

Clinical Evaluation of a Multi-Modal Facial Serum That Addresses Hyaluronic Acid Levels in Skin

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ABSTRACT

Background: Hyaluronic acid (HA), the major glycosaminoglycan present in the human skin, is a key contributor to water retention and mechanical support in skin. The level, size, and functionality of cutaneous HA are known to diminish with age. Topical treatments designed to increase the HA content of skin have been met with limited success. The purpose of this study was to evaluate the tolerance and efficacy of a multi-modal facial serum containing HA, Proxylane (C-Xyloside), purple rice extract, and dipotassium glycyrrhizate in addressing HA levels in skin.

Methods: A 12-week, single center, clinical study was conducted on 59 women with mild to moderate photodamage. Clinical grading to assess the efficacy and tolerability was conducted on the face at baseline and at weeks 4, 8, and 12. Bioinstrumentation measurements were taken, including corneometer, tewameter, ultrasound, and standardized digital imaging. A randomized subset of 20 subjects from the study population had 3 mm punch biopsies collected for quantitative RT-PCR analysis from 2 sites on the face at baseline and week 12. Additionally, a 4-week, single center, clinical study was conducted on the photodamaged forearms of 12 subjects. At both baseline and week 4, a 4 mm punch biopsy was obtained from the subjects' randomized forearms. Biopsy samples were subjected to immunohistochemical staining and analysis of HA content.

Results: Statistically-significant improvements in all facial skin attributes (weeks 4, 8, and 12), stratum corneum hydration (week 12), and transepidermal water loss (week 12) were observed. Tolerability was excellent, with no increases in irritation parameters noted. A significant increase of HA content in skin after 4 weeks of treatment was observed. By PCR analysis, there was a significant increase in hyaluronan synthase 2, as well as a significant increase in collagen type 1a1 after 12 weeks of application.

Conclusion: The findings suggest that this novel topical facial serum is capable of stimulating HA and skin extracellular matrix components, as well as improving skin hydration and skin quality in women with mild to moderate photodamage.

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INTRODUCTION

The earliest work on skin was devoted predominantly to the discreet cellular compartments of skin: the epidermis, dermis, and underlying subcutis. There has been an emerging appreciation for the materials that lie between cells -- the extracellular matrix components -- which have major instructive roles for cellular activities.[3]The major component of skin extracellular matrix (ECM) is the glycosaminoglycan (GAG), hyaluronic acid (HA). Matrix hyaluronan has been implicated in several skin epidermal functions. Since collagen constitutes the main structural element of the ECM — providing tensile strength, regulating cell adhesion, supporting chemotaxis and migration, and directing tissue development[18] — HA serves a critical role in filling space around collagen fibrils, maintaining the extracellular space, and preserving tissue hydration.[1]

The HA content of the epidermis and the dermis helps regulate the cutaneous moisture levels and barrier function of human skin.[1] Hyaluronic acid also plays an important role in skin aging. A decrease in HA levels, reduction in HA size, and loss of HA functionality all directly impact the biomechanical properties of aging skin, resulting in loss of elasticity, firmness, and overall plumpness and volume. In young skin, large amounts of HA are found at the periphery and intersections of collagen and elastin fibers.[2] On the other hand, upon chronic exposure to ultraviolet (UV) radiation, GAGs appear to be deposited on elastotic material and diffusely associated with UV-damaged collagen fibers.[1] Photoaged skin is characterized by reduced HA and increased levels of chondroitin sulfate proteoglycans.[6] Matrix metalloproteinases (MMPs), the main collagen degrading enzymes, are also over-expressed in photoaged skin.[7]

Due to the importance of HA in maintaining skin plumpness and hydration, and its decrease in intrinsically and photoaged skin, a goal in skincare has been to restore lost HA. While a multitude of anti-aging strategies have been developed based on the concept of replenishing the HA content in skin, there is limited evidence to demonstrate a meaningful influence on endogenous HA levels in human skin. In this study we evaluated, in vivo, a unique

topical serum containing a combination of ingredients that has been demonstrated in vitro to stimulate HA synthesis or inhibit its degradation.[8-10] Our results demonstrate that the topical serum is capable of stimulating HA synthesis in vivo, increasing the HA content of skin, and improving both skin hydration and skin appearance in women with mild to moderate photodamage.

MATERIALS AND METHODS

Two human clinical studies were performed. Both studies were approved by an independent institutional review board, and were conducted in accordance with the ICH guidelines. Written informed consent was obtained from all study subjects before enrollment.

The product evaluated in these 2 clinical studies was a topical hydroglycolic serum (H.A. Intensifier, SkinCeuticals Inc., New York, NY) containing the following ingredients: a mixture of fragmented, whole, and encapsulated HA, Proxylane (C-Xyloside, a patented GAG-inducing saccharide derivative), purple rice extract, and dipotassium glycyrrhizate. In earlier in vitro studies, these ingredients have been demonstrated to have a positive effect on GAG and HA synthesis and/or to prevent their degradation.

Forearm Study

Study Design

Twelve healthy, post-menopausal females (absence of menstruation for at least one year), between the ages of 45 and 65 years, with clinical evidence of photodamage on the assigned forearm, were enrolled in a 4-week clinical study to evaluate the efficacy of the topical formulation (H.A. Intensifier, SkinCeuticals Inc., New York, NY). At baseline, a 4 mm punch biopsy was obtained from the forearm, the test product was applied to the subjects' forearm, and subjects were instructed to re-apply the test product on the forearm once daily for 4 weeks. A second 4 mm punch biopsy was obtained 24 hours after the final application of the topical serum.

Biopsy Analysis

Skin biopsies were embedded in optimal cutting temperature (OCT) compound and snap frozen in liquid nitrogen. Two 50 mm sections of each skin biopsy were extracted and HA was quantified by commercially available enzyme-linked immunosorbant assay (ELISA) kit. Total HA in the sample was determined by reference to known standards and normalized to sample volume. HA staining was performed on 10 micron sections of skin biopsies, using biotinylated HA binding protein (HABP) and streptaviden-conjugated horse radish peroxidase. Sections pretreated with hyaluronidase were used as a control for specificity. Following staining, sections were digitally photographed.

Facial Clinