions. Generally speaking, if a product is a sensitizer it will produce a similar reaction under both occlusion and semi-occlusion. Whereas, if the product has caused an irritant reaction, the reactions will be less pronounced under the semi-occlusive condition.

6.0 DOCUMENTATION AND RETENTION OF DATA

The CRFs were designed to identify each subject by subject number and initials, and record demographics, examination results, AEs, and end of study status. Originals or copies of all CRFs,

correspondence, study reports, and all source data will be kept on hard-copy file for a period of 5 years from completion of the study. Storage is maintained either at a [redacted] facility in a secured room accessible only to [redacted] employees, or at an offsite location which provides a secure environment with burglar/fire alarm systems, camera detection and controlled temperature and humidity. Documentation will be available for the Sponsor's review on the premises of [redacted]

7.0 RESULTS & DISCUSSION

One hundred twenty-nine (129) subjects between the ages of 22 and 70 were enrolled. One hundred fifteen (115) subjects completed the study (see Tables 1 and 2 in Appendix I and Data Listings 1 and 2 in Appendix II). The following table summarizes subject enrollment and disposition.

Number enrolled:		129
Number discontinued:		14
Lost to follow-up:	14	
Number completed:		115

Source: Table 1, Appendix 1

There were no adverse events (AEs) reported during the study.

Due to the holiday on 01/01/20, subject cohorts 1 and 2 were scheduled to return on 01/02/20 for their required visit.

A summary of response data is provided in Table 3, Appendix I. Individual listings of dermatological response grades are provided in Data Listing 3, Appendix II.

8.0 CONCLUSION

Under the conditions employed in this study, [redacted] was non-sensitizing and non-irritating.

9.0 REFERENCES

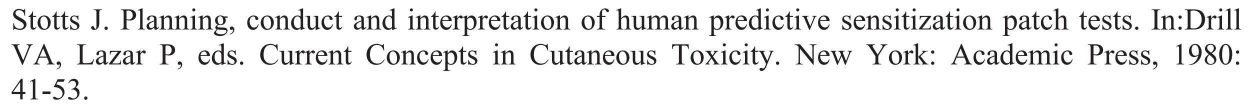
Schwartz L, Peck SM. The patch test in contact dermatitis. Publ Health Pep 1944; 59:2.

Draize JH, Woodward G, Calvary HO. Methods for the study of irritation and toxicology of substances applied topically to the skin and mucous membranes. J Pharmacol Exp Ther 1944; 82: 377-390.

Lanman BM, Elvers WB, Howard CS. The role of human patch testing in a product development program. Joint Conf Cosmet Sci Toilet Goods Assoc 1968; 135-145.

Marzulli FN, Maibach HI. Contact allergy: predictive testing in man. Contact Dermatitis 1976; 2:1.

Zhai H, Maibach HI. Dermatotoxicology. 6th ed. New York:Hemisphere, 1996.



Stotts J. Planning, conduct and interpretation of human predictive sensitization patch tests. In:Drill VA, Lazar P, eds. Current Concepts in Cutaneous Toxicity. New York: Academic Press, 1980: 41-53.

Griffith JF. Predictive and diagnostic testing for contact sensitization. Toxicol Appl Pharmacol, Suppl 1969; 3:90.

Gerberick GF, Robinson MK, Stotts J. An approach to allergic contact sensitization risk assessment of new chemicals and product ingredients. American Journal of Contact Dermatitis 1993; 4(4): 205-211.

APPENDIX I

SUMMARY TABLES

Table 1: Summary of Subject Enrollment and Disposition

	N (%)
Subjects enrolled	129
Subjects completed induction phase	119 (92.2)
Subjects completed all phases	115 (89.1)
Total subjects discontinued	14 (10.9)
Lost to follow-up	14 (10.9)

Note: All percentages are relative to total subjects enrolled.

See data listing 1 for further detail.

Table 2: Summary of Subject Demographics
All Enrolled Subjects

Age	
N (%) 18 to 44	29 (22.5)
N (%) 45 to 65	85 (65.9)
N (%) 66 and up	15 (11.6)
Mean (SD)	53.9 (11.6)
Median	56.7
Range	22.7 to 70.8
Sex	
N (%) Male	51 (39.5)
N (%) Female	78 (60.5)
Race	
Black	80 (62.0)
Caucasian	47 (36.4)
Other	2 (1.6)
Ethnicity	
Hispanic/Latino	17 (13.2)
Not Hispanic/Not Latino	112 (86.8)

See data listing 2 for further detail.

Table 3: Summary of Dermatologic Response Grades
Number of Subjects by Product

	Induction Reading										Challenge Phase		
Response	1	2	3	4	5	6	7	8	9	Make Up	48hr	72hr	96hr(*)
-	123	123	115	113	110	105	107	108	87	31	110	110	
?	0	1	3	6	9	10	8	5	2	0	5	5	
Total evaluable	123	124	118	119	119	115	115	113	89	31	115	115	
Number absent	4	1	6	4	4	5	4	6	30		0	0	
Number discontinued	2	4	5	6	6	9	10	10	10		14	14	

Maximum Elicited Response During Induction
All Subjects Completing Induction (N=119)

Response	n(%) Subjects
-	104 (87.4%)
?	15 (12.6%)

(*) when required

See Table 3.1 for Key to Symbols and Scores

Score or Symbol	Response or Description of Reaction
	Erythema Results
-	No reaction
?	Minimal or doubtful response, slightly different from surrounding normal skin
+	Definite erythema, no edema
++	Definite erythema, definite edema
+++	Definite erythema, definite edema and vesiculation
	Additional Comments
X	Reading not performed due to missed visit or subject discontinuation
D	Damage to epidermis: oozing, crusting and/or superficial erosions
E	Marked/severe erythema
I	Itching
p	Papular response >50%
pv	Papulovesicular response >50%
S	Spreading of reaction beyond patch site
NP	Not patched due to reaction achieved
PD	Patch dislodged
N9G	No ninth grading
NA	Not applied

APPENDIX II

DATA LISTINGS

Data Listing 1: Subject Enrollment and Disposition

	Study Dates						
Subject No.	Screened	1st Applic	Chall Applic	Ended	Last Reading #	Completion Status	Days in Study
001	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
002	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
003	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
004	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
005	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
006	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
007	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
008	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
009	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
010	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
011	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
012	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
013	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
014	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
015	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
016	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
017	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
018	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
019	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
020	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
021	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
022	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
023	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
024	12/30/19	12/30/19	--	02/04/20	I9	L	37
025	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
026	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
027	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
028	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
029	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
030	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
031	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40

Key:
Last Reading # (I=Induction Phase, C=Challenge Phase)
Completion Status (C=Completed, L=Lost to follow-up, S=Voluntary withdrawal, V=Protocol violation, AE=Adverse event, O=Other)

Data Listing 1: Subject Enrollment and Disposition

	Study Dates						
Subject No.	Screened	1st Applic	Chall Applic	Ended	Last Reading #	Completion Status	Days in Study
032	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
033	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
034	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
035	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
036	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
037	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
038	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
039	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
040	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
041	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
042	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
043	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
044	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
045	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
046	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
047	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
048	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
049	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
050	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
051	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
052	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
053	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
054	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
055	12/30/19	12/30/19	--	01/06/20	I1	L	8
056	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
057	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
058	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
059	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
060	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
061	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
062	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40

Key:
Last Reading # (I=Induction Phase, C=Challenge Phase)
Completion Status (C=Completed, L=Lost to follow-up, S=Voluntary withdrawal, V=Protocol violation, AE=Adverse event, O=Other)

Data Listing 1: Subject Enrollment and Disposition

	Study Dates						
Subject No.	**Screened**	**1st Applic**	**Chall Applic**	**Ended**	**Last Reading #**	**Completion Status**	**Days in Study**
063	12/30/19	12/30/19	--	01/06/20	I1	L	8
064	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
065	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
066	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
067	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
068	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
069	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
070	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
071	12/30/19	12/30/19	02/04/20	02/07/20	C	C	40
072	12/30/19	12/30/19	--	02/04/20	I9	L	37
073	01/02/20	01/02/20	02/04/20	02/07/20	C	C	37
074	01/02/20	01/02/20	02/04/20	02/07/20	C	C	37
075	01/02/20	01/02/20	02/04/20	02/07/20	C	C	37
076	01/02/20	01/02/20	02/04/20	02/07/20	C	C	37
077	01/02/20	01/02/20	02/04/20	02/07/20	C	C	37
078	01/02/20	01/02/20	02/04/20	02/07/20	C	C	37
079	01/02/20	01/02/20	--	01/15/20	I5	L	14
080	01/02/20	01/02/20	--	01/15/20	I5	L	14
081	01/02/20	01/02/20	02/04/20	02/07/20	C	C	37
082	01/02/20	01/02/20	02/04/20	02/07/20	C	C	37
083	01/02/20	01/02/20	02/04/20	02/07/20	C	C	37
084	01/02/20	01/02/20	02/04/20	02/07/20	C	C	37
085	01/02/20	01/02/20	02/04/20	02/07/20	C	C	37
086	01/02/20	01/02/20	--	01/08/20	I2	L	7
087	01/02/20	01/02/20	02/04/20	02/07/20	C	C	37
088	01/02/20	01/02/20	--	02/04/20	I9	L	34
089	01/02/20	01/02/20	02/04/20	02/07/20	C	C	37
090	01/03/20	01/03/20	--	01/06/20	I0	L	4
091	01/03/20	01/03/20	02/04/20	02/07/20	C	C	36
092	01/03/20	01/03/20	02/04/20	02/07/20	C	C	36
093	01/03/20	01/03/20	02/04/20	02/07/20	C	C	36

Key:
Last Reading # (I=Induction Phase, C=Challenge Phase)
Completion Status (C=Completed, L=Lost to follow-up, S=Voluntary withdrawal, V=Protocol violation, AE=Adverse event, O=Other)

Data Listing 1: Subject Enrollment and Disposition

	Study Dates						
Subject No.	**Screened**	**1st Applic**	**Chall Applic**	**Ended**	**Last Reading #**	**Completion Status**	**Days in Study**
094	01/03/20	01/03/20	02/04/20	02/07/20	C	C	36
095	01/03/20	01/03/20	02/04/20	02/07/20	C	C	36
096	01/03/20	01/03/20	02/04/20	02/07/20	C	C	36
097	01/03/20	01/03/20	--	01/17/20	I5	L	15
098	01/03/20	01/03/20	--	01/06/20	I0	L	4
099	01/03/20	01/03/20	--	01/13/20	I3	L	11
100	01/03/20	01/03/20	02/04/20	02/07/20	C	C	36
101	01/03/20	01/03/20	02/04/20	02/07/20	C	C	36
102	01/03/20	01/03/20	02/04/20	02/07/20	C	C	36
103	01/03/20	01/03/20	02/04/20	02/07/20	C	C	36
104	01/03/20	01/03/20	02/04/20	02/07/20	C	C	36
105	01/03/20	01/03/20	02/04/20	02/07/20	C	C	36
106	01/03/20	01/03/20	02/04/20	02/07/20	C	C	36
107	01/03/20	01/03/20	02/04/20	02/07/20	C	C	36
108	01/03/20	01/03/20	--	02/04/20	I9	L	33
109	01/03/20	01/03/20	02/04/20	02/07/20	C	C	36
110	01/03/20	01/03/20	02/04/20	02/07/20	C	C	36
111	01/03/20	01/03/20	02/04/20	02/07/20	C	C	36
112	01/03/20	01/03/20	02/04/20	02/07/20	C	C	36
113	01/03/20	01/03/20	02/04/20	02/07/20	C	C	36
114	01/03/20	01/03/20	02/04/20	02/07/20	C	C	36
115	01/03/20	01/03/20	02/04/20	02/07/20	C	C	36
116	01/03/20	01/03/20	02/04/20	02/07/20	C	C	36
117	01/03/20	01/03/20	02/04/20	02/07/20	C	C	36
118	01/03/20	01/03/20	02/04/20	02/07/20	C	C	36
119	01/03/20	01/03/20	02/04/20	02/07/20	C	C	36
120	01/03/20	01/03/20	02/04/20	02/07/20	C	C	36
121	01/03/20	01/03/20	02/04/20	02/07/20	C	C	36
122	01/03/20	01/03/20	02/04/20	02/07/20	C	C	36
123	01/03/20	01/03/20	02/04/20	02/07/20	C	C	36
124	01/03/20	01/03/20	02/04/20	02/07/20	C	C	36
125	01/03/20	01/03/20	--	01/20/20	I6	L	18
126	01/03/20	01/03/20	02/04/20	02/07/20	C	C	36
127	01/03/20	01/03/20	02/04/20	02/07/20	C	C	36
128	01/03/20	01/03/20	02/04/20	02/07/20	C	C	36
129	01/03/20	01/03/20	02/04/20	02/07/20	C	C	36

Key:
Last Reading # (I=Induction Phase, C=Challenge Phase)
Completion Status (C=Completed, L=Lost to follow-up, S=Voluntary withdrawal, V=Protocol violation, AE=Adverse event, O=Other)

Data Listing 3: Dermatologic Response Grades
By Product and Subject

Subject No.	Induction Reading										Challenge Phase		
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**MU**	**48hr**	**72hr**	**96hr(*)**
001	-	-	-	-	-	-	-	-	X	-	-	-	
002	-	-	-	-	-	-	-	-	-		-	-	
003	-	-	-	-	-	-	-	-	-		-	-	
004	-	-	-	-	-	-	X	-	-	-	-	-	
005	-	-	-	-	-	-	-	-	-		-	-	
006	-	-	-	-	-	-	-	-	-		-	-	
007	-	-	-	-	-	-	-	-	-		-	-	
008	-	-	-	-	-	-	-	-	-		-	-	
009	-	-	-	-	-	-	-	-	-		-	-	
010	-	-	-	-	-	-	-	-	-		-	-	
011	-	-	-	-	-	X	-	-	-	-	-	-	
012	-	-	-	-	X	-	-	-	-	-	-	-	
013	-	-	-	-	-	?	?	-	-		-	-	
014	-	-	-	X	-	-	-	-	-	-	-	-	
015	-	-	-	-	-	-	-	-	-		-	-	
016	-	-	?	?	?	?	?	?	-		-	-	
017	-	-	-	-	-	-	X	-	-	-	-	-	
018	-	-	-	-	-	-	-	?	?		-	-	
019	-	-	-	-	-	-	-	-	-		-	-	
020	-	-	-	-	-	-	-	-	-		-	-	
021	-	-	-	-	?	?	?	-	-		-	-	
022	-	-	-	-	-	-	-	-	-		-	-	
023	-	-	-	-	-	-	-	X	-	-	-	-	

See Table 3.1 for Key to Symbols and Scores

MU = Make-up reading for missed induction visit

Data Listing 3: Dermatologic Response Grades
By Product and Subject

Subject No.	Induction Reading										Challenge Phase		
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**MU**	**48hr**	**72hr**	**96hr(*)**
024	-	-	-	-	-	-	-	-	-		X	X	
025	-	-	-	-	-	-	-	-	X	-	-	-	
026	-	-	-	-	-	-	-	X	-	-	-	-	
027	-	-	-	-	-	-	-	X	-	-	-	-	
028	-	-	-	-	-	-	-	-	-		-	-	
029	-	-	-	-	-	X	-	-	-	-	-	-	
030	-	-	-	-	-	-	-	-	-		-	-	
031	-	-	-	-	X	-	-	-	-	-	-	-	
032	-	-	-	-	-	-	-	-	-		-	-	
033	-	-	-	-	-	-	-	-	-		-	-	
034	-	-	-	-	-	-	-	-	-		-	-	
035	-	-	-	-	-	-	-	-	-		-	-	
036	-	-	-	-	-	-	-	-	-		-	-	
037	-	-	-	-	-	-	X	-	-	-	-	-	
038	-	-	-	X	-	-	-	-	-	-	-	-	
039	-	-	-	-	-	-	-	-	-		-	-	
040	-	-	-	-	X	-	-	-	-	-	-	-	
041	-	-	-	-	-	-	-	-	-		-	-	
042	-	-	-	-	X	-	-	-	-	-	-	-	
043	-	-	X	-	-	-	-	-	-	-	-	-	
044	-	-	X	-	-	-	-	-	-	-	-	-	
045	-	-	-	-	-	-	-	-	-		-	-	
046	-	-	-	-	-	X	-	-	-	-	-	-	

Data Listing 3: Dermatologic Response Grades
By Product and Subject

Subject No.	Induction Reading										Challenge Phase		
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**MU**	**48hr**	**72hr**	**96hr(*)**
047	-	-	X	-	-	-	-	-	-	-	?	?	
048	X	-	-	-	-	-	-	-	-	-	-	-	
049	-	-	-	-	-	-	-	-	-		-	-	
050	-	-	-	-	-	-	-	-	-		-	-	
051	-	-	?	?	?	?	?	?	?		-	-	
052	-	-	-	-	-	-	X	-	-	-	-	-	
053	-	-	-	-	-	-	-	-	-		-	-	
054	-	-	-	-	-	-	-	-	-		-	-	
055	-	X	X	X	X	X	X	X	X		X	X	
056	-	-	-	-	-	-	-	-	-		-	-	
057	X	-	-	-	-	-	-	-	-	-	-	-	
058	-	-	-	-	-	-	-	-	-		-	-	
059	-	-	-	-	-	-	-	-	-		-	-	
060	-	-	-	-	-	-	-	-	-		-	-	
061	-	-	-	-	-	X	-	-	-	-	-	-	
062	-	-	-	-	-	X	-	-	-	-	-	-	
063	-	X	X	X	X	X	X	X	X		X	X	
064	-	-	X	-	-	-	-	-	-	-	-	-	
065	-	-	-	-	-	-	-	-	-		-	-	
066	-	-	-	-	-	-	?	?	X	-	-	-	
067	-	-	-	-	-	-	-	-	-		-	-	
068	-	-	-	-	-	-	-	-	-		-	-	
069	-	-	-	-	-	-	-	-	-		-	-	

Data Listing 3: Dermatologic Response Grades
By Product and Subject

Subject No.	Induction Reading										Challenge Phase		
	1	2	3	4	5	6	7	8	9	MU	48hr	72hr	96hr(*)
070	-	-	-	-	-	-	-	-	X	-	-	-	
071	-	-	-	-	-	-	-	-	X	-	-	-	
072	-	X	-	-	-	-	-	-	-	-	X	X	
073	-	-	-	-	-	-	-	-	-		-	-	
074	-	-	-	-	-	-	-	-	-		-	-	
075	-	-	-	-	-	?	?	-	-		-	-	
076	-	-	-	-	-	?	?	?	N9G		-	-	
077	-	-	-	-	-	-	-	-	-		-	-	
078	-	-	-	-	-	-	-	X	-	-	-	-	
079	-	-	X	-	-	X	X	X	X		X	X	
080	-	-	X	-	-	X	X	X	X		X	X	
081	-	-	-	-	-	-	-	-	-		-	-	
082	-	?	?	?	?	-	-	-	-		-	-	
083	-	-	-	-	-	-	-	X	-	N9G	-	-	
084	-	-	-	X	-	-	-	-	-	N9G	-	-	
085	-	-	-	X	-	-	-	-	-	N9G	-	-	
086	X	-	X	X	X	X	X	X	X		X	X	
087	-	-	-	-	-	-	-	X	-	N9G	-	-	
088	-	-	-	-	-	-	-	-	-		X	X	
089	X	-	-	-	-	-	-	-	-	N9G	-	-	
090	X	X	X	X	X	X	X	X	X		X	X	
091	-	-	-	-	-	-	-	-	N9G		-	-	
092	-	-	-	-	-	-	-	-	N9G		-	-	

Data Listing 3: Dermatologic Response Grades
By Product and Subject

Subject No.	Induction Reading 1	2	3	4	5	6	7	8	9	MU	Challenge Phase 48hr	72hr	96hr(*)
093	-	-	-	?	?	-	-	-	N9G		-	-	
094	-	-	-	-	?	?	-	-	N9G		-	-	
095	-	-	-	-	-	-	-	-	N9G		-	-	
096	-	-	-	-	-	-	-	-	N9G		-	-	
097	-	-	-	-	-	X	X	X	X		X	X	
098	X	X	X	X	X	X	X	X	X		X	X	
099	-	-	-	X	X	X	X	X	X		X	X	
100	-	-	-	-	-	-	-	-	N9G		-	-	
101	-	-	-	-	?	-	-	-	N9G		-	-	
102	-	-	-	-	-	-	-	-	-		-	-	
103	-	-	-	?	?	?	?	-	N9G		-	-	
104	-	-	-	-	-	-	-	-	-		-	-	
105	-	-	-	?	?	?	-	-	N9G		-	-	
106	-	-	-	-	-	-	-	-	N9G		-	-	
107	-	-	-	-	-	-	-	-	N9G		?	?	
108	-	-	-	-	-	-	-	-	N9G		X	X	
109	-	-	-	-	-	-	-	-	N9G		?	?	
110	-	-	-	-	-	-	-	-	N9G		?	?	
111	-	-	-	-	-	-	-	-	N9G		-	-	
112	-	-	-	-	-	-	-	-	N9G		-	-	
113	-	-	-	-	-	-	-	-	N9G		-	-	
114	-	-	-	-	-	-	-	-	N9G		-	-	
115	-	-	-	-	-	-	-	-	-		-	-	
116	-	-	-	-	-	-	-	-	N9G		-	-	
117	-	-	-	-	-	-	-	-	N9G		-	-	
118	-	-	-	-	-	-	-	-	N9G		-	-	
119	-	-	-	-	-	?	-	-	N9G		-	-	
120	-	-	-	-	-	-	-	-	N9G		-	-	
121	-	-	-	-	-	-	-	-	-		-	-	
122	-	-	-	-	-	-	-	-	-		-	-	
123	-	-	-	-	-	-	-	-	-		-	-	
124	-	-	-	-	-	-	-	-	-		-	-	
125	-	-	-	-	-	-	X	X	X		X	X	
126	-	-	-	-	-	-	-	-	-		-	-	
127	-	-	-	-	-	-	-	-	-		-	-	
128	-	-	-	-	-	-	-	-	-		?	?	
129	-	-	-	-	-	-	-	-	-		-	-	

Distributed for Comment Only -- Do Not Cite or Quote

formula contains 0.2% Sodium Acetylated Hyaluronate

HUMAN REPEATED INSULT PATCH TEST WITH CHALLENGE

STUDY REFERENCES

INVESTIGATIONAL PRODUCT	
Denomination	
Reference	
Batch number	

SPONSOR	
STUDY MONITOR	
COORDINATING CENTRE	
INVESTIGATING CENTRE	
SCIENTIFIC MANAGER OF THE INVESTIGATING CENTRE	
MAIN INVESTIGATOR	
CO-INVESTIGATORS	

Initiation date of study performance	**13/01/2020**
Completion date of study performance	**22/02/2020**

Date of the study report: 09/03/2020

HUMAN REPEATED INSULT PATCH TEST WITH CHALLENGE

Table of contents

ENGLISH SYNOPSIS 3
SIGNATURES AND DATES 6
I – INITIAL PROTOCOL DESIGN 7
I.1. STUDY OBJECTIVES 7
I.2. ETHICS 7
I.3. COORDINATING CENTRE AND STAFF 10
I.4. INVESTIGATING CENTRE AND STAFF 10
I.5. DATES OF STUDY PERFORMANCE 11
I.6. OVERALL STUDY DESIGN 12
I.7. STUDY POPULATION 14
I.8. INVESTIGATIONAL PRODUCT 19
I.9. CHECKING OF THE SKIN COMPATIBILITY 22
I.10. FOLLOW-UP OF THE TEST SUBJECTS 25
I.11. SUSPENSION OF THE STUDY 25
I.12. ADVERSE EVENTS 26
I.13. RAW DATA RECORDING AND STUDY REPORT FILING 27
I.14. REFERENCE 27
II – PRACTICAL CONDITIONS OF STUDY PERFORMANCE 28
II.1. PROTOCOL ADHERENCE 28
III – RESULTS 30
III.1. RESULTS / DISCUSSION 30
III.2. OVERALL CONCLUSION 31
III.3. QUALITY CONTROL AND QUALITY ASSURANCE 31

HUMAN REPEATED INSULT PATCH TEST WITH CHALLENGE

English synopsis

STUDY OBJECTIVES	Mainly, to confirm that the application of the investigational product under maximising conditions of exposure in a panel of healthy human adult subjects does not induce delayed contact sensitisation Secondarily, to assess the skin compatibility of the investigational product during the study
SPONSOR	
STUDY MONITOR	
COORDINATING CENTRE	
INVESTIGATING CENTRE	
MAIN INVESTIGATOR	
CO-INVESTIGATORS	
TYPE OF THE STUDY	Monocentric randomised study performed in simple blind, corresponding to the "Test Clinique Final de Sécurité" as defined by the France's Agency for the safety of health products (ANSM) Study project previously approved by a survey committee
DATES OF STUDY PERFORMANCE	From January 13th to February 22nd 2020
INVESTIGATIONAL PRODUCT	

English synopsis (continuation)

STUDY POPULATION	**Number of test subjects: 100** valid cases **Specific inclusion criteria: test subjects** ▪ aged from 18 to 70 (the 60-70-year age bracket not exceeding 10% of the total number of subjects) ▪ female / male ▪ with all types of skin on body ▪ with a phototype (Fitzpatrick): II, III or IV **Specific non-inclusion criteria: test subjects** • with personal history of adverse reaction to: ethanol, colophony, rubber, nickel, aluminium, patch materials, adhesive plaster • with family or personal history of atopy
METHODOLOGY	**Application of the investigational product**, in healthy human subjects, by a technician, at the investigating centre, to a skin site on the upper back, under maximising conditions of exposure (under occlusive patch) for a defined time Repeated applications 9 times to the same site (induction site) over a period of 3 consecutive weeks, period necessary to induce a possible allergy (induction period) After a minimal 2-week rest period, with no product application, single application of the investigational product, under patch, to the induction site and to a virgin site and for a defined time, enabling to reveal a possible induced allergy (challenge phase) **Application in parallel of distilled water** under occlusive patch at the same defined times as the investigational product = control site Skin examination of the application site, before the 1st product application of the induction period and the application of the challenge phase and after each patch removal by the same investigator or technician, supervised by the investigator Reporting of the sensations of discomfort directly by the test subjects to the investigator or technician, during the study Assessment of the allergic potential - checking of the skin compatibility: • Accurate description of the skin reactions observed • Evaluation of the allergic reaction according to the ICDRG scale: ?+, (+), (++), (+++) • Calculation of the percentage of reactive test subjects during the challenge phase and the induction period

English synopsis (continuation)

RESULTS

Characteristics of the included panel

Number of included subjects: 110
Number of exclusions: none
Number of withdrawals (reason): 6 (ref. 21a, 24a, 54a,23b, 42b and 43b) - for personal reasons independent from the study
Number of valid cases: 104

- Age: 21 to 64 (Mean: 48) the age bracket 60-70 = 7% of the total number of subjects
- Sex: F/M
- Phototype: II and III
- Skin types on the application site: with all types of skin on body

Checking of the skin compatibility

No reaction was noted on the control site.

For the investigational product:

Induction period			
Type of reaction	**Description of the reaction on the induction site**	**Number and percentage of reactive test subjects**	**Total number and percentage of reactive test subjects**
E: Erythema	None	0 / 0%	0 / 0%
M: Complementary mention	None	0 / 0%	
A: ICDRG scale	None	0 / 0%	

Challenge phase			
Type of reaction	**Description of the reactions on the induction site and the virgin site**	**Number and percentage of reactive test subjects**	**Total number and percentage of reactive test subjects**
E: Erythema	None	0 / 0%	0 / 0%
M: Complementary mention	None	0 / 0%	
A: ICDRG scale	None	0 / 0%	

OVERALL CONCLUSION

Under the experimental conditions adopted:

- During the induction period, the repeated applications of the product under occlusive patch on a panel of 104 test subjects with all types of skin on body induced no reaction of irritation.

- During the challenge phase, the single application of the investigational product to the induction site and virgin site induced no allergic reaction.

Based on these results, the product has a very good skin compatibility and does not show a sensitizing effect.

HUMAN REPEATED INSULT PATCH TEST WITH CHALLENGE

HUMAN REPEATED INSULT PATCH TEST WITH CHALLENGE

I – INITIAL PROTOCOL DESIGN

I.1. STUDY OBJECTIVES

Mainly, this study intended to confirm that the application of the investigational product under maximising conditions of exposure in a panel of healthy human adult subjects did not induce delayed contact sensitisation.

Secondarily, the skin compatibility of the investigational product was assessed during the study.

I.2. ETHICS

I.2.1. Ethical conduct of the study

The study was performed in the spirit of:

- the general principles of medical ethics in clinical research coming from the Declaration of Helsinki (June 1964) and its successive amendments,
- the international recommendations relating to Good Clinical Practices for conducting clinical trials for drugs ICH E6(R2) of 09/11/2016,
- the Directive of the European Parliament and Council 2001/20/EC concerning the harmonization of legislative, statutory and administrative provisions of the member States relating to the application of good clinical practices when conducting clinical trials for drugs for human use – OJ/EC of 01/05/2001,
- the recommendations of Colipa - August 1997: "guidelines for the assessment of human skin compatibility",
- the Romanian Order No. 904/25.07.2006 on approval of rules relating to the implementation of good clinical practice in the conduct of clinical trials on medicinal products for human use.

and was in accordance with the REGULATION (EU) 2016/679 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 27 April 2016 on the protection of natural persons with regard to the processing of personal data and on the free movement of such data

and according to the recommendations of the France's Agency for the safety of health products ("Agence Française de Sécurité Sanitaire des Produits de Santé – AFSSAPS") – December 2008: "Test clinique final de sécurité d'un produit cosmétique en vue de confirmer son absence de potentiel sensibilisant cutané retardé : recommandations aux promoteurs de recherche et aux prestataires de service".

I.2.2. Relevance of the study

On the one hand, the aims of the study were a better knowledge of the skin safety of the investigational product and the confirmation of the absence of allergic potential and the investigational product was not applied under normal conditions of use. So, the test subject had no direct benefit from this study.

On the other hand, the foreseeable risk incurred by the test subjects were a possible allergic reaction to one or several ingredients of the investigational product or a skin irritation due to the finished product applied under maximising conditions (under patch).

Generally in this type of study, the possible adverse effects (as erythema, vesicles…) are limited on the application sites and decrease in some days.

The applications were performed at the investigating centre and supervised by a dermatologist, so the application had to be quickly stopped if necessary, and the clinical follow-up of the reactive test subject(s) had to be done by a competent person.

So, according to the nature and the severity of the possible reaction, the investigator had to define the conduct to be adopted and the suitable steps to ensure the safety of the test subject(s) (for example definitive or temporary exclusion of the test subject(s) concerned from the study, modification of the application conditions of the product…) and had to ensure the clinical follow-up of the test subject(s) concerned, as long as it was necessary.

All the test subjects were included in the study the same day.

So, there was suitability between the aim of the study and its eventual risks and the foreseeable troubles related to the experimental conditions of the protocol.

The skin examination was performed by the investigator or technician, supervised by the investigator, having the appropriate experience.

The experimental conditions of product application created a certain occlusion and favoured the penetration of the ingredients through the skin. If some of them had an allergenic potential, this one was more easily proved by this kind of approach.

The product dose was perfectly controlled and the patch material and the conditions of use of the product were adapted to the product category.

A control site (without investigational product) served as control to take into account the possible effects not directly related to the investigational product but due to the patch material.

The investigational product was tested with other products at the same time, the experimental area chosen (back) enabling to test easily several products (maximum 15 product sites at least 1 cm far apart). The sites of application of the different products and the control site(s) were chosen according to a clockwise distribution, altering of one rank from a test subject to another, to take into account the variability of the skin reactivity according to the site.

The observance of the experimental conditions by the subjects, who took part in the study, was assessed by a questionnaire at the end of the induction period and at the end of the challenge phase.

I.2.3. Survey committee

The study had to be devoid of any foreseeable serious risk for the safety of the test subjects.

According to the procedure of the investigating centre, the protocol, the informed consent form and the preclinical information concerning the investigational product (particularly referring to its safety) were submitted to the opinion of an Institutional Ethics Committee, formed with members belonging to the staff of the investigating centre, but not directly involved in the study.

The Institutional Ethics Committee gave the approval on January 10th 2020.

The study began after the approval of the Institutional Ethics Committee.

I.2.4. Information of the test subject and informed consent form

The information about the study was given to each test subject before the start of the study.

This information was accessible, understandable and suitable for each test subject. It was orally given and then in a written specific document (in Romanian).

This information was completed, if necessary, by the investigator (or the competent person designated by him) who answered all the questions asked by the test subject.

The informed consent form was personal and previous to the start of the study.

It was clear, informed and explicit. It was written and given on the same support as the information on the study, in order to avoid any risk of dispute about its content.

The content of this document particularly specified:

- that the test subject had declared to have a health coverage,
- the aim of the study,
- the study design and the experimental conditions of the study,
- the investigational product conditions of use,
- the approximate number of test subjects involved in the study,
- the expected duration of the study (for the test subject),
- the number of visits to the investigating centre, their dates and their duration,
- the study constraints (obligations, restrictions and troubles),
- the reasonable foreseeable risks,
- that skin site photographs could be taken and in this case, that the test subject would not be recognizable,
- that the test subject could be requested, if necessary, to take part in a complementary test to complete the study,
- the opinion of the Institutional Ethics Committee,
- the person to contact and the contact telephone number,
- that the personal data of the test subject were confidentially treated by the study staff, available for the study monitor and possibly consulted (with the authorization of the test subject) by the auditors, the members of the Institutional Ethics Committee and the Health Authorities (subject to non-divulgation),
- the ban on taking part simultaneously in other clinical studies that could interfere with the current study,
- the amount of the compensation for the constraints to be undergone,
- the form of compensation in case of possible harm caused by the study (all the costs of health care assumed through the investigating centre),

- the follow-up of all test subjects after the end of the study by delivering an oral and written information note,
- the period of exclusion at the end of the study during which the test subject will not be allowed to take part in another clinical study,
- the confidential treatment of the study data,
- that the anonymity of the test subject was and will be preserved,
- the freedom for the test subject to refuse to participate or to stop his participation at any time without any justification and any legal consequences.

This document was previously approved by the Institutional Ethics Committee.

At the beginning of the study, 2 copies of this document were dated and signed simultaneously by the test subject and by the investigator or the competent person designated. One copy was given to the test subject, the other one was kept at the investigating centre.

I.2.5. Confidentiality and identification of the test subject

The information concerning the test subject, required for his recruitment, his inclusion and particularly that related to his health, obtained during the medical examination prior to his admission in the general panel of the investigating centre, formed part of medical secret and was confidentially treated.

The test subject was coded when included in the current study (according to the corresponding procedure of the investigating centre) in order to preserve his anonymity.

If photographs of the skin had to be taken, the test subject had to be non-recognizable.

I.3. COORDINATING CENTRE AND STAFF

The coordinating centre ensured the liaison between the sponsor and the investigating centre.

I.4. INVESTIGATING CENTRE AND STAFF

I.4.2. Technical staff

The test was performed by a competent investigator and a trained and qualified technical staff.

I.5. DATES OF STUDY PERFORMANCE

Initiation date of study performance: 13/01/2020

Completion date of study performance: 22/02/2020

I.6. OVERALL STUDY DESIGN

I.6.1. Type of the study

This monocentric clinical study was randomized and performed in simple blind, in a panel of healthy human subjects.

This study corresponds to the "Test Clinique Final de Sécurité" as defined by the France's Agency for the safety of health products (ANSM).

The test subject was used as own control.

I.6.2. General principle of the study

The study was performed on the basis of the protocol – version n°1 (08/01/2020).

The investigational product had to be applied in **100** test subjects, by a technician, at the investigating centre, under maximized conditions of exposure (under patch) for a defined time. The applications had to be repeated 9 times to the same site (induction site) over a period of 3 consecutive weeks, period necessary to induce a possible allergy (induction period).

After a minimal 2-week rest period, with no product application, a single application of the investigational product, under patch, to the induction site and to a virgin site and for a defined time, enabling to reveal a possible induced allergy (challenge phase), had to be performed.

A skin examination of the application site had to be performed before the 1st product application of the induction period and the application of the challenge phase and after each patch removal, by the same investigator or technician, supervised by the investigator.

The sensations of discomfort had to be directly reported by the test subjects to the investigator or technician, during the study.

The results of the skin compatibility were descriptively expressed.

Since sensitisation is not a matter of quantification, the possible reactions had to be classified as allergic or not, according to the observation done during the challenge phase compared with the observation done during the induction period.

I.6.3. Chronology of the study

Induction period: 3 consecutive weeks			
Operations at the investigating centre	**Experimental times**		
	D1	**D3 - D5 - D8 - D10 - D12 - D15 - D17 - D19**	**D22**
Pre-inclusion: Delivery of the informed consent form Signature of the informed consent form Checking of the inclusion and non-inclusion criteria	●		
Clinical examination of the application site and questioning of the test subject by the investigator or technician supervised by the investigator, before product application	●		
Final inclusion	●		
Application of the investigational product under patch to the defined induction site	●	●	
Removal of the patch at the investigating centre		●	●
Clinical examination of the application site and questioning of the test subject by the investigator or technician supervised by the investigator		●	●
Control of the observance			●

Rest period : 2 consecutive weeks at least (4 weeks at the most)	**No product application**

Challenge phase: 1 week			
Operations at the investigating centre	**Experimental times**		
	D36	**D38**	**D40**
Clinical examination of the application site and questioning of the test subject by the investigator or technician supervised by the investigator, before product application	●		
Application of the investigational product under patch to the defined induction site and to a defined virgin site	●		
Removal of the patch at the investigating centre		●	
Clinical examination of the application sites and questioning of the test subject by the investigator or technician supervised by the investigator		●	●
Control of the observance			●

I.7. STUDY POPULATION

I.7.1. Constitution of the panel of test subjects and mode of recruitment

The investigating centre has at its disposal a general panel of subjects constantly renewed. These subjects come from all social categories. They either volunteer spontaneously to the investigating centre or reply to a direct call from the latter. Prior to their admission in this general panel, they are subjected to a medical examination and a detailed medical and cosmetological questionnaire, performed by a general practitioner according to the internal procedure of the investigating centre.

All the data concerning the panel are computerized and on paper.

For the study, the test subjects were selected from this general panel on the basis of inclusion criteria and non-inclusion criteria specific to the study and on their ability to respect the constraints required by the protocol. They were definitely included in the study after a specific questioning and a clinical examination.

I.7.2. Number of test subjects

The number of test subjects with exploitable data (valid cases[(1)]) at the end of the study had to be at least **100**.

[(1)]valid case = test subject that respected the protocol with no significant deviation which could have some influence on the study results.

The number of test subjects necessary to allow a reliable prediction of the sensitising potential of an investigational product depends on the methods used. The statistical considerations involved in extrapolating from a small test population to a large number of users were discussed in the following publication:

- Henderson C.R., Riley E., Certain statistical considerations in patch testing, J. Invest. Dermatology, 1945, 6, pp. 227-232

It is obvious that studies with numbers of test subjects sufficient to obtain statistically valid data applicable to several thousand consumers are not feasible. Therefore, the value of predictive patch testing does not lie in the precision of the prediction but in screening out the rare sensitising products.

So, referring to the experience acquired in the field of contact allergy to cosmetic products, the number of test subjects, empirically defined in the protocol, was sufficient to confirm, before product launching, the absence of allergenic potential of the investigational product and to achieve the study objectives.

At the beginning of the study, complementary test subjects (+10) had to be included to answer the demand and to compensate the possible withdrawals or exclusions from the study independent of the investigational product.

The test subjects excluded from the study for reasons dependent of the investigational product had to be taken into account in the study results and did not have to be replaced.

If during the study, there was a risk not to have the required number of valid cases (great number of withdrawals…), the study monitor had to be informed and an additional quota of subjects had to be possibly included to reach the target.

At the end of the study, in spite of the precautions taken by the investigating centre, if the number of valid cases was less than the number of test subjects requested by the sponsor, the study monitor had to be informed.

I.7.3. Inclusion criteria

I.7.3.1. General inclusion criteria

According to the protocol, had to be included in the study, the subjects:

- suitable to participate in the study (after the clinical examination and questioning) and corresponding to the quality of "healthy subject" as defined in the corresponding procedure of the investigating centre,
- declaring to have a health coverage,
- signing an "informed consent form" for this study,
- certifying not to take part in another clinical study that could interfere with the current study,
- certifying the truth of the personal information declared to the investigator,
- capable of following directions and reliable to respect the constraints of the protocol (living not too far from the investigating centre, no linguistic and intellectual barrier),
- free to ensure the visits to the investigating centre,
- declaring not to have exposed themselves to a risk of pregnancy for at least 3 months before the beginning of the study and committing themselves to use effective contraceptive method throughout the study (for the women of childbearing potential),

I.7.3.2. Specific inclusion criteria

Subjects:

- aged from 18 to 70 (the 60-70-year age bracket not exceeding 10% of the total number of subjects),
- female and/or male,
- with all types of skin on body,
- with a phototype (Fitzpatrick): II, III or IV.

I.7.4. Non inclusion criteria

I.7.4.1 General non inclusion criteria

According to the protocol, did not have to be included in the study, the subjects:

- being in exclusion period,
- deprived of freedom by administrative or legal decision or under guardianship,
- who could not be contacted in case of emergency,
- admitted in a residential care,

- planning an hospitalisation during the study,
- belonging to the staff of the investigating centre,
- being of age but protected by law,
- having received vaccination within the 3 weeks prior to the study or intending to be vaccinated during the course of the study,
- with documented history of contact allergy,
- with person al history of adverse reactions to the same type of product as the investigational product
- exhibiting skin marks and/or moles and/or freckles in too great quantity, hyperpilosity on the experimental area able to interfere with the assessment of the possible skin reactions,
- with still visible eczematous reaction, scar or pigmentary after-effects of previous tests on the experimental area,
- under treatment, prior to the study, able to interfere with the interpretation of the study results, particularly:
  - systemic retinoids (isotretinoin per os …) within the 6 months,
  - other systemic anti-acne medication within the 3 months,
  - topical retinoids within the 2 months,
  - other topical anti-acne medication within the month,
  - anti-acne cosmetic products within the 2 weeks (excluding face anti acne products),
  - topical or systemic medication with anti-inflammatory or antihistamine products within the 2 weeks,
  - antibiotics within the 2 weeks,
  - medication for malignancy (of any kind) within the 5 years,
  - desensitisation treatment within the 6 months,
- foreseeing, during the study, a treatment able to interfere with the interpretation of the study results (systemic or topical anti-acne medication, anti-acne cosmetic products, topical or systemic medication with anti-inflammatory or antihistamine, antibiotics, desensitisation treatment, …),
- having had a fever lasting more than 24 hours, within the 8 days prior to the study,
- breastfeeding or pregnant or planning a pregnancy during the study (for the women of childbearing potential),
- having started or changed oestrogen-progesterone contraception or hormonal treatment, within the 3 months prior to the study or foreseeing it for the duration of the study,
- having had any invasive aesthetic cares on chest and back (peeling, laser…) by a dermatologist within the 2 months prior to the study or foreseeing it for the duration of the study,
- having had any non-invasive aesthetic cares on chest and back (scrub, skin cleansing…) by an aesthetician within the month prior to the study or foreseeing it for the duration of the study,
- having received excessive or intensive exposure to sunlight (natural or artificial) within the month prior to the study or foreseeing UV exposures for the duration of the study,
- under treatment with PUVA or UVB within the month prior to the study,
- having participated in a human repeated insult patch test with challenge with or without sun exposure 4 months prior to the study,

- having already participated in 5 clinical studies involving patch test, including 3 human repeated patch tests with or without challenge within the year prior to the study,
- foreseeing bath (in bathtub, sea or swimming-pool), sauna or Turkish bath during the study period,
- regularly practicing intensive sport causing sweating and requiring frequent showers.

I.7.4.2. Specific non-inclusion criteria

Subjects:

- with personal history of adverse reaction to: colophony, ethanol, rubber, nickel, aluminium, patch materials, adhesive plaster,
- with family or personal history of atopy.

I.7.5. Specific information concerning the test subjects and medication

Skin reactivity, history of atopy, contraception (type) and possible current medication were documented at the inclusion by the technician, supervised by the investigator or the competent person designated, in the collective case report form (CRF).

No medication likely to interfere with the study was allowed during the study; however, if the health state of the subjects justified some medication (particularly anti-inflammatory drugs), any information relating to this concomitant medication had to be carefully documented in the case report form.

The investigator had to exclude the test subjects taking concomitant medication likely to interfere with the study and the interpretation of the results.

I.7.6. Exclusion criteria

According to the study protocol and to the procedures of the investigating centre, had to be excluded from the study, the test subjects:

- who did not comply with the protocol and created deviation resulting in un-exploitable results,
- who took part in another clinical study that could interfere with the current study,
- who had adverse event (for example: inter-current disease requiring a concomitant medication interfering with the study and the interpretation of the results or severe skin intolerance to the investigational product), incompatible with a good protocol observance.

The temporary or definitive discontinuations decided by the investigator and their dates and reasons had to be carefully documented in the collective case report form.

I.7.7. Withdrawal criteria

According to the study protocol and to the procedures of the investigating centre, had to be considered as withdrawals, the test subjects:

- who discontinued the study for personal reasons independent of the study (for example: moving house, new job),
- who did not come to the investigating centre for the checking in spite of phone calling.

The withdrawals and their dates and reasons had to be carefully documented by the investigator in the collective case report form (CRF).

I.7.8. Study constraints imposed on the test subjects

The constraints defined by the procedures of the investigating centre and partly in the study protocol, imposed on the test subjects during the study, were the following ones:

- if justified and asked by the investigator, participation in a complementary test (additional visits to the investigating centre),
- exclusion period at the end of the study (according to the corresponding procedure of the investigating centre and 4 months minimum before starting a human repeated insult patch test with challenge, 1 month minimum before starting another type of study),
- no participation in another clinical study that could interfere with the current study,
- if justified, description of any concomitant medical treatment not excluded by the inclusion and non-inclusion criteria,
- no drug liable to interfere with the study and the interpretation of the results, *e.g.* aspirin (except low dose maintenance therapy), products containing aspirin, antihistamine drugs, anti-inflammatory drugs, antibiotics… (however, if therapeutic requirement: possible exclusion from the study),
- neither initiation of an hormonal treatment nor change of the usual hormonal treatment,
- no change of the mode of contraception,
- no significant change in lifestyle: diet, smoking, sport,…,
- visit to the investigating centre (13/14 times) and respect of the dates and hours of visits,
- neither anti-acne nor anti seborrheic local treatment,
- neither invasive body aesthetic cares (peeling, laser…) nor non-invasive body aesthetic cares (scrub, skin cleansing…) on chest and back, by a dermatologist or an aesthetician in Beauty Salon,
- no application of cosmetic care products to the back,
- no change in usual body hygiene products,
- no introduction of new cosmetic products,

- no intensive sun or UVA exposure (U.V. lamps) during the study and 2 weeks after the end of the study,
- no wearing of too tight or restraining clothes liable to produce frictions on the experimental area and to cause the un-sticking of the patch(es),
- neither Turkish bath nor sauna nor bath (in bathtub or swimming-pool or sea), liable to cause excessive sweating and/or the un-sticking of the patch(es),
- during shower, protection of the experimental area (no violent projection of water, no application of soap, very gentle wiping if necessary) to avoid the un-sticking of the patch(es) or the appearance of inter-current skin irritation,
- no intensive sport liable to cause excessive sweating and the un-sticking of the patch(es),
- no vaccination.

The test subjects were questioned at the end of the induction period and at the end of the challenge phase about the respect of the study constraints. These data were documented in the case report form (CRF). The investigator had to assess the importance of the possible deviations in comparison with the experimental conditions required at the beginning of the study and their incidence on the validity of the results.

I.8. INVESTIGATIONAL PRODUCT

I.8.1. Identification of the investigational product

Denomination	
Cosmetic category	
Formula number	
Batch number	
Galenic form and organoleptic characteristics	
Normal foreseeable conditions of use	

I.8.2. Coding and storage

The product units were sent to the investigating centre. Upon receipt, the investigating centre noted the date of product receipt and checked the supplied quantities.

The product units were coded and labelled in Romanian, according to the corresponding procedure of the investigating centre.

Number and type of product units	7 plastic flasks
Content of product unit	50ml

Before starting the study, the storage of the investigational products units was carried out according to the conditions defined by the sponsor, in the product storage area and a product sample (one product unit) was taken and kept in the sample storage area of the investigating centre for at least 3 years after the end of the study then destroyed, according to the corresponding procedure of the investigating centre.

Apart from the specific demand of the sponsor, the used and unused product units will be kept at least 2 weeks after the sending of the final report then destroyed, according to the corresponding procedure of the investigating centre.

I.8.3. Information concerning the investigational product

The investigational product units had to be supplied with a certificate that particularly referred to:

- the compliance of the ingredients of the investigational product formula with the European Regulation N° 1223/2009 of the European Parliament and Council on cosmetic products,

- the safety of the finished investigational product and the absence of foreseeable serious risk for the health of the test subjects.

The qualitative formula of the product had to be supplied to the coordinating centre and the investigating centre by the study monitor.

I.8.4. Experimental conditions of application of the investigational product

I.8.4.1. Induction period

The skin site had to be defined by the technician in charge of the study, on the upper back of the test subjects, on a surface free from scars, moles, freckles and any other skin anomaly, and avoiding the areas of friction with clothes.

The quantity of investigational product had to be measured, by the technician in charge of the study with a single use syringe and put into the patch.

Before patching, the skin site had to be wiped with a cotton pad.

The patch containing the investigational product had to be applied, by the technician in charge of the study, on the defined skin site.

The experimental conditions of application had to be the following ones:

Patch material	Experimental conditions of use of the investigational product	Quantity to be applied
Occlusive patch Finn Chamber standard®: aluminium cupula kept in position by an hypoallergenic adhesive: Scanpor® (inner diameter: 8 mm, surface : 50 mm²) *Switch to semi-occlusive patch if reactions ≥ 2 occurs*	As supplied	20 µl

An occlusive patch containing 20 µl of distilled water had to be applied in parallel as control to eliminate, when the results were interpreted, the possible inter-current effects due to the patch material.

The induction consisted of 9 patches applied 3 times a week (for example: Monday, Wednesday and Friday) for a 3 week period.

Patches applied on Mondays and Wednesdays had to be worn for 48 hours ± 4 hours and patches applied on Fridays had to be worn for 72 hours ± 4 hours.

The application was possibly performed on other days of the week, subject to the respect of the three 72-hour contact patches and the six 48-hour contact patches.

Induction period				
Operations	**Experimental times**			
	D1	**D3 - D5 D10 - D12 D17 - D19**	**D8 - D15**	**D22**
Application of the investigational product under patch to the defined induction site	●	●	●	/
Removal of the patch at the investigating centre	/	● After 48h of contact	● After 72h of contact	● After 72h of contact

The applications had to be repeated on the same site, except in the case of significant irritation/sensitisation reaction.

In case of moderate or severe skin erythema or mild erythema with oedema/infiltration, the study monitor had to be quickly informed and the product application had to be stopped to the induction site defined and continued to a new adjacent site (the change of site being done once only).

In case of suspected allergic reaction the product did not have to be applied again and the case had to be quickly discussed with the coordinating centre and the study monitor. Then, the decision to reapply or not the product had to be jointly taken by the investigator and the study monitor.

During the induction period, the technician had to precisely locate the test site to be able to retrieve it after the rest period, according to the procedure of the investigating centre.

I.8.4.2. Rest period

No product application had to be performed for a period of 2 weeks minimum (4 weeks maximum) following the end of the induction period.

The test subjects had to inform the investigator of any reaction occurring during this period.

I.8.4.3. Challenge phase

The challenge patches had to be applied once after the rest period. The investigational product and the control product had to be applied using the same patching conditions as those used for the induction period, on 2 sites: a virgin site and the induction site, symmetrically located, if possible. The patches had to be removed 48 hours ± 4 hours after application.

Challenge phase			
Operations	**Experimental times**		
	D36	**D38**	**D40**
Application of the investigational product under patch to the defined induction site and to a defined virgin site	●	/	/
Removal of the patch at the investigating centre	/	● After 48h of contact	/

I.9. CHECKING OF THE SKIN COMPATIBILITY

I.9.1. Recording of the skin reactions

Skin examinations of the application sites had to be performed visually, by the same investigator or technician, supervised by the investigator, under standard "daylight" source:

- during the induction period:
  - before patching on D1
  - 15 to 30 minutes after patch removal (or more, if redness appeared after the removal of the adhesive) on D3, D5, D8, D10, D12, D15, D17, D19, D22
- during the challenge phase:
  - before patching on D36
  - 15 to 30 minutes after patch removal on D38 (or more, if redness appeared after the removal of the adhesive)
  - 48h +/- 4 hours after patch removal on D40

In case of delayed skin reaction occurring after the 96h grading, the test subject had to contact the investigating centre and the site had to be re-examined by the investigator as long as necessary until reactions disappear.

All adverse reactions had to be graded until resolution.

Concurrently with the clinical examinations performed, the test subjects had to be questioned about the possible sensations of discomfort they feel.

In case of strong sensations of discomfort felt during the patch wearing at home, the test subjects had to inform by phone the investigator. If necessary, the patch was removed and a skin examination was quickly performed by the investigator (before the next planned visit to the investigating centre).

In case of application to a new adjacent site, the original test site had to be scored in parallel with the new test site until completion of the study and the skin scores of this original test site had to be distinctly documented.

Digital photographs of the skin had to be systematically taken when justified (adverse effects).

All the data were recorded in the collective case report form (CRF).

I.9.2. Expression of the results

All the reactions had to be accurately described at each experimental time using the criteria and the scale hereafter.

E = Erythema d= diffuse p = punctuated peri = peripheral	**0** – no visible erythema **0.5** – very slight erythema – barely perceptible **1** – mild erythema – faint pink **2** – moderate erythema – well defined **3** – severe erythema **4** – caustic effect – erosive aspect and/or necrotic aspect

If any visible response, the investigator had to proceed to palpation to assess infiltration / oedema.

M = Complementary mention : Other reactions	
Sv	Soap effect (shiny skin with possibly wrinkles)
D	Desquamation
Dr	Dryness
Hy	Hypopigmentation
C	Skin coloration – hyperpigmentation
Oe	Homogeneous infiltration / oedema
P	Papules
V	Vesicles
Pe	Petechiae
Fr	Follicular reaction
I	Itching at the test site
S	Spreading beyond the patch area (infiltration or erythema)
F	Fissuring
Cr	Exudation and/or Surface encrustation
B	Bullae
Sc	Scab
He	Heating
Pu	Pustules
/	No reaction

The other visible clinical signs had to be presented descriptively

M = Complementary mention: Additional comments	
NA	Product not applied
T	Tape reaction
L	Loss of patch during the first 12 hours
N9G	No 9th grading
X	Succeeding patch not applied and succeeding grade (in brackets) denotes a residual reaction
Abs	Test subject absent

For the induction period and the challenge phase, the results were expressed in percentage of reactive test subjects: for this calculation only the visible signs of reactivity were taken into account: erythema, oedema, vesicle, bulla, papule…

I.9.3. Interpretation of the results

I.9.3.1. Allergy

All the test subjects included in the study were taken into account to appreciate the skin allergic potential of the investigational product as long as they were submitted at least to one post application examination at the defined time or else.

The nature, intensity, appearance period from the application, disappearance period from the application, location (induction site and/or virgin site) of the skin reaction and the phase of the study were taken into account for the interpretation of the results.

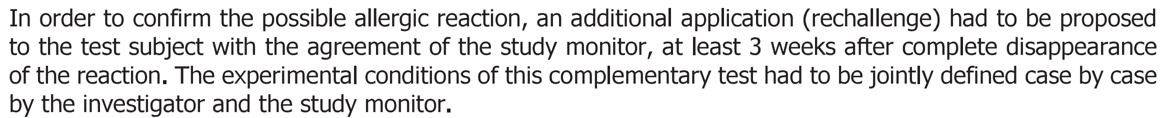
A = ICDRG scale	
IR	Irritation reaction
-	No allergic reaction
?+	Doubtful reaction (only slight erythema)
(+)	Weak positive reaction (without vesicle): slight erythema and infiltration with presence of small papular elevations, possibly papules
(++)	Strong positive reaction: erythema, papules, vesicles, infiltration
(+++)	Extreme positive reaction: intense erythema, oedema, coalescent vesicles (bullae)

A site where erythema was graded 2 or more during the challenge phase (with or without infiltration) had to be evaluated on subsequent days to note whether the reaction diminished or increased, in order to differentiate between an allergic and an irritant reaction. A quick decreasing reaction could be indicative of irritation. A reaction with infiltration / oedema that persisted or increased over time usually could indicate an allergic reaction.

If the possible allergic reaction was observed during the induction period, it could be the revelation of an allergy previously contracted or the revelation of an allergy precociously induced by the investigational product.

If the possible allergic reaction was observed during the challenge phase (similar responses observed on the virgin site and on the induction site), it could be the revelation of an allergy induced during the induction period by the investigational product.

In case of suspected allergic reaction, the study monitor had to be quickly informed.



In order to confirm the possible allergic reaction, an additional application (rechallenge) had to be proposed to the test subject with the agreement of the study monitor, at least 3 weeks after complete disappearance of the reaction. The experimental conditions of this complementary test had to be jointly defined case by case by the investigator and the study monitor.

I.9.3.2. Irritation

All the test subjects included in the study were taken into account to appreciate the skin irritant potential of the investigational product as long as they were submitted at least to one post application examination at the defined time or any other time.

To appreciate the skin irritant potential, the interpretation of the results was based on the experience of the investigator in this field. The skin compatibility of the investigational product was classified as: very good, good, moderate or bad, in the study conditions.

If justified in case of reactivity in some test subjects, a complementary study will be possibly carried out in these test subjects, after agreement of the sponsor. The experimental conditions of this study will be defined by the investigator, case by case.

I.10. FOLLOW-UP OF THE TEST SUBJECTS

The test subjects having, in totality or partially, participated in this study received at the end of the study an oral and written information, asking them to contact the investigating centre in case of any abnormal skin reaction, occurring with the use of a cosmetic product after the study.

The test subjects having presented a confirmed allergic reaction during the study were definitely crossed off the general panel of the investigating centre.

An information note was given to them with the following information:

- title and objective of the study, and study reference of the investigating centre,
- dates of the study and of the complementary test(s) performed,
- description of the reaction, conclusion of the investigator, imputability of the investigational product,
- category of the investigational product,
- when identified, substance(s) responsible for the reaction or suspected substances if the link cannot be established with certainty between the reaction and the product ingredients: INCI name(s) and function(s),
- type of products containing or likely to contain the concerned substance(s),
- recommendations and cautions for the future use of certain categories of cosmetic products, advices in case of reaction (general practitioner / dermatologist / allergologist consultation),
- necessity to inform the investigating centre in case of future reaction with cosmetic products.

The investigating centre has to keep a copy of this document and an updated record of these test subjects.

I.11. SUSPENSION OF THE STUDY

The investigator had to stop the study if it showed a risk for the health or the integrity of the test subjects.

The date of the suspension and the reasons had to be carefully documented by the investigator in the case report form.

The coordinating centre had to inform promptly the study monitor, by phone, fax or e-mail.

The sponsor was able to stop the study at any time for administrative reasons or other ones.

I.12. ADVERSE EVENTS

I.12.1. Definitions

Any topical product can induce, when used in Human, according to individual sensitivities, a local and minor reactivity, defined as follows: any slight local reaction of intolerance or sensation of discomfort, occurring in a test subject during a clinical study, completely reversible, expected, due to the investigational product and which does not question the observance of the study protocol or the good implementation of the study.

- **adverse event**: any harmful event with or without relationship with the investigational product, occurring in a test subject during a clinical study.
- **suspicion of adverse effect**: any adverse event with a quite possible relationship with the investigational product.
- **adverse effect**: any harmful and unwanted reaction, due to the investigational product, occurring in a test subject during a clinical study.
- **unexpected adverse effect**: any adverse effect due to the investigational product, the nature, the intensity and/or the evolution of which do not agree with the product information.
- **serious adverse event / effect**: any adverse event or adverse effect that causes death, endangers test subject's life, induces an hospitalisation or the prolongation of the hospitalisation, causes severe and lasting incapacity or handicap or induces congenital anomaly or malformation.

I.12.2. Data collection

The investigator had to accurately describe the adverse event and had to appreciate its seriousness. According to the corresponding procedure of the investigating centre, he had to define the link of causality between this event and the investigational product, on the basis of the symptoms, the chronology, the results of the possible specific complementary tests undertaken and any available information.

The imputability of the product had to be assessed according to the scale: very likely, likely, possible, questionable, excluded (in accordance with the recommendations of the European Council in its resolution ResAP (2006)1 of 08/11/2006 and the method of imputability of the adverse effects linked to the cosmetic products published by AFSSAPS in December 2009).

I.12.3. Conduct to be adopted in case of adverse event

Faced with an adverse event, the investigator had to freely define, case by case, the conduct to be adopted and the suitable steps to ensure the safety of the test subject concerned and of the other test subjects included in the study.

In case of suspicion of adverse effect (with a quite possible relationship with the investigational product), the investigator had to ensure the clinical follow-up of the test subject concerned, as long as necessary.

I.12.4. Communication with the study monitor

According to the corresponding procedure, the serious adverse events and the adverse effects had to be notified as soon as possible and within 24 hours at the latest, by the investigating centre to the study monitor, by phone, fax or e-mail.

The investigator had to send an adverse event form to the study monitor and to the coordinating centre.

If justified, the investigator had to give to the study monitor and to the coordinating centre complementary information when available.

I.12.5. Communication with the Institutional Ethics Committee

According to the corresponding procedure of the investigating centre, the serious adverse events and the adverse effects had to be notified, as soon as possible, by the investigator to the Institutional Ethics Committee.

I.13. RAW DATA RECORDING AND STUDY REPORT FILING

All the data gathered during the study were recorded accurately, legibly and indelibly by the investigator or the technician in charge of the study, under his control, in the collective case report form.

Each page of this document was initialled by the technician; the whole was verified and validated by the investigator.

The content of this study report took into account the recommendations of the Colipa related to the assessment of the efficacy of cosmetic products (May 2008) and the explanatory note related to the structure and the content of the reports of clinical studies – ICH E3, of 28/11/1995 and is in accordance with the French decree of 25/08/2006 concerning the content of the final report and of the final report summary of a biomedical research on cosmetic product.

At the end of the study, the information concerning the investigational product, the information concerning the test subjects (collective CRF, informed consent forms) were filed and will be kept for 10 years, in the filing area of the investigating centre and the information related to the conduct of the study (protocol signed by the sponsor, copy of this study report….) were filed and will be kept for 10 years, in the filing area of the investigating centre and /or the coordinating centre.

At the end of this period, the sponsor will choose among 3 options:

- return of the study documentation to the sponsor,
- filing of the study documentation in the filing area of the investigating and/or coordinating centre, based on a specific contract,
- destruction of the study documentation (after sponsor's written and signed authorization).

I.14. REFERENCE

The methodology used was an adaptation from that described by Marzulli and Maibach (Human Repeated Insult Patch Test for delayed contact hypersensitivity: HRIPT)

- Marzulli F.N., Maibach H.I., Contact allergy: predictive testing in man, Contact Dermatitis, 1976, 2, pp. 1-17
- V.T. Politano, A.M. Api / Regulatory Toxicology and Pharmacology 52 (2008) 35–38

II – PRACTICAL CONDITIONS OF STUDY PERFORMANCE

II.1. PROTOCOL ADHERENCE

Preamble: No serious adverse effect or intercurrent event justified the suspension of the study.

II.1.1. Study population

Number of test subjects included in the study	110	
Withdrawals	**Test subjects concerned**	**Date and reasons**
	Ref. 21a	15/01/2020 - for personal reasons independent from the study
	Ref. 24a	15/01/2020 - for personal reasons independent from the study
	Ref. 54a	24/01/2020 -- for personal reasons independent from the study
	Ref. 23b	20/01/2020 -- for personal reasons independent from the study
	Ref. 42b	24/01/2020 -- for personal reasons independent from the study
	Ref. 43b	17/01/2020 -- for personal reasons independent from the study
Exclusion	**Test subjects concerned**	**Date and reasons**
	None	Not applicable
Valid cases	**104**	

The number of recruited test subjects took into account the inclusion criteria, the constraints of the study and the period of the study performance.

At the beginning of the study, complementary test subjects (+10) were included to compensate the possible withdrawals or exclusions from the study independent of the investigational product.

II.1.1.2. Inclusion and non-inclusion criteria

All the test subjects corresponded to the inclusion and non-inclusion criteria.

The individual typological characteristics of the test subjects are reported in **Appendices 1**, and recapitulated belo for the whole panel:

Age (years old)	Included test subjects	Valid cases
Minimum	19	21
Maximum	64	64
Mean	47	48
Median	49	50

60-70 age bracket	Included test subjects	Valid cases
Number of test subjects	7	7
% of test subjects	6%	7%

Criteria	Included test subjects		Valid cases	
	Nb	%	Nb	%
Phototype				
II	28	25%	26	25%
III	82	75%	78	75%
Sex				
Female	89	81%	87	84%
Male	21	19%	17	16%

II.1.1.3. Specific information concerning the test subjects

The answers of the test subjects concerning the skin reactivity, the history of atopy, contraception (type) and the current medication are reported in **Appendices 2.**

II.1.1.4. Study constraints imposed on the test subjects

All the constraints of the study, defined in the protocol, were respected by the test subjects who completed the study.

The answers of the test subjects concerning the respect of the constraints defined in the protocol were reported in the CRF.

II.1.2. INVESTIGATIONAL PRODUCT

Experimental conditions of application of the investigational product

All the experimental conditions of application at the investigating centre were respected, as defined in the protocol, except for the challenge phase:

The challenge phase was performed on D37, D39, D41 instead of D36, D38 and D40 due to administrative reasons.

This minor deviation has no impact on the study results.

II.1.3. CHECKING OF THE SKIN COMPATIBILITY: RECORDING OF THE SKIN REACTIONS

All the skin examinations and questioning of the test subjects were performed in accordance with the conditions defined in the protocol.

III – RESULTS

III.1. RESULTS / DISCUSSION

III.1.1. Checking of the skin compatibility

For the investigational product, the individual data of the skin examination and questioning of the test subjects are reported in **Appendices 3.**

Induction period			
Type of reaction	**Description of the reaction on the induction site**	**Number and percentage of reactive test subjects**	**Total number and percentage of reactive test subjects**
E: Erythema	None	0 / 0%	0 / 0%
M: Complementary mention	Tape reaction	1 / 1% Ref. 9b	
A: ICDRG scale	None	0 / 0%	

Challenge phase			
Type of reaction	**Description of the reactions on the induction site and the virgin site**	**Number and percentage of reactive test subjects**	**Total number and percentage of reactive test subjects**
E: Erythema	None	0 / 0%	0 / 0%
M: Complementary mention	None	0 / 0%	
A: ICDRG scale	None	0 / 0%	

For the control product, the individual data of the skin examination and questioning of the test subjects are reported in **Appendices 4.**

Induction period			
Type of reaction	**Description of the reaction on the induction site**	**Number and percentage of reactive test subjects**	**Total number and percentage of reactive test subjects**
E: Erythema	None	0 / 0%	0 / 0%
M: Complementary mention	Tape reaction	1 / 1% Ref. 9b	
A: ICDRG scale	None	0 / 0%	

Challenge phase			
Type of reaction	**Description of the reactions on the induction site and the virgin site**	**Number and percentage of reactive test subjects**	**Total number and percentage of reactive test subjects**
E: Erythema	None	0 / 0%	0 / 0%
M: Complementary mention	None	0 / 0%	
A: ICDRG scale	None	0 / 0%	

III.2. OVERALL CONCLUSION

Under the experimental conditions adopted:

- During the induction period, the repeated applications of the product under occlusive patch on a panel of 104 test subjects with all types of skin on body induced no reaction of irritation.

- During the challenge phase, the single application of the investigational product to the induction site and virgin site induced no allergic reaction.

Based on these results, the product has a very good skin compatibility and does not show a sensitizing effect.

III.3. QUALITY CONTROL AND QUALITY ASSURANCE

The study was performed in compliance with the procedures of the investigating centre, established according to the regulations in force.

The investigator, in charge of the performance of the study, made sure of the quality of the work of the technical staff, particularly concerning the respect of the protocol and its appendices, the collection of raw data, the management of the investigational product.

The personnel of the Quality Assurance department controlled that the study documentation was present, dated and signed.

The personnel of the Quality Assurance department regularly controls that the protocol and working procedures relevant to this type of study are duly applied.

APPENDICES

INVESTIGATIONAL PRODUCT:
SKIN EXAMINATION AND QUESTIONING DURING THE INDUCTION PHASE

***E: Erythema**: **0** = no visible erythema, **0.5** = very slight erythema – barely perceptible, **1** = mild erythema – faint pink, **2** = moderate erythema – well defined, **3** = severe erythema, **4** = caustic effect – erosive aspect and/or necrotic aspect*
d= diffuse / p = punctuated / peri = peripheral

***M: Additional comments/Others reactions: H or Oe** = Homogeneous infiltration / oedema, **P** = Papules, **V** = Vesicles, **B** = Bullae, **Pe** = Petechiae, **S:** Spreading beyond the patch, **SV** = Soap effect (shiny skin with possibly wrinkles), **F** = Fissuring , **D** = Desquamation, **Dr** = Dryness, **C** = Skin coloration, hyperpigmentation, **HY** = Hypopigmentation, **Fr** = Follicular reaction, **NA** = Product not applied, **T**= Tape reaction, **I** = Itching at the test site, **Cr** = Exsudation and/or Surface encrustation, **Sc** = Scab, **Pr** = Pruritus, **He** = Heating, **Pu** = Pustules, * = Additional free comments, **N9G** = No 9th grade, **X** = Succeeding patch not applied and succeeding grade (in brackets) denotes a residual reaction, **Abs or "–"** = Subject absent, **MU** = Make-up patch*

/: no reaction

***A: ICDRG scale**: **IR** = Irritation reaction, **-** = No allergic reaction, **?+ =** Doubtful reaction (only slight erythema), **(+) =** Weak positive reaction (without vesicle): slight erythema and infiltration with presence of small papular elevations, possibly papules, **(++) =** Strong positive reaction: erythema, papules, vesicles, infiltration, **(+++) =** Extreme positive reaction: intense erythema, oedema, coalescent vesicles (bullae)*

Test subjects reference	Type of reaction	Experimental times									
		D1	D3	D5	D8	D10	D12	D15	D17	D19	D22
1a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
2a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
3a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
4a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
5a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
6a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
7a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
8a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
9a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
10a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
11a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
12a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
13a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									

Distributed for Comment Only -- Do Not Cite or Quote

INVESTIGATIONAL PRODUCT:
SKIN EXAMINATION AND QUESTIONING DURING THE INDUCTION PHASE

Test subjects reference	Type of reaction	Experimental times									
		D1	D3	D5	D8	D10	D12	D15	D17	D19	D22
14a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
15a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
16a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
17a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
18a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
19a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
20a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
21a	E	0									
	M	/									
	A	-									
22a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
23a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
24a	E	0									
	M	/									
	A	-									
25a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
26a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
27a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
28a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
29a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
30a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									

Legends: [grey] *Withdrawal*

Appendix 3-1/3

INVESTIGATIONAL PRODUCT:

SKIN EXAMINATION AND QUESTIONING DURING THE INDUCTION PHASE

Test subjects reference	Type of reaction	Experimental times									
		D1	D3	D5	D8	D10	D12	D15	D17	D19	D22
31a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
32a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
33a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
34a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
35a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
36a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
37a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
38a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
39a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
40a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
41a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
42a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
43a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
44a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
45	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
46a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
47a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									

INVESTIGATIONAL PRODUCT:

SKIN EXAMINATION AND QUESTIONING DURING THE INDUCTION PHASE

Test subjects reference	Type of reaction	Experimental times									
		D1	D3	D5	D8	D10	D12	D15	D17	D19	D22
48a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
49a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
50a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
51a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
52a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
53a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
54a	E	0	0	0	0	0					
	M	/	/	/	/	/					
	A	-									
55a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
56a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
1b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
2b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
3b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
4b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
5b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
6b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
7b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
8b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									

Legends: (grey) *Withdrawal*

Appendix 3-1/5

INVESTIGATIONAL PRODUCT:

SKIN EXAMINATION AND QUESTIONING DURING THE INDUCTION PHASE

Test subjects reference	Type of reaction	Experimental times									
		D1	D3	D5	D8	D10	D12	D15	D17	D19	D22
9b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
10b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
11b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
12b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
13b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
14b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
15b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
16b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
17b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
18b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
19b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
20b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
21b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
22b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
23b	E	0	0	0							
	M	/	/	/							
	A	-									
24b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
25b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									

Legends: [grey box] *Withdrawal*

INVESTIGATIONAL PRODUCT:

SKIN EXAMINATION AND QUESTIONING DURING THE INDUCTION PHASE

Test subjects reference	Type of reaction	Experimental times									
		D1	D3	D5	D8	D10	D12	D15	D17	D19	D22
26b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
27b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
28b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
29b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
30b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
31b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
32b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
33b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
34b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
35b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
36b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
37b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
38b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
39b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
40b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
41b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
42b	E	0	0	0	0	0					
	M	/	/	/	/	/					
	A	-									

Legends: (grey box) *Withdrawal*

Appendix 3-1/7

INVESTIGATIONAL PRODUCT:

SKIN EXAMINATION AND QUESTIONING DURING THE INDUCTION PHASE

Test subjects reference	Type of reaction	Experimental times									
		D1	D3	D5	D8	D10	D12	D15	D17	D19	D22
43b	E	0	0								
	M	/	/								
	A	-									
44b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
45b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
46b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
47b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
48b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
49b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
50b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
51b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
52b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
53b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
54b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									

Legends: (grey) *Withdrawal*

Appendix 3-2/1

INVESTIGATIONAL PRODUCT:
SKIN EXAMINATION AND QUESTIONING DURING THE CHALLENGE PHASE

***E: Erythema**: **0** = no visible erythema, **0.5** = very slight erythema – barely perceptible, **1** = mild erythema – faint pink, **2** = moderate erythema – well defined, **3** = severe erythema, **4** = caustic effect – erosive aspect and/or necrotic aspect*
d= diffuse / p = punctuated / peri = peripheral

M: Additional comments/Others reactions: H or Oe** = Homogeneous infiltration / oedema, **P** = Papules, **V** = Vesicles, **B** = Bullae, **Pe** = Petechiae, **S:** Spreading beyond the patch, **SV** = Soap effect (shiny skin with possibly wrinkles), **F** = Fissuring , **D** = Desquamation, **Dr** = Dryness, **C** = Skin coloration, hyperpigmentation, **HY** = Hypopigmentation, **Fr** = Follicular reaction, **NA** = Product not applied, **T**= Tape reaction, **I** = Itching at the test site, **Cr** = Exsudation and/or Surface encrustation, **Sc** = Scab, **Pr** = Pruritus, **He** = Heating, **Pu** = Pustules, ** = Additional free comments, **N9G** = No 9th grade, **X** = Succeeding patch not applied and succeeding grade (in brackets) denotes a residual reaction, **Abs or "–"** = Subject absent, **MU** = Make-up patch*

/: no reaction

***A: ICDRG scale**: **IR** = Irritation reaction, **-** = No allergic reaction, **?+** = Doubtful reaction (only slight erythema), **(+)** = Weak positive reaction (without vesicle): slight erythema and infiltration with presence of small papular elevations, possibly papules, **(++)** = Strong positive reaction: erythema, papules, vesicles, infiltration, **(+++)** = Extreme positive reaction: intense erythema, oedema, coalescent vesicles (bullae)*

Test subjects reference	Type of reaction	Experimental times					
		Induction site			Virgin site		
		D37	D39	D41	D37	D39	D41
1a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
2a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
3a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
4a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
5a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
6a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
7a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
8a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
9a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
10a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
11a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
12a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
13a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					

Appendix 3-2/2

INVESTIGATIONAL PRODUCT:
SKIN EXAMINATION AND QUESTIONING DURING THE CHALLENGE PHASE

Test subjects reference	Type of reaction	Experimental times					
		Induction site			Virgin site		
		D37	D39	D41	D37	D39	D41
14a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
15a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
16a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
17a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
18a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
19a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
20a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
21a	E						
	M						
	A						
22a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
23a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
24a	E						
	M						
	A						
25a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
26a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
27a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
28a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
29a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					

Legends: *Withdrawal* (shaded)

Distributed for Comment Only -- Do Not Cite or Quote

INVESTIGATIONAL PRODUCT:

SKIN EXAMINATION AND QUESTIONING DURING THE CHALLENGE PHASE

Test subjects reference	Type of reaction	Experimental times					
		Induction site			Virgin site		
		D37	D39	D41	D37	D39	D41
30a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
31a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
32a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
33a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
34a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
35a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
36a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
37a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
38a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
39a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
40a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
41a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
42a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
43a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
44a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
45a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					

INVESTIGATIONAL PRODUCT:
SKIN EXAMINATION AND QUESTIONING DURING THE CHALLENGE PHASE

Test subjects reference	Type of reaction	Experimental times					
		Induction site			Virgin site		
		D37	D39	D41	D37	D39	D41
46a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
47a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
48a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
49a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
50a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
51a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
52a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
53a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
54a	E						
	M						
	A						
55a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
56a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
1b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
2b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
3b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
4b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
5b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					

Legends: [grey box] *Withdrawal*

Appendix 3-2/5

INVESTIGATIONAL PRODUCT:
SKIN EXAMINATION AND QUESTIONING DURING THE CHALLENGE PHASE

Test subjects reference	Type of reaction	Experimental times					
		Induction site			Virgin site		
		D37	D39	D41	D37	D39	D41
6b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
7b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
8b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
9b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
10b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
11b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
12b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
13b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
14b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
15b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
16b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
17b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
18b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
19b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
20b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
21b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					

Appendix 3-2/6

INVESTIGATIONAL PRODUCT:
SKIN EXAMINATION AND QUESTIONING DURING THE CHALLENGE PHASE

Test subjects reference	Type of reaction	Experimental times					
		Induction site			Virgin site		
		D37	D39	D41	D37	D39	D41
22b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
23b	E						
	M						
	A						
24b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
25b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
26b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
27b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
28b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
29b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
30b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
31b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
32b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
33b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
34b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
35b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
36b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
37b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					

Legends: *Withdrawal*

INVESTIGATIONAL PRODUCT:
SKIN EXAMINATION AND QUESTIONING DURING THE CHALLENGE PHASE

Test subjects reference	Type of reaction	Experimental times					
		Induction site			Virgin site		
		D37	D39	D41	D37	D39	D41
38b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
39b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
40b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
41b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
42b	E						
	M						
	A						
43b	E						
	M						
	A						
44b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
45b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
46b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
47b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
48b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
49b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
50b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
51b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
52b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
53b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
54b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					

Legends: [grey box] *Withdrawal*

Appendix 4-1/1

CONTROL PRODUCT: DISTILLED WATER
SKIN EXAMINATION AND QUESTIONING DURING THE INDUCTION PHASE

***E: Erythema**: **0** = no visible erythema, **0.5** = very slight erythema – barely perceptible, **1** = mild erythema – faint pink, **2** = moderate erythema – well defined, **3** = severe erythema, **4** = caustic effect – erosive aspect and/or necrotic aspect*
d= diffuse / p = punctuated / peri = peripheral

***M: Additional comments/Others reactions: H or Oe** = Homogeneous infiltration / oedema, **P** = Papules, **V** = Vesicles, **B** = Bullae, **Pe** = Petechiae, **S:** Spreading beyond the patch, **SV** = Soap effect (shiny skin with possibly wrinkles), **F** = Fissuring , **D** = Desquamation, **Dr** = Dryness, **C** = Skin coloration, hyperpigmentation, **HY** = Hypopigmentation, **Fr** = Follicular reaction, **NA** = Product not applied, **T**= Tape reaction, **I** = Itching at the test site, **Cr** = Exsudation and/or Surface encrustation, **Sc** = Scab, **Pr** = Pruritus, **He** = Heating, **Pu** = Pustules, * = Additional free comments, **N9G** = No 9th grade, **X** = Succeeding patch not applied and succeeding grade (in brackets) denotes a residual reaction, **Abs or "–"** = Subject absent, **MU** = Make-up patch*

/: no reaction

***A: ICDRG scale**: **IR** = Irritation reaction, **-** = No allergic reaction, **?+ =** Doubtful reaction (only slight erythema), **(+) =** Weak positive reaction (without vesicle): slight erythema and infiltration with presence of small papular elevations, possibly papules, **(++) =** Strong positive reaction: erythema, papules, vesicles, infiltration, **(+++) =** Extreme positive reaction: intense erythema, oedema, coalescent vesicles (bullae)*

Test subjects reference	Type of reaction	Experimental times									
		D1	D3	D5	D8	D10	D12	D15	D17	D19	D22
1a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
2a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
3a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
4a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
5a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
6a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
7a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
8a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
9a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
10a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
11a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
12a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
13a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									

Appendix 4-1/2

CONTROL PRODUCT: DISTILLED WATER
SKIN EXAMINATION AND QUESTIONING DURING THE INDUCTION PHASE

Test subjects reference	Type of reaction	Experimental times									
		D1	D3	D5	D8	D10	D12	D15	D17	D19	D22
14a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
15a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
16a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
17a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
18a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
19a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
20a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
21a	E	0									
	M	/									
	A	-									
22a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
23a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
24a	E	0									
	M	/									
	A	-									
25a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
26a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
27a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
28a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
29a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
30a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									

Legends: (grey) *Withdrawal*

Appendix 4-1/3

CONTROL PRODUCT: DISTILLED WATER

SKIN EXAMINATION AND QUESTIONING DURING THE INDUCTION PHASE

Test subjects reference	Type of reaction	Experimental times									
		D1	D3	D5	D8	D10	D12	D15	D17	D19	D22
31a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
32a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
33a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
34a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
35a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
36a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
37a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
38a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
39a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
40a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
41a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
42a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
43a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
44a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
45	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
46a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
47a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									

Appendix 4-1/4

CONTROL PRODUCT: DISTILLED WATER
SKIN EXAMINATION AND QUESTIONING DURING THE INDUCTION PHASE

Test subjects reference	Type of reaction	Experimental times									
		D1	D3	D5	D8	D10	D12	D15	D17	D19	D22
48a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
49a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
50a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
51a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
52a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
53a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
54a	E	0	0	0	0	0					
	M	/	/	/	/	/					
	A	-									
55a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
56a	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
1b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
2b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
3b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
4b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
5b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
6b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
7b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
8b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									

Legends: (grey) *Withdrawal*

CONTROL PRODUCT: DISTILLED WATER
SKIN EXAMINATION AND QUESTIONING DURING THE INDUCTION PHASE

Test subjects reference	Type of reaction	Experimental times									
		D1	D3	D5	D8	D10	D12	D15	D17	D19	D22
9b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
10b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
11b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
12b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
13b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
14b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
15b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
16b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
17b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
18b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
19b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
20b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
21b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
22b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
23b	E	0	0	0							
	M	/	/	/							
	A	-									
24b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
25b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									

Legends: [grey] *Withdrawal*

CONTROL PRODUCT: DISTILLED WATER
SKIN EXAMINATION AND QUESTIONING DURING THE INDUCTION PHASE

Test subjects reference	Type of reaction	Experimental times									
		D1	D3	D5	D8	D10	D12	D15	D17	D19	D22
26b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
27b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
28b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
29b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
30b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
31b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
32b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
33b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
34b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
35b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
36b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
37b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
38b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
39b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
40b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
41b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
42b	E	0	0	0	0	0					
	M	/	/	/	/	/					
	A	-									

Legends: [grey box] *Withdrawal*

CONTROL PRODUCT: DISTILLED WATER
SKIN EXAMINATION AND QUESTIONING DURING THE INDUCTION PHASE

Test subjects reference	Type of reaction	Experimental times									
		D1	D3	D5	D8	D10	D12	D15	D17	D19	D22
43b	E	0	0								
	M	/	/								
	A	-									
44b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
45b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
46b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
47b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
48b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
49b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
50b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
51b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
52b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
53b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									
54b	E	0	0	0	0	0	0	0	0	0	0
	M	/	/	/	/	/	/	/	/	/	/
	A	-									

Legends: [grey box] *Withdrawal*

Appendix 4-2/1

CONTROL PRODUCT: DISTILLED WATER
SKIN EXAMINATION AND QUESTIONING DURING THE CHALLENGE PHASE

***E: Erythema**: **0** = no visible erythema, **0.5** = very slight erythema – barely perceptible, **1** = mild erythema – faint pink, **2** = moderate erythema – well defined, **3** = severe erythema, **4** = caustic effect – erosive aspect and/or necrotic aspect*
d= diffuse / p = punctuated / peri = peripheral

M: Additional comments/Others reactions: H or Oe** = Homogeneous infiltration / oedema, **P** = Papules, **V** = Vesicles, **B** = Bullae, **Pe** = Petechiae, **S:** Spreading beyond the patch, **SV** = Soap effect (shiny skin with possibly wrinkles), **F** = Fissuring , **D** = Desquamation, **Dr** = Dryness, **C** = Skin coloration, hyperpigmentation, **HY** = Hypopigmentation, **Fr** = Follicular reaction, **NA** = Product not applied, **T**= Tape reaction, **I** = Itching at the test site, **Cr** = Exsudation and/or Surface encrustation, **Sc** = Scab, **Pr** = Pruritus, **He** = Heating, **Pu** = Pustules, ** = Additional free comments, **N9G** = No 9th grade, **X** = Succeeding patch not applied and succeeding grade (in brackets) denotes a residual reaction, **Abs or "–"** = Subject absent, **MU** = Make-up patch*

/: no reaction

***A: ICDRG scale**: **IR** = Irritation reaction, **-** = No allergic reaction, **?+** = Doubtful reaction (only slight erythema), **(+)** = Weak positive reaction (without vesicle): slight erythema and infiltration with presence of small papular elevations, possibly papules, **(++)** = Strong positive reaction: erythema, papules, vesicles, infiltration, **(+++)** = Extreme positive reaction: intense erythema, oedema, coalescent vesicles (bullae)*

Test subjects reference	Type of reaction	Experimental times					
		Induction site			Virgin site		
		D37	D39	D41	D37	D39	D41
1a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
2a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
3a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
4a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
5a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
6a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
7a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
8a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
9a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
10a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
11a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
12a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
13a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					

Distributed for Comment Only -- Do Not Cite or Quote

CONTROL PRODUCT: DISTILLED WATER
SKIN EXAMINATION AND QUESTIONING DURING THE CHALLENGE PHASE

Test subjects reference	Type of reaction	Experimental times					
		Induction site			Virgin site		
		D37	D39	D41	D37	D39	D41
14a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
15a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
16a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
17a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
18a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
19a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
20a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
21a	E						
	M						
	A						
22a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
23a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
24a	E						
	M						
	A						
25a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
26a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
27a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
28a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
29a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					

Legends: [grey box] *Withdrawal*

Appendix 4-2/3

CONTROL PRODUCT: DISTILLED WATER
SKIN EXAMINATION AND QUESTIONING DURING THE CHALLENGE PHASE

Test subjects reference	Type of reaction	Experimental times					
		Induction site			Virgin site		
		D37	D39	D41	D37	D39	D41
30a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
31a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
32a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
33a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
34a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
35a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
36a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
37a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
38a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
39a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
40a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
41a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
42a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
43a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
44a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
45a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					

Appendix 4-2/4

CONTROL PRODUCT: DISTILLED WATER
SKIN EXAMINATION AND QUESTIONING DURING THE CHALLENGE PHASE

Test subjects reference	Type of reaction	Experimental times					
		Induction site			Virgin site		
		D37	D39	D41	D37	D39	D41
46a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
47a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
48a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
49a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
50a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
51a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
52a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
53a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
54a	E						
	M						
	A						
55a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
56a	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
1b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
2b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
3b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
4b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
5b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					

Legends: (grey) *Withdrawal*

Appendix 4-2/5

CONTROL PRODUCT: DISTILLED WATER
SKIN EXAMINATION AND QUESTIONING DURING THE CHALLENGE PHASE

Test subjects reference	Type of reaction	Experimental times					
		Induction site			Virgin site		
		D37	D39	D41	D37	D39	D41
6b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
7b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
8b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
9b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
10b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
11b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
12b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
13b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
14b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
15b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
16b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
17b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
18b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
19b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
20b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
21b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					

CONTROL PRODUCT: DISTILLED WATER
SKIN EXAMINATION AND QUESTIONING DURING THE CHALLENGE PHASE

Test subjects reference	Type of reaction	Experimental times					
		Induction site			Virgin site		
		D37	D39	D41	D37	D39	D41
22b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
23b	E						
	M						
	A						
24b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
25b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
26b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
27b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
28b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
29b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
30b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
31b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
32b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
33b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
34b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
35b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
36b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
37b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					

Legends: [grey box] *Withdrawal*

CONTROL PRODUCT: DISTILLED WATER
SKIN EXAMINATION AND QUESTIONING DURING THE CHALLENGE PHASE

Test subjects reference	Type of reaction	Experimental times					
		Induction site			Virgin site		
		D37	D39	D41	D37	D39	D41
38b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
39b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
40b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
41b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
42b	E						
	M						
	A						
43b	E						
	M						
	A						
44b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
45b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
46b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
47b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
48b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
49b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
50b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
51b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
52b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
53b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					
54b	E	0	0	0	0	0	0
	M	/	/	/	/	/	/
	A	-					

Legends: [grey box] *Withdrawal*

REPEATED INSULT PATCH TEST

formula contains 1.5% Sodium Hyaluronate

CONDUCTED FOR:

DATE OF ISSUE:

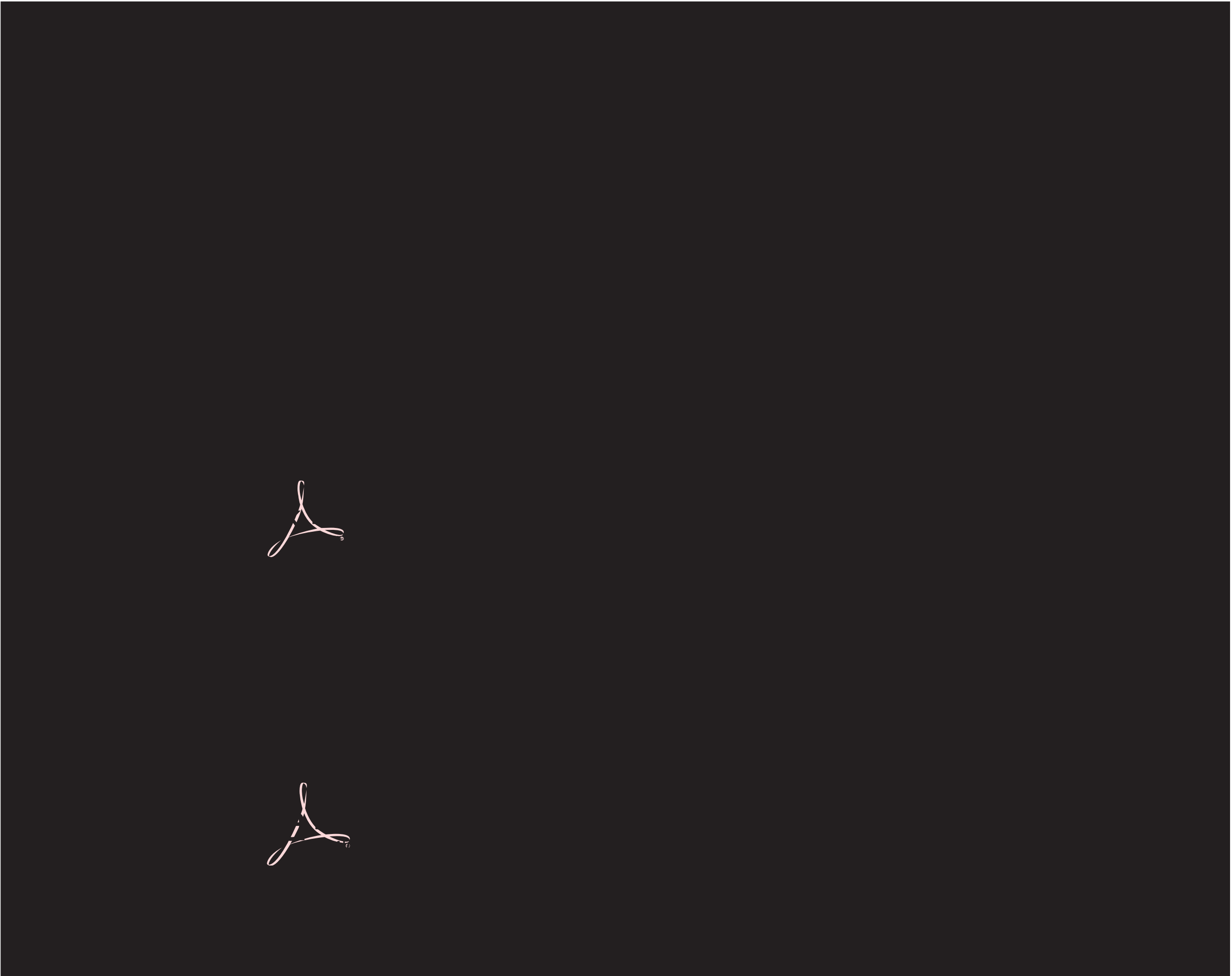
May 17, 2019

TABLE OF CONTENTS

SIGNATURES 1
STATEMENT OF QUALITY CONTROL 1
TITLE OF STUDY 2
SPONSOR 2
STUDY MATERIAL 2
DATE STUDY INITIATED 2
DATE STUDY COMPLETED 2
DATE OF ISSUE 2
INVESTIGATIVE PERSONNEL 2
CLINICAL SITES 2
SUMMARY 3
1.0 OBJECTIVE 4
2.0 RATIONALE 4
3.0 STUDY DESIGN 4
3.1 STUDY POPULATION 4
3.1.1 Inclusion Criteria 4
3.1.2 Exclusion Criteria 4
3.1.3 Informed Consent 5
3.2 DESCRIPTION OF STUDY 5
3.2.1 Outline of Study Procedures 5
3.2.2 Study Flow Chart 6
3.2.3 Definitions Used for Grading Responses 7
3.2.4 Evaluation of Responses 7
4.0 NATURE OF STUDY MATERIAL 8
4.1 STUDY MATERIAL SPECIFICATIONS 8
4.2 STORAGE, HANDLING, AND DOCUMENTATION OF STUDY MATERIAL 8
4.3 APPLICATION OF STUDY MATERIAL 8
4.4 DESCRIPTION OF PATCH CONDITIONS 8
5.0 INTERPRETATION 8
6.0 DOCUMENTATION AND RETENTION OF DATA 9
7.0 RESULTS AND DISCUSSION 9
8.0 CONCLUSION 10
9.0 REFERENCES 10

APPENDICES

I SUMMARY TABLES
II DATA LISTINGS
III INFORMED CONSENT DOCUMENTS



STATEMENT OF QUALITY CONTROL

The Quality Control Unit of the Dermatological Safety Department conducted a 100% review of all study-related documents. The protocol was reviewed prior to the start of the study, and the medical screening forms and informed consent documents were reviewed in-process of the study. The regulatory binder and study data were reviewed post-study to ensure accuracy. The study report was reviewed and accurately reflects the data for this study.

---

[1] ICH Topic E6 “Note for guidance on Good Clinical Practices (CPMP/ICH/135/95)” – ICH Harmonised Tripartite Guideline for Good Clinical Practices having reached Step 5 of the ICH Process at the ICH Steering Committee meeting on 1 May 1996.

TITLE OF STUDY

Repeated Insult Patch Test

SPONSOR

STUDY MATERIAL

DATE STUDY INITIATED

March 11, 2019

DATE STUDY COMPLETED

April 26, 2019

DATE OF ISSUE

May 17, 2019

INVESTIGATIVE PERSONNEL

SUMMARY

One study material, [redacted] was evaluated neat to determine its ability to sensitize the skin of volunteer subjects with normal skin using an occlusive repeated insult patch study. One hundred ninety-eight (198) subjects completed the study. This is a deviation from the protocol-specified requirement of 200 completed subjects. In the opinion of the Principal Investigator, this deviation did not affect the validity of the study.

Under the conditions employed in this study, there was no evidence of sensitization [redacted]

1.0 OBJECTIVE

The objective of this study was to determine the ability of the study material to cause sensitization by repeated topical applications to the skin of humans under controlled patch study conditions.

2.0 RATIONALE

Substances that come into contact with human skin need to be evaluated for their propensity to irritate and/or sensitize. Once an appropriate pre-clinical safety evaluation has been performed, a reproducible, standardized, quantitative patch evaluation procedure must be used to demonstrate that a particular material can be applied safely to human skin without significant risk of adverse reactions. The method herein employed is generally accepted for such a purpose.

Repeated insult patch evaluation is a modified predictive patch study that can detect weak sensitizers that require multiple applications to induce a cell-mediated (Type IV) immune response sufficient to cause an allergic reaction. Irritant reactions may also be detected using this evaluation method, although this is not the primary purpose of this procedure. Results are interpreted according to interpretive criteria based upon published works, as well as the clinical experience of Inc. These interpretive criteria are periodically reviewed and amended as new information becomes available.

3.0 STUDY DESIGN

3.1 STUDY POPULATION

A sufficient number of subjects were enrolled to provide 200 completed subjects. In the absence of any sensitization reactions in this sample size (200 evaluable subjects), a 95% upper confidence bound on the population rate of sensitization would be 1.5%.

3.1.1 Inclusion Criteria

Individuals eligible for inclusion in the study were those who:

1. Were males or females, 18 to 70 years of age, in general good health;
2. Were free of any systemic or dermatologic disorder which, in the opinion of the investigative personnel, would have interfered with the study results or increased the risk of adverse events (AEs);
3. Were of any skin type or race, providing the skin pigmentation would allow discernment of erythema;
4. Had completed a medical screening procedure; and
5. Had read, understood, and signed an informed consent (IC) agreement.

3.1.2 Exclusion Criteria

Individuals excluded from participation in the study were those who:

1. Had any visible skin disease at the study site which, in the opinion of the investigative personnel, would have interfered with the evaluation;

2. Were receiving systemic or topical drugs or medication which, in the opinion of the investigative personnel, would have interfered with the study results;
3. Had psoriasis and/or active atopic dermatitis/eczema;
4. Were females who were pregnant, planning to become pregnant during the study, or breast-feeding;
5. Had a known sensitivity to cosmetics, skin care products, or topical drugs as related to the material being evaluated; and/or
6. Were participating in another study or had been recruited to participate in another study concurrently.

3.1.3 Informed Consent

A properly executed IC document was obtained from each subject prior to entering the study. The signed IC document is maintained in the study file. In addition, the subject was provided with a copy of the IC document (see Appendix III).

3.2 DESCRIPTION OF STUDY

3.2.1 Outline of Study Procedures

Subjects participated in the study over a 6-week period involving 3 phases: (1) Induction, (2) Rest, and (3) Challenge. Prior to study entry, the subjects were screened to assure that they met the inclusion/exclusion criteria. Informed consent was obtained. Each subject was provided with a schedule of the study activities. All subjects were told to avoid wetting the patches and were asked not to engage in activities that caused excessive perspiration. They were instructed to notify the staff if they experienced any discomfort beyond mild itching or observed any adverse changes at the patch sites, while on the study or within 2 weeks of completing the study.

The Induction Phase consisted of 9 applications of the study material and subsequent evaluations of the patch sites. Prior to application of the patches, the sites were outlined with a skin marker, eg, gentian violet. The subjects were required to remove the patches approximately 24 hours after application. They returned to the facility at 48-hour intervals to have the sites evaluated and identical patches applied to the same sites. Patches applied on Friday were removed by subjects after 24 hours. The sites were evaluated on the following Monday, ie, 72 hours after patch application.[2]

Following the 9th evaluation, the subjects were dismissed for a Rest Period of approximately 10-15 days.

Subjects who were absent once during the Induction Phase received a make-up (MU) patch at the last induction visit. The MU applications were graded 48 hours later at the MU visit, or were recorded as N9G (no ninth grading).

The Challenge Phase was initiated during the 6th week of the study. Identical patches were applied to sites previously unexposed to the study material. The patches were removed by subjects after

[2] A Monday or Friday holiday could result in evaluation at 96 hours after patch application.

24 hours and the sites graded after additional 24-hour and 48-hour periods (ie, 48 and 72 hours after application). Rechallenge was performed whenever there was evidence of possible sensitization.

To be considered a completed case, a subject must have had 9 applications and no fewer than 8 subsequent readings during Induction, and a single application and 2 readings at Challenge. Only completed cases were used to assess sensitization.

3.2.2 Study Flow Chart

WEEK 1

DAY	ACTIVITIES
1[3]	Staff obtained informed consent, reviewed completed medical screening form, applied patches
2	Subject removed patches
3	Staff graded sites, applied patches
4	Subject removed patches
5	Staff graded sites, applied patches
6	Subject removed patches

WEEK 2

DAY	ACTIVITIES
1	Staff graded sites, applied patches
2-6	Same as Week 1

WEEK 3

DAY	ACTIVITIES
1-6	Same as Week 2

WEEK 4

DAY	ACTIVITIES
1	Staff graded sites; applied make-up (MU) induction patches, if required
2	Subject removed MU patches
3	Staff graded MU induction sites at MU visit
2-7	Rest Period

WEEK 5

DAY	ACTIVITIES
1-7	Rest Period

[3] Study flow starting with Week 1, Day 1, was altered when enrollment occurred on Wednesday or Friday. Study flow could be altered if a holiday occurred during the study.

WEEK 6

DAY	ACTIVITIES
1	Staff applied patches
2	Subject removed patches
3	Staff graded sites
4	Staff graded sites

3.2.3 Definitions Used for Grading Responses

The symbols found in the scoring scales below were used to express the response observed at the time of examination:

SYMBOL		REACTION
-	=	No reaction
?	=	Minimal or doubtful response, slightly different from surrounding normal skin
+	=	Definite erythema, no edema
++	=	Definite erythema, definite edema
+++	=	Definite erythema, definite edema and vesiculation

SPECIAL NOTATIONS

E	=	Marked/severe erythema
S	=	Spreading of reaction beyond patch site (ie, reaction where material did not contact skin)
p	=	Papular response > 50%
pv	=	Papulovesicular response > 50%
D	=	Damage to epidermis: oozing, crusting and/or superficial erosions
I	=	Itching
X	=	Subject absent
PD	=	Patch dislodged
NA	=	Not applied
NP	=	Not patched (due to reaction achieved)
N9G	=	No ninth grading

3.2.4 Evaluation of Responses

All responses were graded by a trained dermatologic evaluator meeting [redacted] certification requirements to standardize the assignment of response grades.

4.0 NATURE OF STUDY MATERIAL

4.1 STUDY MATERIAL SPECIFICATIONS

Identification : [redacted]
Amount Applied : 0.2mL

4.2 STORAGE, HANDLING, AND DOCUMENTATION OF STUDY MATERIAL

Receipt of the material used in this study was documented in a general logbook, which serves as a permanent record of the receipt, storage, and disposition of all study material received by [redacted]. On the basis of information provided by the Sponsor, the study material was considered reasonably safe for evaluation on human subjects. A sample of the study material was reserved and will be stored for a period of 6 months. All study material was kept in a locked product storage room accessible to clinical staff members only. At the conclusion of the clinical study, the remaining study material was discarded or returned to the Sponsor and the disposition documented in the logbook.

4.3 APPLICATION OF STUDY MATERIAL

All study material was supplied by the Sponsor. Material was applied in an amount proportionate to the patch type or as requested by the Sponsor, generally 0.2 mL or g or an amount sufficient to cover the 2 cm x 2 cm patch. The patches were applied to the infrascapular area of the back, either to the right or left of the midline, or to the upper arm.

4.4 DESCRIPTION OF PATCH CONDITIONS

Material evaluated under occlusive patch conditions is applied to a 2 cm x 2 cm Webril™ pad attached to a non-porous, plastic film adhesive bandage (3M medical tape). The patches are secured with hypoallergenic tape (Micropore), as needed.

Material evaluated under semi-occlusive patch conditions is applied to a 2 cm x 2 cm Webril™ pad. The pads are affixed to the skin with hypoallergenic tape (Micropore).

5.0 INTERPRETATION

Sensitization is characterized by an acute allergic contact dermatitis. Typical sensitization reactions begin with an immunologic response in the dermis resulting in erythema, edema formation, and secondary epidermal damage (vesiculation), sometimes extending beyond the patch site and often accompanied by itching. Sensitization reactions tend to be delayed. The reaction typically becomes evident between 24 and 48 hours, peaks at 48-72 hours and subsequently subsides. The reaction is often greater at 72 hours than at 48 hours. The severity of the reaction is generally greater during the Challenge Phase of a Repeated Insult Patch Test (RIPT) than that seen during Induction.

Irritant reactions are characterized as a non-immunologic, localized, superficial, exudative, inflammatory response of the skin due to an externally applied material. The typical initial reaction does not develop much edema or vesiculation but results in scaling, drying, cracking, oozing, crusting, and erosions. The reaction is usually sharply delineated, not spreading beyond the patch site. Irritant reactions are typically evident by 24 hours and diminish over the next 48-72 hours.

Removal of the offending agent results in gradual improvement of the epidermal damage. The reaction seen at 72 hours is, therefore, less severe than that seen at 48 hours. Finally, the severity of the reaction experienced in the Challenge Phase is generally similar to that seen during Induction.

If the results of the study indicate the likelihood of sensitization, the recommended practice is to rechallenge the subjects who have demonstrated sensitization-like reactions to confirm that these reactions are, indeed, associated with the product. Our preferred Rechallenge procedure involves the application of the product to naive sites, under both occlusive and semi-occlusive patch conditions. Use of the semi-occlusive patch condition helps to differentiate irritant and sensitization reactions. Generally speaking, if a product is a sensitizer it will produce a similar reaction under both occlusion and semi-occlusion. Whereas, if the product has caused an irritant reaction, the reactions will be less pronounced under the semi-occlusive condition.

6.0 DOCUMENTATION AND RETENTION OF DATA

The case report forms (CRFs) were designed to identify each subject by subject number and initials, and to record demographics, examination results, AEs, and end of study status. Originals or copies of all CRFs, correspondence, study reports, and all source data will be kept on hard-copy file for a minimum of 5 years from completion of the study. Storage was maintained either at a facility in a secured room accessible only to employees, or at an offsite location which provided a secure environment with burglar/fire alarm systems, camera detection and controlled temperature and humidity. Documentation will be available for the Sponsor's review on the premises of

7.0 RESULTS AND DISCUSSION

Two hundred thirty-eight (238) subjects between the ages of 18 and 70 were enrolled. One hundred ninety-eight (198) subjects completed the study. This is a deviation from the protocol-specified requirement of 200 completed subjects. In the opinion of the Principal Investigator, this deviation did not affect the validity of the study. (see Tables 1 and 2 in Appendix I and Data Listings 1 and 2 in Appendix II). The following table summarizes subject enrollment and disposition.

Number enrolled:		238
Number discontinued:		40
Lost to follow-up:	31	
Voluntary withdrawal:	9	
Number completed:		198

Source: Table 1, Appendix I

There were no adverse events (AEs) reported during these studies.

A summary of response data is provided in Table 3, Appendix I. Individual dermatological response grades are provided in Data Listing 3, Appendix II.

8.0 CONCLUSION

Under the conditions employed in this study, there was no evidence of sensitization to

9.0 REFERENCES

Schwartz L, Peck SM. The patch test in contact dermatitis. Publ Health Pep 1944; 59:2.

Draize JH, Woodward G, Calvary HO. Methods for the study of irritation and toxicology of substances applied topically to the skin and mucous membranes. J Pharmacol Exp Ther 1944; 82: 377-390.

Lanman BM, Elvers WB, Howard CS. The role of human patch testing in a product development program. Joint Conf Cosmet Sci Toilet Goods Assoc 1968; 135-145.

Marzulli FN, Maibach HI. Contact allergy: predictive testing in man. Contact Dermatitis 1976; 2:1.

Zhai H, Maibach HI. Dermatotoxicology. 6th ed. New York:Hemisphere, 1996.

Stotts J. Planning, conduct and interpretation of human predictive sensitization patch tests. In:Drill VA, Lazar P, eds. Current Concepts in Cutaneous Toxicity. New York: Academic Press, 1980:41-53.

Griffith JF. Predictive and diagnostic testing for contact sensitization. Toxicol Appl Pharmacol, Suppl 1969; 3:90.

Gerberick GF, Robinson MK, Stotts J. An approach to allergic contact sensitization risk assessment of new chemicals and product ingredients. American Journal of Contact Dermatitis 1993; 4(4): 205-211.

APPENDIX I

SUMMARY TABLES

Table 1: Summary of Subject Enrollment and Disposition

	N (%)
Subjects enrolled	119
Subjects completed induction phase	110 (92.4)
Subjects completed all phases	107 (89.9)
Total subjects discontinued	12 (10.1)
Lost to follow-up	7 (5.9)
Voluntary withdrawal	5 (4.2)

Note: All percentages are relative to total subjects enrolled.

See data listing 1 for further detail.

Table 2: Summary of Subject Demographics
All Enrolled Subjects

Age	
N (%) 18 to 44	18 (15.1)
N (%) 45 to 65	84 (70.6)
N (%) 66 and up	17 (14.3)
Mean (SD)	54.9 (12.3)
Median	56.7
Range	19.3 to 70.7
Sex	
N (%) Male	14 (11.8)
N (%) Female	105 (88.2)
Race	
Asian	2 (1.7)
Black	6 (5.0)
Caucasian	110 (92.4)
Other	1 (0.8)
Ethnicity	
Hispanic/Latino	9 (7.6)
Not Hispanic/Not Latino	110 (92.4)

See data listing 2 for further detail.

Distributed for Comment Only -- Do Not Cite or Quote

Table 3: Summary of Dermatologic Response Grades
Number of Subjects by Product

	Induction Reading										Challenge Phase		
Response	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**Make Up**	**48hr**	**72hr**	**96hr(*)**
-	116	116	109	107	107	110	109	109	106	2	106	106	
?	0	0	0	0	0	0	0	0	0	0	1	1	
Total evaluable	116	116	109	107	107	110	109	109	106	2	107	107	
Number absent	0	0	6	7	6	2	1	1	4		0	0	
Number discontinued	3	3	4	5	6	7	9	9	9		12	12	

Maximum Elicited Response During Induction
All Subjects Completing Induction (N=110)

Response	n(%) Subjects
-	110 (100.0%)

(*) when required

See Table 3.1 for Key to Symbols and Scores

Table 3.1: Key To Symbols and Scores

Score or Symbol	Response or Description of Reaction
	Erythema Results
-	No reaction
?	Minimal or doubtful response, slightly different from surrounding normal skin
+	Definite erythema, no edema
++	Definite erythema, definite edema
+++	Definite erythema, definite edema and vesiculation
	Additional Comments
X	Reading not performed due to missed visit or subject discontinuation
D	Damage to epidermis: oozing, crusting and/or superficial erosions
E	Marked/severe erythema
I	Itching
p	Papular response >50%
pv	Papulovesicular response >50%
S	Spreading of reaction beyond patch site
NP	Not patched due to reaction achieved
PD	Patch dislodged
N9G	No ninth grading
NA	Not applied

Table 1: Summary of Subject Enrollment and Disposition

	N (%)
Subjects enrolled	119
Subjects completed induction phase	94 (79.0)
Subjects completed all phases	91 (76.5)
Total subjects discontinued	28 (23.5)
Lost to follow-up	24 (20.2)
Voluntary withdrawal	4 (3.4)

Note: All percentages are relative to total subjects enrolled.

See data listing 1 for further detail.

Table 2: Summary of Subject Demographics
All Enrolled Subjects

Age	
N (%) 18 to 44	38 (31.9)
N (%) 45 to 65	76 (63.9)
N (%) 66 and up	5 (4.2)
Mean (SD)	48.6 (12.9)
Median	51.9
Range	18.2 to 70.3
Sex	
N (%) Male	33 (27.7)
N (%) Female	86 (72.3)
Race	
Black	58 (48.7)
Caucasian	57 (47.9)
Other	4 (3.4)
Ethnicity	
Hispanic/Latino	28 (23.5)
Not Hispanic/Not Latino	91 (76.5)

See data listing 2 for further detail.

Table 3: Summary of Dermatologic Response Grades
Number of Subjects by Product

	Induction Reading										Challenge Phase		
Response	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**Make Up**	**48hr**	**72hr**	**96hr(*)**
-	103	99	96	91	94	91	94	93	92	20	91	91	
?	0	0	0	1	1	0	0	0	0	0	0	0	
Total evaluable	103	99	96	92	95	91	94	93	92	20	91	91	
Number absent	8	7	7	6	3	6	1	2	2		0	0	
Number discontinued	8	13	16	21	21	22	24	24	25		28	28	

Maximum Elicited Response During Induction
All Subjects Completing Induction (N= 94)

Response	n(%) Subjects
-	93 (98.9%)
?	1 (1.1%)

(*) when required

See Table 3.1 for Key to Symbols and Scores

Table 3.1: Key To Symbols and Scores

Score or Symbol	Response or Description of Reaction
	Erythema Results
-	No reaction
?	Minimal or doubtful response, slightly different from surrounding normal skin
+	Definite erythema, no edema
++	Definite erythema, definite edema
+++	Definite erythema, definite edema and vesiculation
	Additional Comments
X	Reading not performed due to missed visit or subject discontinuation
D	Damage to epidermis: oozing, crusting and/or superficial erosions
E	Marked/severe erythema
I	Itching
p	Papular response >50%
pv	Papulovesicular response >50%
S	Spreading of reaction beyond patch site
NP	Not patched due to reaction achieved
PD	Patch dislodged
N9G	No ninth grading
NA	Not applied

Distributed for Comment Only -- Do Not Cite or Quote

APPENDIX II

DATA LISTINGS

Data Listing 1: Subject Enrollment and Disposition

	Study Dates						
Subject No.	**Screened**	**1st Applic**	**Chall Applic**	**Ended**	**Last Reading #**	**Completion Status**	**Days in Study**
001	03/11/19	03/11/19	--	03/25/19	I5	L	15
002	03/11/19	03/11/19	04/15/19	04/18/19	C	C	39
003	03/11/19	03/11/19	04/15/19	04/18/19	C	C	39
004	03/11/19	03/11/19	04/15/19	04/18/19	C	C	39
005	03/11/19	03/11/19	04/15/19	04/18/19	C	C	39
006	03/11/19	03/11/19	04/15/19	04/18/19	C	C	39
007	03/11/19	03/11/19	04/15/19	04/18/19	C	C	39
008	03/11/19	03/11/19	04/15/19	04/18/19	C	C	39
009	03/11/19	03/11/19	04/15/19	04/18/19	C	C	39
010	03/11/19	03/11/19	04/15/19	04/18/19	C	C	39
011	03/11/19	03/11/19	--	03/13/19	I0	S	3
012	03/11/19	03/11/19	04/15/19	04/18/19	C	C	39
013	03/11/19	03/11/19	04/15/19	04/18/19	C	C	39
014	03/11/19	03/11/19	04/15/19	04/18/19	C	C	39
015	03/11/19	03/11/19	--	03/15/19	I0	L	5
016	03/11/19	03/11/19	04/15/19	04/18/19	C	C	39
017	03/11/19	03/11/19	04/15/19	04/18/19	C	C	39
018	03/11/19	03/11/19	04/15/19	04/16/19	I9	L	37
019	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
020	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
021	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
022	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
023	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
024	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
025	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
026	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
027	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
028	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
029	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
030	03/15/19	03/15/19	--	04/01/19	I6	L	18
031	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35

Key:
Last Reading # (I=Induction Phase, C=Challenge Phase)
Completion Status (C=Completed, L=Lost to follow-up, S=Voluntary withdrawal, V=Protocol violation, AE=Adverse event, O=Other)

Data Listing 1: Subject Enrollment and Disposition

	Study Dates						
Subject No.	**Screened**	**1st Applic**	**Chall Applic**	**Ended**	**Last Reading #**	**Completion Status**	**Days in Study**
032	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
033	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
034	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
035	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
036	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
037	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
038	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
039	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
040	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
041	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
042	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
043	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
044	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
045	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
046	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
047	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
048	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
049	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
050	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
051	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
052	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
053	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
054	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
055	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
056	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
057	03/15/19	03/15/19	--	04/15/19	I9	S	32
058	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
059	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
060	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
061	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
062	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35

Key:
Last Reading # (I=Induction Phase, C=Challenge Phase)
Completion Status (C=Completed, L=Lost to follow-up, S=Voluntary withdrawal, V=Protocol violation, AE=Adverse event, O=Other)

Data Listing 1: Subject Enrollment and Disposition

	Study Dates						
Subject No.	**Screened**	**1st Applic**	**Chall Applic**	**Ended**	**Last Reading #**	**Completion Status**	**Days in Study**
063	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
064	03/15/19	03/15/19	--	03/25/19	I2	L	11
065	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
066	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
067	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
068	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
069	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
070	03/15/19	03/15/19	--	03/27/19	I3	L	13
071	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
072	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
073	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
074	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
075	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
076	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
077	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
078	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
079	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
080	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
081	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
082	03/15/19	03/15/19	--	04/03/19	I6	L	20
083	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
084	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
085	03/15/19	03/15/19	--	04/15/19	I9	S	32
086	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
087	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
088	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
089	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
090	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
091	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
092	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
093	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35

Key:
Last Reading # (I=Induction Phase, C=Challenge Phase)
Completion Status (C=Completed, L=Lost to follow-up, S=Voluntary withdrawal, V=Protocol violation, AE=Adverse event, O=Other)

Data Listing 1: Subject Enrollment and Disposition

	Study Dates						
Subject No.	**Screened**	**1st Applic**	**Chall Applic**	**Ended**	**Last Reading #**	**Completion Status**	**Days in Study**
094	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
095	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
096	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
097	03/15/19	03/15/19	--	03/18/19	I0	S	4
098	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
099	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
100	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
101	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
102	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
103	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
104	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
105	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
106	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
107	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
108	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
109	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
110	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
111	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
112	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
113	03/15/19	03/15/19	--	03/29/19	I4	S	15
114	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
115	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
116	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
117	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
118	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35
119	03/15/19	03/15/19	04/15/19	04/18/19	C	C	35

Key:
Last Reading # (I=Induction Phase, C=Challenge Phase)
Completion Status (C=Completed, L=Lost to follow-up, S=Voluntary withdrawal, V=Protocol violation, AE=Adverse event, O=Other)

Data Listing 3: Dermatologic Response Grades
By Product and Subject

Subject No.	Induction Reading										Challenge Phase		
	1	2	3	4	5	6	7	8	9	MU	48hr	72hr	96hr(*)
001	-	-	X	-	-	X	X	X	X		X	X	
002	-	-	-	-	-	-	-	-	-		-	-	
003	-	-	-	-	-	-	-	-	-		-	-	
004	-	-	-	-	-	-	-	-	-		-	-	
005	-	-	-	-	-	-	-	-	-		-	-	
006	-	-	-	-	-	-	-	-	-		-	-	
007	-	-	-	-	-	-	-	-	-		-	-	
008	-	-	-	-	-	-	-	-	-		-	-	
009	-	-	-	X	-	-	-	-	-	-	-	-	
010	-	-	-	-	-	-	-	-	-		-	-	
011	X	X	X	X	X	X	X	X	X		X	X	
012	-	-	-	-	-	-	-	X	-	-	-	-	
013	-	-	-	-	-	-	-	-	-		-	-	
014	-	-	-	-	-	-	-	-	-		-	-	
015	X	X	X	X	X	X	X	X	X		X	X	
016	-	-	-	-	-	-	-	-	-		-	-	
017	-	-	-	-	-	-	-	-	-		-	-	
018	-	-	-	-	-	-	-	-	-		X	X	
019	-	-	-	-	-	-	-	-	-		-	-	
020	-	-	-	-	-	-	-	-	-		-	-	
021	-	-	-	-	-	-	-	-	-		-	-	
022	-	-	-	-	-	-	-	-	-		-	-	
023	-	-	-	-	-	-	-	-	-		-	-	

See Table 3.1 for Key to Symbols and Scores

MU = Make-up reading for missed induction visit

Data Listing 3: Dermatologic Response Grades
By Product and Subject

Subject No.	Induction Reading										Challenge Phase		
	1	2	3	4	5	6	7	8	9	MU	48hr	72hr	96hr(*)
024	-	-	-	-	-	-	-	-	-		-	-	
025	-	-	-	-	-	-	-	-	-		-	-	
026	-	-	-	-	-	-	-	-	-		-	-	
027	-	-	-	-	X	-	-	-	-	N9G	-	-	
028	-	-	-	-	-	-	-	-	N9G		-	-	
029	-	-	-	-	X	-	-	-	-	N9G	-	-	
030	-	-	-	X	-	-	X	X	X		X	X	
031	-	-	-	-	-	-	-	-	-		-	-	
032	-	-	-	-	-	-	-	-	-		-	-	
033	-	-	-	-	-	-	-	-	-		-	-	
034	-	-	-	-	-	-	-	-	-		-	-	
035	-	-	-	-	-	-	-	-	-		-	-	
036	-	-	-	-	-	-	X	-	-	N9G	-	-	
037	-	-	-	-	-	-	-	-	-		-	-	
038	-	-	-	-	-	-	-	-	-		-	-	
039	-	-	-	-	-	-	-	-	-		-	-	
040	-	-	-	-	-	-	-	-	-		-	-	
041	-	-	-	-	-	-	-	-	-		-	-	
042	-	-	-	-	X	-	-	-	-	N9G	-	-	
043	-	-	-	-	X	-	-	-	-	N9G	-	-	
044	-	-	-	-	-	-	-	-	-		-	-	
045	-	-	-	-	-	-	-	-	-		-	-	
046	-	-	-	-	-	-	-	-	-		-	-	

Distributed for Comment Only -- Do Not Cite or Quote

Data Listing 3: Dermatologic Response Grades
By Product and Subject

Subject No.	Induction Reading										Challenge Phase		
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**MU**	**48hr**	**72hr**	**96hr(*)**
047	-	-	-	-	-	-	-	-	-		-	-	
048	-	-	-	-	-	-	-	-	-		-	-	
049	-	-	-	-	-	-	-	-	-		-	-	
050	-	-	-	-	-	-	-	-	-		-	-	
051	-	-	-	-	-	-	-	-	-		-	-	
052	-	-	-	-	-	-	-	-	-		-	-	
053	-	-	-	-	-	-	-	-	-		-	-	
054	-	-	-	-	-	-	-	-	-		-	-	
055	-	-	-	-	-	-	-	-	-		-	-	
056	-	-	-	-	-	X	-	-	-	N9G	-	-	
057	-	-	-	-	-	-	-	-	-		X	X	
058	-	-	-	-	-	-	-	-	-		-	-	
059	-	-	-	-	-	-	-	-	-		-	-	
060	-	-	-	-	-	-	-	-	-		-	-	
061	-	-	-	-	-	-	-	-	-		-	-	
062	-	-	-	-	-	-	-	-	-		-	-	
063	-	-	-	-	-	-	-	-	-		-	-	
064	-	-	X	X	X	X	X	X	X		X	X	
065	-	-	-	-	-	X	-	-	-	N9G	-	-	
066	-	-	-	-	-	-	-	-	-		-	-	
067	-	-	-	-	-	-	-	-	-		-	-	
068	-	-	-	X	-	-	-	-	-	N9G	-	-	
069	-	-	-	-	-	-	-	-	-		-	-	

Distributed for Comment Only -- Do Not Cite or Quote

Data Listing 3: Dermatologic Response Grades
By Product and Subject

Subject No.	Induction Reading										Challenge Phase		
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**MU**	**48hr**	**72hr**	**96hr(*)**
070	-	-	-	X	X	X	X	X	X		X	X	
071	-	-	-	-	-	-	-	-	-		-	-	
072	-	-	-	-	-	-	-	-	-		-	-	
073	-	-	-	-	-	-	-	-	-		-	-	
074	-	-	-	-	-	-	-	-	-		-	-	
075	-	-	-	X	-	-	-	-	-	N9G	-	-	
076	-	-	-	-	-	-	-	-	N9G		-	-	
077	-	-	-	-	-	-	-	-	-		-	-	
078	-	-	-	-	-	-	-	-	-		-	-	
079	-	-	-	-	-	-	-	-	-		-	-	
080	-	-	-	-	-	-	-	-	-		-	-	
081	-	-	X	-	-	-	-	-	-	N9G	-	-	
082	-	-	-	-	-	-	X	X	X		X	X	
083	-	-	-	-	-	-	-	-	-		-	-	
084	-	-	-	-	-	-	-	-	-		-	-	
085	-	-	X	-	-	-	-	-	-	N9G	X	X	
086	-	-	-	-	-	-	-	-	-		-	-	
087	-	-	-	-	-	-	-	-	-		-	-	
088	-	-	X	-	-	-	-	-	-	N9G	-	-	
089	-	-	-	-	-	-	-	-	-		-	-	
090	-	-	-	X	-	-	-	-	-	N9G	-	-	
091	-	-	X	-	-	-	-	-	-	N9G	-	-	
092	-	-	-	-	-	-	-	-	-		-	-	

Data Listing 3: Dermatologic Response Grades
By Product and Subject

Subject No.	Induction Reading										Challenge Phase		
	1	2	3	4	5	6	7	8	9	MU	48hr	72hr	96hr(*)
093	-	-	-	-	-	-	-	-	-		-	-	
094	-	-	-	-	-	-	-	-	-		-	-	
095	-	-	-	-	-	-	-	-	-		-	-	
096	-	-	-	-	-	-	-	-	N9G		-	-	
097	X	X	X	X	X	X	X	X	X		X	X	
098	-	-	-	-	-	-	-	-	-		-	-	
099	-	-	-	-	-	-	-	-	-		?	?	
100	-	-	-	-	-	-	-	-	-		-	-	
101	-	-	-	-	-	-	-	-	-		-	-	
102	-	-	-	-	-	-	-	-	-		-	-	
103	-	-	-	-	-	-	-	-	-		-	-	
104	-	-	X	-	-	-	-	-	-	N9G	-	-	
105	-	-	-	-	-	-	-	-	-		-	-	
106	-	-	-	-	-	-	-	-	-		-	-	
107	-	-	-	-	-	-	-	-	-		-	-	
108	-	-	-	X	-	-	-	-	-	N9G	-	-	
109	-	-	-	-	-	-	-	-	-		-	-	
110	-	-	-	-	-	-	-	-	-		-	-	
111	-	-	-	-	-	-	-	-	-		-	-	
112	-	-	-	-	-	-	-	-	N9G		-	-	
113	-	-	-	-	X	X	X	X	X		X	X	
114	-	-	-	-	-	-	-	-	-		-	-	
115	-	-	-	-	-	-	-	-	-		-	-	
116	-	-	-	-	-	-	-	-	-		-	-	
117	-	-	-	-	X	-	-	-	-	N9G	-	-	
118	-	-	-	-	X	-	-	-	-	N9G	-	-	
119	-	-	-	X	-	-	-	-	-	N9G	-	-	

Data Listing 1: Subject Enrollment and Disposition

	Study Dates						
Subject No.	**Screened**	**1st Applic**	**Chall Applic**	**Ended**	**Last Reading #**	**Completion Status**	**Days in Study**
001	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
002	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
003	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
004	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
005	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
006	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
007	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
008	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
009	03/18/19	03/18/19	--	04/03/19	I6	L	17
010	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
011	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
012	03/18/19	03/18/19	--	03/27/19	I2	S	10
013	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
014	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
015	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
016	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
017	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
018	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
019	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
020	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
021	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
022	03/18/19	03/18/19	--	03/25/19	I1	L	8
023	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
024	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
025	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
026	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
027	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
028	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
029	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
030	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
031	03/18/19	03/18/19	--	03/25/19	I1	L	8

Key:
Last Reading # (I=Induction Phase, C=Challenge Phase)
Completion Status (C=Completed, L=Lost to follow-up, S=Voluntary withdrawal, V=Protocol violation, AE=Adverse event, O=Other)

Data Listing 1: Subject Enrollment and Disposition

	Study Dates						
Subject No.	**Screened**	**1st Applic**	**Chall Applic**	**Ended**	**Last Reading #**	**Completion Status**	**Days in Study**
032	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
033	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
034	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
035	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
036	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
037	03/18/19	03/18/19	--	03/22/19	I0	L	5
038	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
039	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
040	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
041	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
042	03/18/19	03/18/19	--	03/25/19	I1	L	8
043	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
044	03/18/19	03/18/19	--	03/25/19	I2	L	8
045	03/18/19	03/18/19	04/23/19	04/23/19	I9	L	37
046	03/18/19	03/18/19	04/23/19	04/23/19	I9	L	37
047	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
048	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
049	03/18/19	03/18/19	--	03/22/19	I0	L	5
050	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
051	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
052	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
053	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
054	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
055	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
056	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
057	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
058	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
059	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
060	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
061	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
062	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40

Key:
Last Reading # (I=Induction Phase, C=Challenge Phase)
Completion Status (C=Completed, L=Lost to follow-up, S=Voluntary withdrawal, V=Protocol violation, AE=Adverse event, O=Other)

Data Listing 1: Subject Enrollment and Disposition

	Study Dates						
Subject No.	**Screened**	**1st Applic**	**Chall Applic**	**Ended**	**Last Reading #**	**Completion Status**	**Days in Study**
063	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
064	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
065	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
066	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
067	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
068	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
069	03/18/19	03/18/19	--	03/22/19	I0	L	5
070	03/18/19	03/18/19	04/23/19	04/26/19	C	C	40
071	03/18/19	03/18/19	--	03/25/19	I1	L	8
072	03/22/19	03/22/19	04/23/19	04/26/19	C	C	36
073	03/22/19	03/22/19	04/23/19	04/26/19	C	C	36
074	03/22/19	03/22/19	--	04/23/19	I9	S	33
075	03/22/19	03/22/19	04/23/19	04/26/19	C	C	36
076	03/22/19	03/22/19	04/23/19	04/26/19	C	C	36
077	03/22/19	03/22/19	04/23/19	04/26/19	C	C	36
078	03/22/19	03/22/19	04/23/19	04/26/19	C	C	36
079	03/22/19	03/22/19	--	03/29/19	I1	L	8
080	03/22/19	03/22/19	--	03/27/19	I0	S	6
081	03/22/19	03/22/19	04/23/19	04/26/19	C	C	36
082	03/22/19	03/22/19	04/23/19	04/26/19	C	C	36
083	03/22/19	03/22/19	--	04/05/19	I5	L	15
084	03/22/19	03/22/19	04/23/19	04/26/19	C	C	36
085	03/22/19	03/22/19	04/23/19	04/26/19	C	C	36
086	03/22/19	03/22/19	04/23/19	04/26/19	C	C	36
087	03/22/19	03/22/19	--	04/12/19	I8	L	22
088	03/22/19	03/22/19	--	03/29/19	I2	S	8
089	03/22/19	03/22/19	04/23/19	04/26/19	C	C	36
090	03/22/19	03/22/19	04/23/19	04/26/19	C	C	36
091	03/22/19	03/22/19	--	03/27/19	I0	L	6
092	03/22/19	03/22/19	--	03/27/19	I0	L	6
093	03/22/19	03/22/19	04/23/19	04/26/19	C	C	36

Key:
Last Reading # (I=Induction Phase, C=Challenge Phase)
Completion Status (C=Completed, L=Lost to follow-up, S=Voluntary withdrawal, V=Protocol violation, AE=Adverse event, O=Other)

Data Listing 1: Subject Enrollment and Disposition

	Study Dates						
Subject No.	**Screened**	**1st Applic**	**Chall Applic**	**Ended**	**Last Reading #**	**Completion Status**	**Days in Study**
094	03/22/19	03/22/19	04/23/19	04/26/19	C	C	36
095	03/22/19	03/22/19	04/23/19	04/26/19	C	C	36
096	03/22/19	03/22/19	04/23/19	04/26/19	C	C	36
097	03/22/19	03/22/19	--	04/03/19	I3	L	13
098	03/22/19	03/22/19	04/23/19	04/26/19	C	C	36
099	03/22/19	03/22/19	04/23/19	04/26/19	C	C	36
100	03/22/19	03/22/19	04/23/19	04/26/19	C	C	36
101	03/22/19	03/22/19	04/23/19	04/26/19	C	C	36
102	03/22/19	03/22/19	04/23/19	04/26/19	C	C	36
103	03/22/19	03/22/19	04/23/19	04/26/19	C	C	36
104	03/22/19	03/22/19	04/23/19	04/26/19	C	C	36
105	03/22/19	03/22/19	--	04/01/19	I3	L	11
106	03/22/19	03/22/19	04/23/19	04/26/19	C	C	36
107	03/22/19	03/22/19	04/23/19	04/26/19	C	C	36
108	03/22/19	03/22/19	04/23/19	04/26/19	C	C	36
109	03/22/19	03/22/19	04/23/19	04/26/19	C	C	36
110	03/22/19	03/22/19	04/23/19	04/26/19	C	C	36
111	03/22/19	03/22/19	04/23/19	04/26/19	C	C	36
112	03/22/19	03/22/19	--	04/01/19	I3	L	11
113	03/22/19	03/22/19	04/23/19	04/26/19	C	C	36
114	03/22/19	03/22/19	04/23/19	04/26/19	C	C	36
115	03/22/19	03/22/19	--	04/03/19	I3	L	13
116	03/25/19	03/25/19	--	03/27/19	I0	L	3
117	03/22/19	03/22/19	--	04/03/19	I3	L	13
118	03/22/19	03/22/19	--	03/27/19	I0	L	6
119	03/22/19	03/22/19	--	04/08/19	I6	L	18

Key:
Last Reading # (I=Induction Phase, C=Challenge Phase)
Completion Status (C=Completed, L=Lost to follow-up, S=Voluntary withdrawal, V=Protocol violation, AE=Adverse event, O=Other)

Data Listing 3: Dermatologic Response Grades
By Product and Subject

Subject No.	Induction Reading										Challenge Phase		
	1	2	3	4	5	6	7	8	9	MU	48hr	72hr	96hr(*)
001	-	-	-	-	-	-	-	-	-		-	-	
002	-	-	-	-	-	-	-	-	-		-	-	
003	-	-	-	-	-	-	-	-	-		-	-	
004	X	-	-	-	-	-	-	-	-	-	-	-	
005	-	-	-	-	-	-	-	-	-		-	-	
006	-	-	-	-	-	-	-	-	-		-	-	
007	-	-	-	-	-	-	-	-	-		-	-	
008	-	-	-	-	-	-	-	-	-		-	-	
009	-	-	-	X	-	-	X	X	X		X	X	
010	-	-	-	-	-	-	-	-	-		-	-	
011	-	-	-	-	-	-	-	-	-		-	-	
012	-	-	X	X	X	X	X	X	X		X	X	
013	-	-	-	-	-	-	-	-	-		-	-	
014	-	-	-	-	-	-	-	-	-		-	-	
015	-	-	-	X	-	-	-	-	-	-	-	-	
016	-	-	-	-	-	-	X	-	-	-	-	-	
017	-	-	-	-	-	-	-	-	-		-	-	
018	-	-	-	-	-	-	-	-	-		-	-	
019	-	-	-	-	-	-	-	-	-		-	-	
020	-	-	-	-	-	-	-	-	-		-	-	
021	-	-	-	-	-	-	-	-	-		-	-	
022	-	X	X	X	X	X	X	X	X		X	X	
023	-	-	-	-	-	-	-	-	-		-	-	

See Table 3.1 for Key to Symbols and Scores

MU = Make-up reading for missed induction visit

Data Listing 3: Dermatologic Response Grades
By Product and Subject

Subject No.	Induction Reading										Challenge Phase		
	1	2	3	4	5	6	7	8	9	MU	48hr	72hr	96hr(*)
024	-	-	-	-	-	-	-	-	-		-	-	
025	-	-	-	-	-	-	-	-	-		-	-	
026	-	-	-	-	-	-	-	-	-		-	-	
027	-	-	-	-	-	-	-	-	-		-	-	
028	-	-	-	-	-	X	-	-	-	-	-	-	
029	X	-	-	-	-	-	-	-	-	-	-	-	
030	-	-	-	-	-	-	-	-	-		-	-	
031	-	X	X	X	X	X	X	X	X		X	X	
032	-	X	-	-	-	-	-	-	-	-	-	-	
033	-	-	-	-	-	-	-	-	-		-	-	
034	-	-	-	-	-	-	-	-	-		-	-	
035	-	-	X	-	-	-	-	-	-	-	-	-	
036	-	-	-	-	-	-	-	-	-		-	-	
037	X	X	X	X	X	X	X	X	X		X	X	
038	-	-	X	-	-	-	-	-	-	-	-	-	
039	-	X	-	-	-	-	-	-	-	-	-	-	
040	-	-	-	-	-	X	-	-	-	-	-	-	
041	-	-	-	-	-	-	-	X	-	-	-	-	
042	-	X	X	X	X	X	X	X	X		X	X	
043	-	-	-	-	-	-	-	-	-		-	-	
044	X	-	X	X	X	X	X	X	X		X	X	
045	-	-	-	-	-	-	-	-	-		X	X	
046	-	-	-	-	-	-	-	-	-		X	X	

Distributed for Comment Only -- Do Not Cite or Quote

Data Listing 3: Dermatologic Response Grades
By Product and Subject

Subject No.	Induction Reading										Challenge Phase		
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**MU**	**48hr**	**72hr**	**96hr(*)**
047	-	-	-	-	-	-	-	-	-		-	-	
048	-	X	-	-	-	-	-	-	-	-	-	-	
049	X	X	X	X	X	X	X	X	X		X	X	
050	-	-	-	-	-	-	-	-	-		-	-	
051	-	X	-	-	-	-	-	-	-	-	-	-	
052	-	-	-	-	-	-	-	-	-		-	-	
053	-	-	-	-	-	-	-	-	-		-	-	
054	-	-	-	-	-	-	-	-	-		-	-	
055	-	-	-	-	-	-	-	-	-		-	-	
056	-	-	-	-	-	-	-	-	-		-	-	
057	-	-	-	-	-	X	-	-	-	-	-	-	
058	-	-	-	-	-	-	-	-	-		-	-	
059	X	-	-	-	-	-	-	-	-	-	-	-	
060	-	-	-	-	-	-	-	X	-	-	-	-	
061	-	-	-	-	-	-	-	-	X	-	-	-	
062	-	-	-	-	-	-	-	-	-		-	-	
063	-	-	-	-	X	-	-	-	-	-	-	-	
064	-	-	-	-	-	-	-	-	-		-	-	
065	-	-	-	-	-	-	-	-	-		-	-	
066	-	-	-	-	-	-	-	-	-		-	-	
067	-	-	-	-	X	-	-	-	-	-	-	-	
068	-	-	X	-	-	-	-	-	-	N9G	-	-	
069	X	X	X	X	X	X	X	X	X		X	X	

Data Listing 3: Dermatologic Response Grades
By Product and Subject

Subject No.	Induction Reading										Challenge Phase		
	1	2	3	4	5	6	7	8	9	MU	48hr	72hr	96hr(*)
070	-	-	X	-	-	-	-	-	-	-	-	-	
071	-	X	X	X	X	X	X	X	X		X	X	
072	-	-	-	-	-	X	-	-	-	N9G	-	-	
073	-	-	X	-	-	-	-	-	-	N9G	-	-	
074	-	-	X	-	-	-	-	-	-	N9G	X	X	
075	-	X	-	?	?	-	-	-	-	N9G	-	-	
076	-	-	-	-	-	-	-	-	-		-	-	
077	-	-	-	-	-	-	-	-	-		-	-	
078	-	-	-	-	-	-	-	-	-		-	-	
079	-	X	X	X	X	X	X	X	X		X	X	
080	X	X	X	X	X	X	X	X	X		X	X	
081	-	-	-	-	-	-	-	-	-		-	-	
082	-	-	-	-	X	-	-	-	-	N9G	-	-	
083	-	-	-	X	-	X	X	X	X		X	X	
084	-	-	-	-	-	-	-	-	-		-	-	
085	-	-	-	-	-	-	-	-	-		-	-	
086	-	-	-	-	-	-	-	-	-		-	-	
087	-	-	-	-	-	X	-	-	X		X	X	
088	X	-	X	X	X	X	X	X	X		X	X	
089	-	-	-	-	-	-	-	-	-		-	-	
090	-	-	X	-	-	-	-	-	-	N9G	-	-	
091	X	X	X	X	X	X	X	X	X		X	X	
092	X	X	X	X	X	X	X	X	X		X	X	

Data Listing 3: Dermatologic Response Grades
By Product and Subject

Subject No.	Induction Reading										Challenge Phase		
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**MU**	**48hr**	**72hr**	**96hr(*)**
093	-	-	-	X	-	-	-	-	-	N9G	-	-	
094	-	-	-	-	-	-	-	-	-		-	-	
095	-	-	-	-	-	-	-	-	-		-	-	
096	-	-	-	-	-	-	-	-	N9G		-	-	
097	-	-	-	X	X	X	X	X	X		X	X	
098	-	-	-	-	-	-	-	-	-		-	-	
099	-	-	-	-	-	-	-	-	-		-	-	
100	-	-	-	-	-	-	-	-	-		-	-	
101	-	-	-	-	-	-	-	-	-		-	-	
102	X	-	-	-	-	-	-	-	-	N9G	-	-	
103	-	-	-	X	-	-	-	-	-	N9G	-	-	
104	-	-	-	-	-	-	-	-	-		-	-	
105	X	-	-	X	X	X	X	X	X		X	X	
106	-	-	-	-	-	-	-	-	-		-	-	
107	X	-	-	-	-	-	-	-	-	N9G	-	-	
108	-	-	-	-	-	X	-	-	-	N9G	-	-	
109	-	-	-	-	-	-	-	-	-		-	-	
110	-	X	-	-	-	-	-	-	-	N9G	-	-	
111	-	-	-	-	-	-	-	-	-		-	-	
112	-	X	-	X	X	X	X	X	X		X	X	
113	-	-	-	-	-	-	-	-	-		-	-	
114	-	-	-	-	-	-	-	-	-		-	-	
115	-	-	-	X	X	X	X	X	X		X	X	
116	X	X	X	X	X	X	X	X	X		X	X	
117	-	-	-	X	X	X	X	X	X		X	X	
118	X	X	X	X	X	X	X	X	X		X	X	
119	-	-	-	X	-	-	X	X	X		X	X	

Memorandum

TO: Bart Heldreth, Ph.D.
Executive Director - Cosmetic Ingredient Review

FROM: Carol Eisenmann, Ph.D.
Personal Care Products Council

DATE: December 1, 2022

SUBJECT: Sodium Hyaluronate, Hydrolyzed Sodium Hyaluronate and Hydrolyzed Hyaluronic Acid

Anonymous. 2022. Composition and impurities data of Sodium Hyaluronate.

Anonymous. 2022. Manufacturing Process of Sodium Hyaluronate.

Anonymous. 2022. Manufacturing Process of Low Molecular Weight Sodium Hyaluronate.

Anonymous. 2022. Sodium Hyaluronate Toxicity Summary.

Anonymous. 2022. Composition and impurities data of Hydrolyzed Sodium Hyaluronate.

Anonymous. 2022. Manufacturing Process of Hydrolyzed Sodium Hyaluronate (5-10 KDa).

Anonymous. 2022. Hydrolyzed Sodium Hyaluronate flow chart (1-5 KDa).

Anonymous. 2022. Manufacturing technique flow chart Hydrolyzed Sodium Hyaluronate (<1 KDa).

Anonymous. 2022. Hydrolyzed Sodium Hyaluronate Toxicity Summary.

Anonymous. 2022. Composition and impurities data of Hydrolyzed Hyaluronic Acid.

Anonymous. 2022. Manufacturing Process of Hydrolyzed Hyaluronic Acid.

Distributed for Comment Only -- Do Not Cite or Quote

Composition and impurities data of Sodium Hyaluronate

Product Name : Sodium Hyaluronate

INCI Name: Sodium Hyaluronate

Molecular Weight: $(C_{14}H_{20}NO_{11}Na)_n$ *Mw* ≧1.0M Da

Items	Results	Method
Assay	≥95%	Carbazole method
Bacterial Endotoxins	＜0.5EU/mg	Gel method
Protein	＜0.05%	Bradford method
Chlorides	＜0.5%	Colorimetric method
Nucleic acids	The absorbance at 260nm< 0.5	European Pharmacopoeia
Total metals	≤20 ppm	ChP2020

Product Name : Low Molecular Weight Sodium Hyaluronate

INCI Name: Sodium Hyaluronate

Molecular Weight: $(C_{14}H_{20}NO_{11}Na)_n$ 0.1M-1.0M Da

Items	Results	Method
Assay	≥95%	Carbazole method
Bacterial Endotoxins	＜0.5EU/mg	Gel method
Protein	＜0.05%	Bradford method
Chlorides	＜0.5%	Colorimetric method
Nucleic acids	The absorbance at 260nm< 0.5	European Pharmacopoeia
Total metals	≤20 ppm	ChP2020

Distributed for Comment Only -- Do Not Cite or Quote

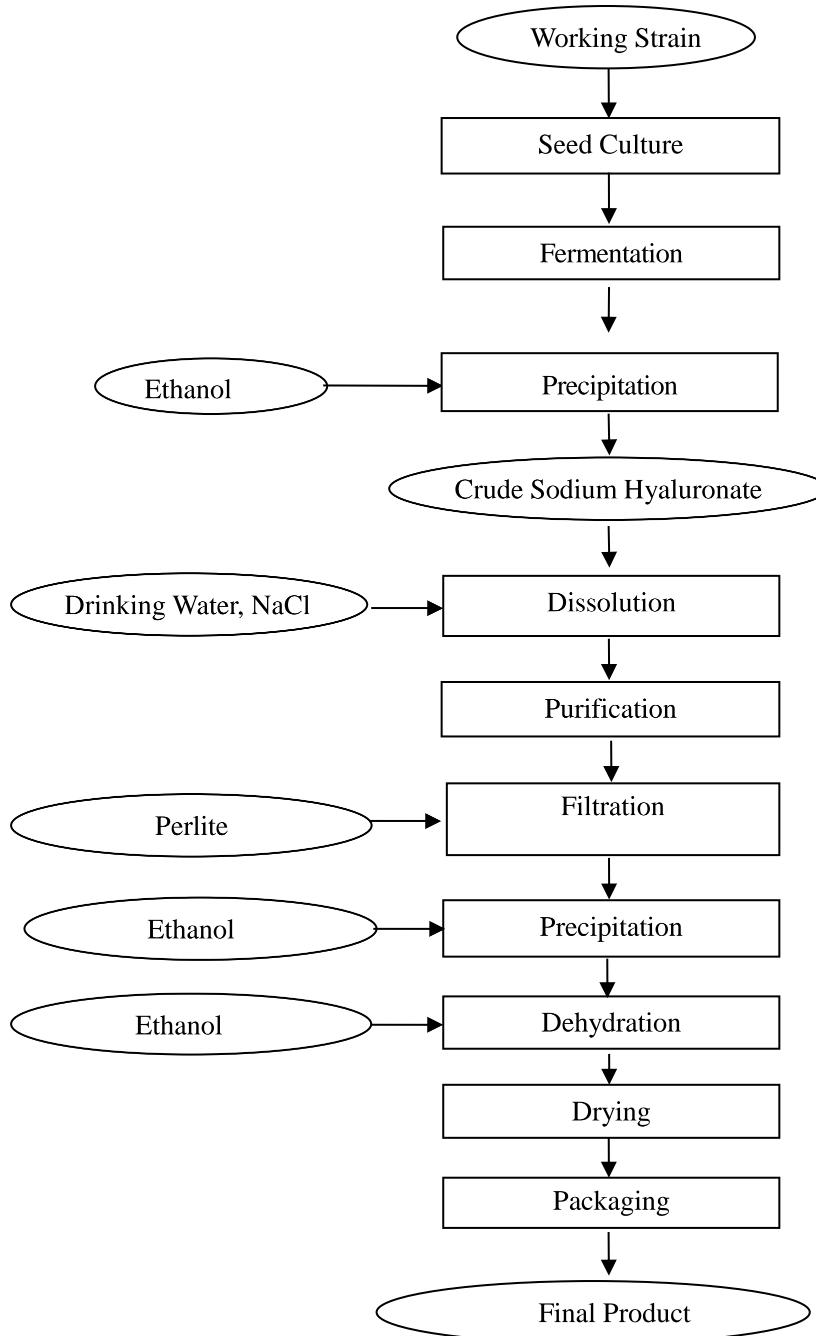
Manufacturing Process of Sodium Hyaluronate

Product Name : Sodium Hyaluronate

INCI Name: Sodium Hyaluronate

Molecular Weight: $(C_{14}H_{20}NO_{11}Na)_n$ $Mw \geqq 1.0$M Da

1. Flow Chart



2. Description of the Manufacturing Process

Sodium Hyaluronate was manufactured via fermentation using the bacterial strain *Streptococcus equi* subsp. *zooepidemicus*. This process begins with the preparation of a seed broth prepared from

Distributed for Comment Only -- Do Not Cite or Quote

seed culture, which is transferred to a fermenter containing sterilized fermentation medium. Following fermentation, the broth is mixed with ethanol. The crude Sodium Hyaluronate precipitate is dissolved in drinking water and filtered to remove the dead bacteria and other impurities. The resulting filtrate is precipitated, dehydrated, and dried, yielding the final product-Sodium Hyaluronate.

Distributed for Comment Only -- Do Not Cite or Quote

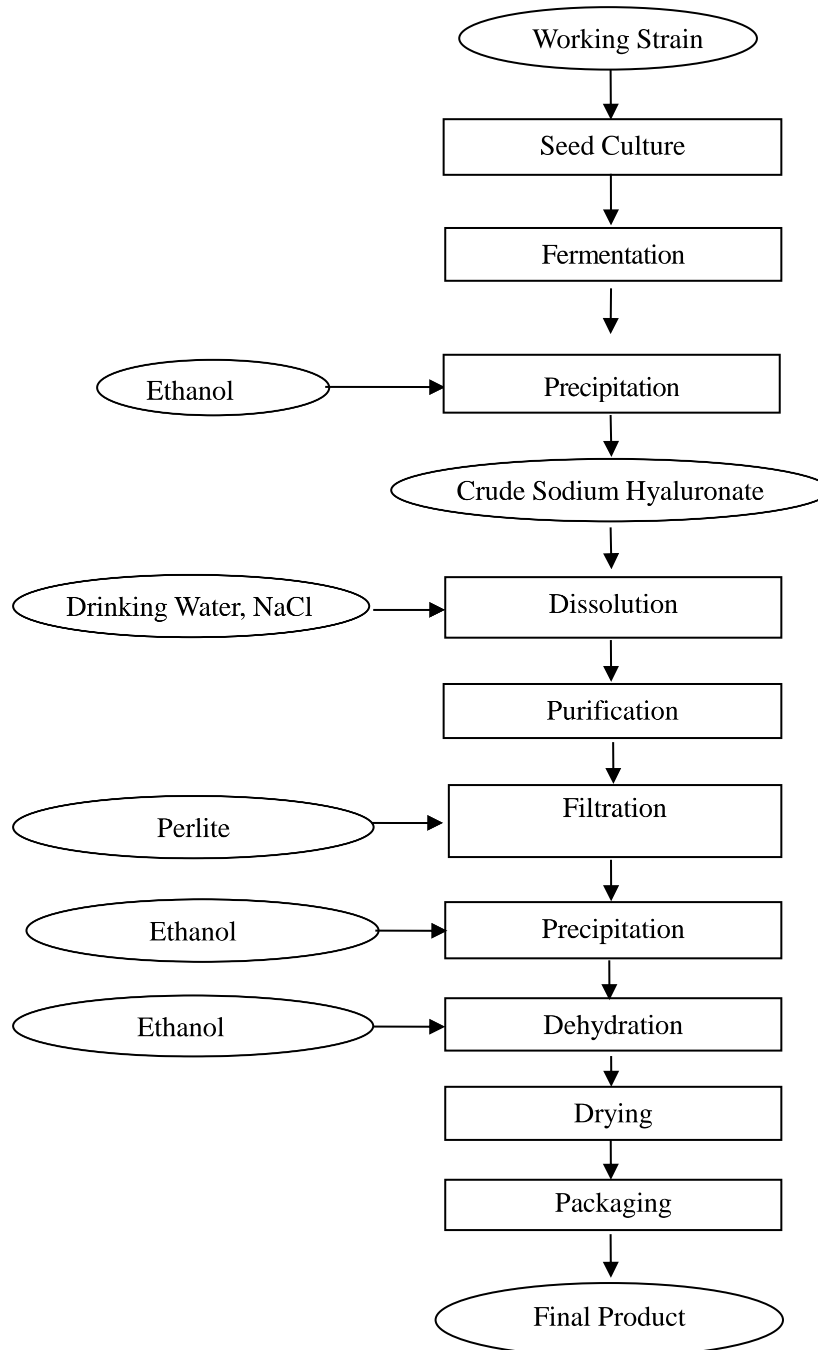
Manufacturing Process of Low Molecular Weight Sodium Hyaluronate

Product Name : Low Molecular Weight Sodium Hyaluronate

INCI Name: Sodium Hyaluronate

Molecular Weight: $(C_{14}H_{20}NO_{11}Na)_n$ 0.1M-1.0M Da

1. Flow Chart



2. Description of the Manufacturing Process

Sodium Hyaluronate was manufactured via fermentation using the bacterial strain *Streptococcus equi* subsp. *zooepidemicus*. This process begins with the preparation of a seed broth prepared from

Distributed for Comment Only -- Do Not Cite or Quote

seed culture, which is transferred to a fermenter containing sterilized fermentation medium. Following fermentation, the broth is degraded and then mixed with ethanol. The crude Sodium Hyaluronate precipitate is dissolved in drinking water and filtered to remove the dead bacteria and other impurities. The resulting filtrate is precipitated, dehydrated, and dried, yielding the final product- Low Molecular Weight Sodium Hyaluronate.

Distributed for Comment Only -- Do Not Cite or Quote

Sodium Hyaluronate toxicity summary

1 Food Grade Sodium Hyaluronate

1.1 Ames (Jan. 2007)
Sodium Hyaluronate (Food Grade) was determined to be non-genotoxic in several Ames assays when tested at up to 5 mg/plate, with and without metabolic activation (assays performed in S. typhimurium strands TA97a, TA98, TA100 and TA102).

1.2 Micronucleus test in bone marrow (Jan. 2007)
The Sodium Hyaluronate (Food Grade) was administrated to mice via oral gavage twice at an interval of 24hr. 50 mice weighting 25~30g were randomly assigned to 5 groups, and each group included 5 male and 5 female mice. 40mg/kg BW cyclophosphamide was used as positive control. Distilled water was used as negative control. The test substance Sodium Hyaluronate (Food Grade) was gavaged at dose level of 20.0ml/kg BW. The mice were sacrificed 6 hours after the last administration. The bone marrow cells were flushed into tubes with fetal bovine serum to prepare bone marrow smear specimens. The specimens were fixed with methanol and stained with Giemsa technique. Percentage of polychromatic erythrocytes (PEC) was calculated based on the observations of 200 erythrocytes per animal. And percentage of micronucleated PEC was calculated based on the observations of 1000 PECs per animal. Picked out epididymis, removed fat, cut in normal saline, centrifuged at 1000rpm for 7 min, removed the supermatant, prepared smear specimens. Malformation rate was calculated based on the observations of 1000 sperms per animal.
The results: Sodium Hyaluronate (Food Grade) has no mutagenic toxicity to somatic chromosome of mice.

1.3 Sperm malformation test in mice (Jan. 2007)
For Sodium Hyaluronate (Food Grade), fifty young adult male mice weighting 30~35g were randomly assigned to 5 groups with 10 in each group. 40mg/kg BW cyclophosphamide was used as positive control. Distilled water was used as negative control. The test substance was gavaged at dose level of 20.0ml/kg BW. The mice were gavaged one time per day for 5 days. The mice were sacrificed 30 days after the last administration.
The results: Sodium Hyaluronate (Food Grade) has no influence to sperm malformation rate of mice, that is, sodium hyaluronate has no mutagenic toxicity to germ cells of mice.

2. Cosmetic Grade Sodium Hyaluronate

2.1 Ames (Dec. 2016)
Sodium Hyaluronate was determined to be non-genotoxic in several Ames assays when tested at up to 1 mg/plate, with and without metabolic activation (assays performed in S. typhimurium strands TA97a, TA98, TA100, TA102, and TA1535).

2.2 Eye irritation (Feb. 2017)

Distributed for Comment Only -- Do Not Cite or Quote

Based on "the Chorioallantoic Membrane Vascular Assay (CAMVA)" and "Bovine Corneal Opacity and Permeability Test (BCOP)", "100% Sodium Hyaluronate" is predicted as non-irritant/ slightly irritant.

2.3 Human Repeated Insult Patch Test (HRIPT, Jan. 2017)

Based on the test population of 100 subjects and under the conditions of HRIPT study, the investigational product identified as Sodium Hyaluronate (0.2%) did not demonstrate a potential for dermal irritation or inducing sensitization.

2.4 Phototoxicity test (Feb. 2017)

Accroding to the OECD TG432 *In vitro* 3T3 NRU Phototoxicity test, it is predicted "Sodium Hyaluronate (125 μg/mL)" is no phototoxicity.

2.5 Skin Irritation test (Oct. 2019)

Based on the Reconstructed Human Epidermis (Episkin, OECD tg439) model, the "Sodium Hyaluronate (undiluted)" belong to No Category (UN GHS classification).

2.6 Skin sensitisation test (Oct. 2019)

According OECD TG442E (2018), under the condition of cell activation, the results of Sodium Hyaluronate (the highest concentration-1mg/mL) , the mean of $REI_{CD54}<200$ and mean $RFI_{CD86}<150$, and the cell activity was greater than 50%, the skin sensitization prediction based on H-CLAT was negative.

Composition and impurities data of Hydrolyzed Sodium Hyaluronate

INCI Name: Hydrolyzed Sodium Hyaluronate,

Molecular Weight: $(C_{14}H_{20}NO_{11}Na)_n$ 5 K-10K Da (intrinsic viscosity method)

Items	Results	Method
Assay	≥95%	HPLC method
Bacterial Endotoxins	＜0.5EU/mg	Gel method
Protein	＜0.05%	Bradford method
Chlorides	＜0.5%	Colorimetric method
Nucleic acids	The absorbance at 260nm< 0.5	European Pharmacopoeia
Total metals	≤20 ppm	ChP2020

INCI Name: Hydrolyzed Sodium Hyaluronate,

Molecular Weight: $(C_{14}H_{20}NO_{11}Na)_n$，1 K-5 K Da (intrinsic viscosity method)

Items	Results	Method
Assay	≥95%	HPLC method
Bacterial Endotoxins	＜0.5EU/mg	Gel method
Protein	＜0.05%	Bradford method
Chlorides	＜0.5%	Colorimetric method
Nucleic acids	The absorbance at 260nm< 0.5	European Pharmacopoeia
Total metals	≤20 ppm	ChP2020

INCI Name: Hydrolyzed Sodium Hyaluronate

Molecular Weight: $(C_{14}H_{20}NO_{11}Na)_n$ Mw<1K Da (Muti-angle laser light-scattering method, GPC-MALLS method)

Items	Results	Method
Assay	≥95%	HPLC method
Bacterial Endotoxins	＜0.5EU/mg	Gel method
Protein	＜0.05%	Bradford method
Chlorides	＜0.5%	Colorimetric method
Nucleic acids	The absorbance at 260nm< 0.5	European Pharmacopoeia
Total metals	≤10 ppm	ChP2020

All the results of Multi-Pesticide Suite 190+ items is negative (detection limit=0.01ppm) based on the EN 15662:2018 method.

Distributed for Comment Only -- Do Not Cite or Quote

Manufacturing Process of Hydrolyzed Sodium Hyaluronate

INCI Name: Hydrolyzed Sodium Hyaluronate

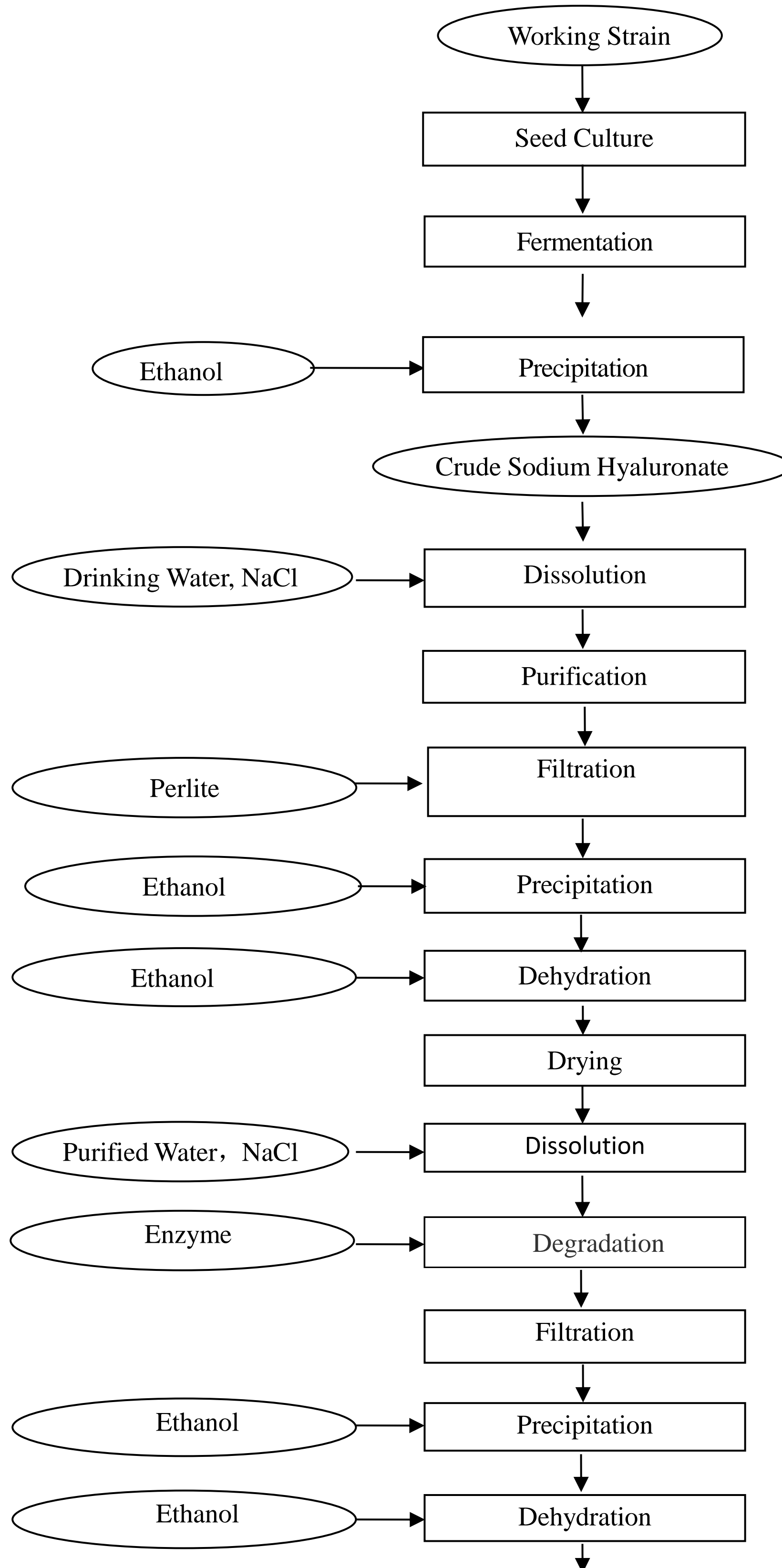
Molecular Weight: $(C_{14}H_{20}NO_{11}Na)_n$ *Mw*: 5K-10K Da

1. Description of the Manufacturing Process

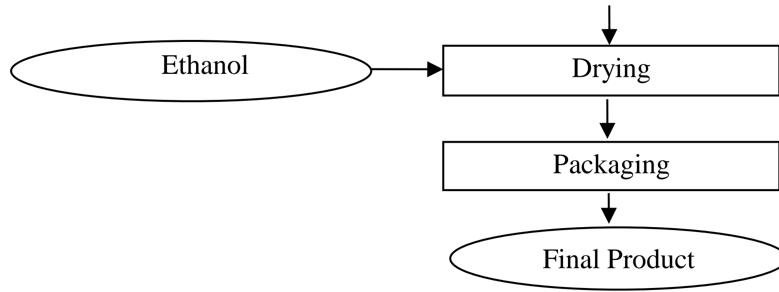
Sodium Hyaluronate manufactured for food and cosmetic use was reported to be manufactured via fermentation using the bacterial strain *Streptococcus equi* subsp. *zooepidemicus*. This process begins with the preparation of a seed broth prepared from seed culture, which is transferred to a fermenter containing sterilized fermentation medium. Following fermentation, the broth is mixed with ethanol. The crude Sodium Hyaluronate precipitate is dissolved in water and filtered to remove impurities and inactivated fragments. The resulting filtrate is precipitated, dehydrated, and dried, yielding the HA product. This product is dissolved in purified water, add enzyme and the HA solution is degraded, heated, filtered, precipitated, dehydrated, and dried, yielding the Hydrolyzed Sodium Hyaluronate.

2. Flow Chart:

(Next page)


Working Strain
Seed Culture
Fermentation
Ethanol
Precipitation
Crude Sodium Hyaluronate
Drinking Water, NaCl
Dissolution
Purification
Perlite
Filtration
Ethanol
Precipitation
Ethanol
Dehydration
Drying
Purified Water，NaCl
Dissolution
Enzyme
Degradation
Filtration
Ethanol
Precipitation
Ethanol
Dehydration

Distributed for Comment Only -- Do Not Cite or Quote



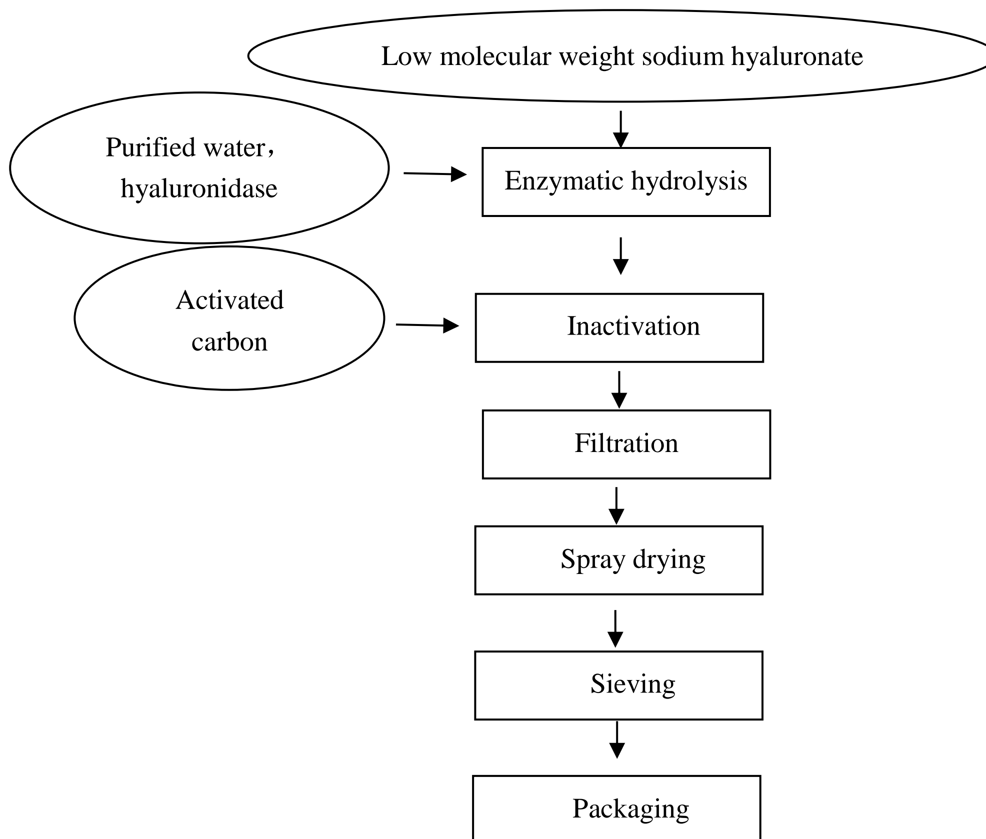
Hydrolyzed Sodium Hyaluronate flow chart

INCI Name: Hydrolyzed Sodium Hyaluronate

Molecular Weight: $(C_{14}H_{20}NO_{11}Na)_n$， 1 K-5 K Da (intrinsic viscosity method)

This product is hydrolyzed by hyaluronidase from low molecular weight sodium hyaluronate. The production process includes dissolution, enzymatic hydrolysis, inactivation (high temperature), filtration, spray drying, sieving and packaging.

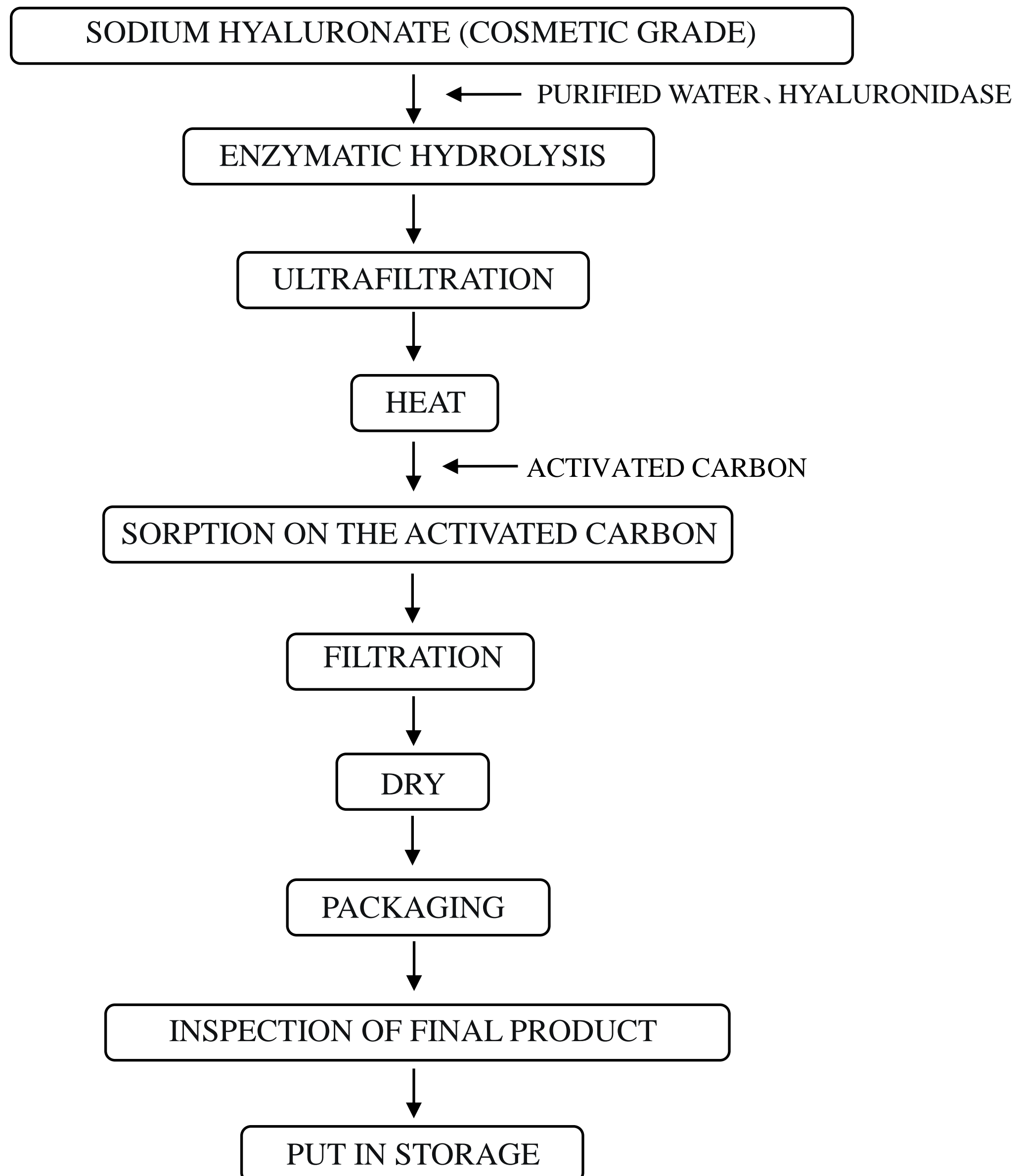
The reagents used are low molecular weight sodium hyaluronate, hyaluronidase, hydrochloric acid. Among them, hyaluronidase is prepared by *Bacillus* which specific activity is ≥500 U/mg. The amount of hyaluronidase that is required to produce microHA is 6.0×10^4 ~8.0×10^4 U/kg. The protein introduced into this product by hyaluronidase is less than 0.16 mg/kg.



MANUFACTURING TECHNIQUE FLOW CHART

INCI Name: Hydrolyzed Sodium Hyaluronate

Molecular Weight: $(C_{14}H_{20}NO_{11}Na)_n$ Mw<1K Da (Muti-angle laser light-scattering method, GPC-MALLS method)



This products (INCI: Hydrolyzed Sodium Hyaluronate) is a low molecular weight hydrolyzed sodium hyaluronate (MW＜1KDa）prepared by enzymolysis of Sodium Hyaluronate. Its substrate is cosmetics grade Sodium Hyaluronate (MW＞1000KDa). In the process of enzymatic hydrolysis, purified water and hyaluronase are added. When the enzymatic hydrolysis reaction is completed, the enzymolysis solution undergoes purification such as ultrafiltration to remove the hyaluronidase. The ultrafiltration filter is heated to denaturate and deactivate the remaining hyaluronidase. Activated carbon is added to the enzymolysis solution to absorb the denaturated hyaluronidase, and the residual hyaluronidase is removed by the removal of activated carbon through multistage filtration. The resulting filtrate is dried, yielding the final product.

The substrate Sodium Hyaluronate manufactured was reported to be manufactured via fermentation using the bacterial strain *Streptococcus equi* subsp. *zooepidemicus*.[1] This process begins with the preparation of a seed broth prepared from seed culture, which is transferred to a fermenter containing sterilized fermentation medium. Following fermentation, the broth is mixed with ethanol. The crude Sodium Hyaluronate precipitate is dissolved in water and filtered to remove impurities and inactivated fragments. The resulting filtrate is precipitated, dehydrated, and dried, yielding the final product.

1. United States Pharmacopeial Convention. Food Chemicals Codex *9th ed*. In: (USP) USP, ed. Rockville, MD 2014. Accessed June 6, 2022

Distributed for Comment Only -- Do Not Cite or Quote

Hydrolyzed Sodium Hyaluronate toxicity summary

INCI Name: Hydrolyzed Sodium Hyaluronate,

The product and Molecular Weight:

- First product, 5 K-10K Da (intrinsic viscosity method)
- Second product，1 K-5 K Da (intrinsic viscosity method)
- Third product, Mw<1K Da (Muti-angle laser light-scattering method, GPC-MALLS method[1])

1 Hydrolyzed Sodium Hyaluronate (5 K-10K Da)

1.1 Ames (Dec. 2016)

Hydrolyzed Sodium Hyaluronate (5 K-10K Da) was considered NOT mutagenic in several Ames assays when tested at up to 5 mg/plate, with and without metabolic activation (assays performed in S. typhimurium strands TA97a, TA98, TA100 and TA102).

1.2 Human Repeated Insult Patch Test (HRIPT, Oct. 2016)

Based on the test population of 55 subjects and under the conditions of HRIPT study, the investigational product identified as Hydrolyzed Sodium Hyaluronate (0.5% miniHA™) was found to be NON-PRIMARY and NON-PRIMARY SENSITISER (Three were no observations of erythema or related effects for any of the test participants).

1.3 Cytotoxicity test

According to method of the cytotoxicity test (GB/T16886.5, GB/T14233.2), this test was carried out. Based the Fibroblast L929 model and the cytotoxicity grades of U.S. Pharmacopoeia (toxic=relative growth rate, RGR<50%), miniHA™ at concentration of 3% (w/v) could be considered as non-toxic whose RGR is more than 70%.

1.4 Human Skin closed Patch test

30 healthy persons were as volunteers according to the method of China Hygienic Standard for Cosmetics (2007). As the results, all the cutaneous reactions of the 30 volunteers are negative (Grade 0) with 1.0% miniHA™.

2. Hydrolyzed Sodium Hyaluronate (1 K-5 K Da)

2.1 Human Skin closed patch test (Sensitive skin model, April, 2020)

With reference to China Safety and Techinical Standards for Cosmetics, Ministry of Health, 2015 edition. 32 sensitive skin subjects was selected. All the subjects were negative (Grade 0) after removal of the patch for 24h and 48h with 0.5% Hydrolyzed Sodium Hyaluronate.

2.2 Cytotoxicity test

Distributed for Comment Only -- Do Not Cite or Quote

According to method of the cytotoxicity test (GB/T16886.5, GB/T14233.2), this test was carried out. Based the Human keratinocytes (HaCaT) model and the cytotoxicity grades of U.S. Pharmacopoeia (toxic=relative growth rate, RGR<50%), microHA™ at concentration of 1% (w/v) could be considered as non-toxic whose RGR is more than 98%.

2.3 Human Skin closed Patch test
30 healthy persons were as volunteers according to the method of China Safety and Technical standards for Cosmetics (2015). As the results, all the cutaneous reactions of the 30 volunteers are negative (Grade 0) with 0.5% and 2% microHA™.

3. Hydrolyzed Sodium Hyaluronate (Mw<1K Da)
3.1 Ames (Sept. 2022)
Hydrolyzed Sodium Hyaluronate was determined to be non-genotoxic in several Ames assays when tested at up to 5 mg/plate, with and without metabolic activation (assays performed in S. typhimurium strands TA97a, TA98, TA100, TA102, and TA1535).

2.2 Eye irritation (Sept. 2021)
Based on "the Chorioallantoic Membrane Vascular Assay (CAMVA)", it is verified Hydrolyzed Sodium Hyaluronate is predicted as non-irritant/ slightly irritant because of the RC_{50}=11%.

2.3 Phototoxicity test (Sept. 2021)
Accroding to the OECD TG432 *In vitro* 3T3 NRU Phototoxicity test, it is predicted the PIF value of "Hydrolyzed Sodium Hyaluronate (up to 128mg/mL)" was less than 1.0, thus the Hydrolyzed Sodium Hyaluronate is no phototoxicity.

2.4 Skin Irritation test (Sept. 2021)
Based on the Reconstructed Human Epidermis (Epikutis®, OECD TG439) model, the "Hydrolyzed Sodium Hyaluronate without attenuation" belong to No Category (UN GHS classification) with a 97.31% tissue viability, further the tissue viability of Hydrolyzed Sodium Hyaluronate was 96.97% when the mass percentage was 1%.

2.5 Human Skin closed Patch test (Aug. 2022)
30 healthy persons were as volunteers according to the method of China Safety and Technical standards for Cosmetics (2015). As the results, all the cutaneous reactions of the 30 volunteers are negative (Grade 0) with 1% Hydrolyzed Sodium Hyaluronate.

[1] Ying Han, Dejie Li, etc. Impact of Refractive Index Increment on the Determination of Molecular Weight of Hyaluronic Acid by Muti-Angle Laser Light-Scattering Technique, *Scientific Reports* (2020) 10:1858 | https://doi.org/10.1038/s41598-020-58992-7.

Distributed for Comment Only -- Do Not Cite or Quote

Composition and impurities data of Hydrolyzed Hyaluronic Acid

Product Name: Hydrolyzed Hyaluronic Acid

INCI Name: Hydrolyzed Hyaluronic Acid

Molecular Weight: 37K~56K Da (intrinsic viscosity method)

Items	Results	Method
Assay	≥90%	Carbazole method
Bacterial Endotoxins	＜0.5EU/mg	Gel method
Protein	＜0.05%	Bradford method
Chlorides	＜0.5%	Colorimetric method
Nucleic acids	The absorbance at 260nm< 0.5	European Pharmacopoeia
Total metals	<20 ppm	ChP2020, 0821, second method
Arsenic	<2 ppm	ChP2020, 0822, first method

Distributed for Comment Only -- Do Not Cite or Quote

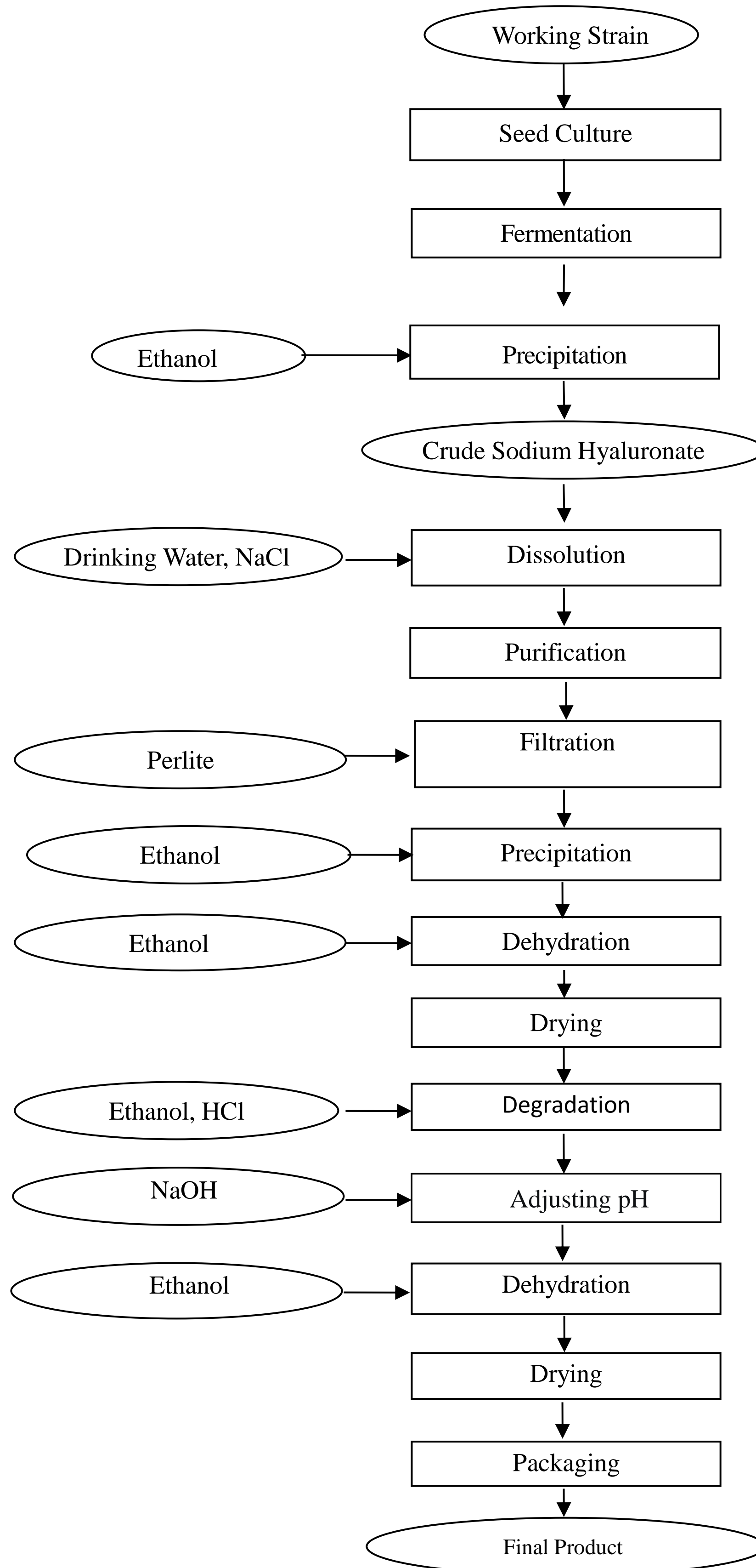
Manufacturing Process of Hydrolyzed Hyaluronic Acid

Product Name : Hydrolyzed Hyaluronic Acid
INCI Name: Hydrolyzed Hyaluronic Acid
Molecular Weight: $(C_{14}H_{21}NO_{11})n$ (379.3)n Mw: 37K~56K Da

1. Description of the Manufacturing Process

Sodium Hyaluronate manufactured for food use was reported to be manufactured via fermentation using the bacterial strain *Streptococcus equi* subsp. *zooepidemicus*. This process begins with the preparation of a seed broth prepared from seed culture, which is transferred to a fermenter containing sterilized fermentation medium. Following fermentation, the broth is mixed with ethanol. The crude Sodium Hyaluronate precipitate is dissolved in water and filtered to remove impurities and inactivated fragments. The resulting filtrate is precipitated, dehydrated, and dried, yielding the HA product. This product “Hydrolyzed Hyaluronic Acid” is mixed with ethanol and HCl. When Molecular weight of HA is degraded to the set point, adjust pH by addition of NaOH solution. At last, it is dehydrated, and dried, yielding the Hydrolyzed Hyaluronic Acid.

2. Flow Chart: (Next Page)


Working Strain
Seed Culture
Fermentation
Ethanol
Precipitation
Crude Sodium Hyaluronate
Drinking Water, NaCl
Dissolution
Purification
Perlite
Filtration
Ethanol
Precipitation
Ethanol
Dehydration
Drying
Ethanol, HCl
Degradation
NaOH
Adjusting pH
Ethanol
Dehydration
Drying
Packaging
Final Product

Memorandum

TO: Bart Heldreth, Ph.D.
Executive Director - Cosmetic Ingredient Review

FROM: Carol Eisenmann, Ph.D.
Personal Care Products Council

DATE: January 3, 2023

SUBJECT: Sodium Hyaluronate

Spera Nexus, Inc. 2022. Statement composition Sodium Hyaluronate.

Spera Nexus, Inc. 2022. Flow chart hyaluronate IW series (Sodium Hyaluronate).

Spera Nexus, Inc. 2022. Instruction of hyaluronate IW series (Sodium Hyaluronate).

Spera Nexus, Inc. 2022. Safety test of hyaluronate IW series (Sodium Hyaluronate).

Distributed for Comment Only -- Do Not Cite or Quote

SPERA NEXUS, Inc. 8-2, NIHONBASHI HONCHO 4-CHOME, CHUO-KU TOKYO 103-0023 JAPAN

Statement

SPERA NEXUS, Inc. hereby states that:

To our knowledge, the composition and impurities of Hyaluronate IW200, Hyaluronate IW120, and Hyaluronate IW90 are as follows,

Composition /Impurities	Content
Sodium Hyaluronate	100%
Residual Solvents Ethanol	< 5000ppm
Total Heavy Metals	< 20ppm
As	< 2 ppm
Protein	< 0.1%

SPERA NEXUS, Inc.
Shizuoka Plant

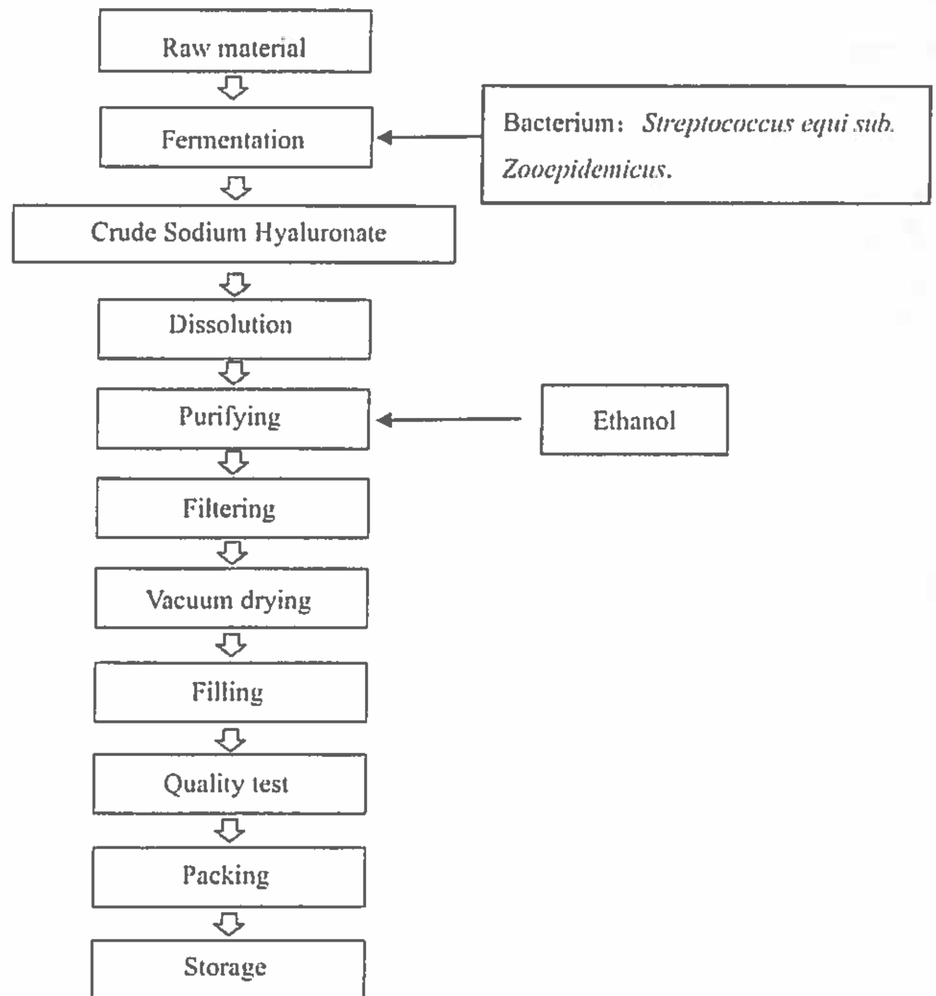
Yusuke Ikeda
Quality Assurance Manager

Document number : N-QU-MP-1510-13-84
Prepared on Nov. 18, 2022

Distributed for Comment Only -- Do Not Cite or Quote

SPERA NEXUS, Inc. 8-2, NIHONBASHI HONCHO 4-CHOME, CHUO-KU TOKYO 103-0023 JAPAN

Flow Chart of Hyaluronate IW series



SPERA NEXUS, Inc.
Shizuoka Plant

Yusuke Ikeda
Quality Assurance Manager

Document number : N-QU-MP-1510-13-85
Prepared on Nov. 18, 2022

Distributed for Comment Only -- Do Not Cite or Quote

SPERA NEXUS, Inc. 8-2, NIHONBASHI HONCHO 4-CHOME, CHUO-KU TOKYO 103-0023 JAPAN

Instruction of Hyaluronate IW series

INCI Name : Sodium Hyaluronate

Description	Produced by fermentation technology
Functions	Moisturizing, lubricating, thickening
Application	Skin care, hair care, oral care products, etc.
Usage	Soluble in water, the dissolution process is slow relatively because of high molecular weight and high viscosity. 1. Dissolving directly. Prepare water or aqueous phase solution, add HA slowly with stirring continuously till completely dissolved. 2. Dissolving after pre-treating. Add HA into alcohols (such as glycerin, butanediol, etc.) and stir till dispersing evenly, add water and stir till completely dissolved.
Recommend dosage	0.1%-0.5%
Recommend temperature	Dissolution temperature should be less than 80°C.
pH stability or others	5.0-8.0
Compatibilities	Solvents: glycerinum, butanediol, etc. Surfactants: non-ionic type, ampholytic type Preservatives: phenoxyethanol, parabens, polyhydric alcohols (such as hexanediol), etc.
Incompatibilities	Cationic compounds, such as cationic emulgator, cationic preservatives, etc
Store condition	Kept airtight, 2~8℃, three years.

SPERA NEXUS, Inc.
Shizuoka Plant

Yusuke Ikeda
Quality Assurance Manager

Document number : N-QU-MP-1510-13-86
Prepared on Nov. 18, 2022

Distributed for Comment Only -- Do Not Cite or Quote

Safety test of Hyaluronate IW series

Sodium-Hyaluronate

Item	Test method	Test concentration	Result
Mutagenicity	AMES Bacterial Reverse Mutation : OECD TG471	<-S9mix> *S. typhimurium* TA98, TA100, TA1535, TA1537, *E. coli* WP2 *uvrA* 5000, 2500, 1250, 625, 313 μ g/plate <+S9mix> *S. typhimurium* TA98, TA100, TA1535, TA1537 1250, 625, 313, 156, 78.1, 39.1 μ g/plate *E. coli* WP2 *uvrA* 5000, 2500, 1250, 625, 313 μ g/plate	Negative
Repeated Insult Patch Test	50 people	0.2%	Negative

SPERA NEXUS, Inc.
Shizuoka Plant

Yusuke Ikeda
Quality Assurance Manager

Document number : N-QU-MP-1510-13-87
Prepared on Nov. 18, 2022
Revision date Dec. 28,2022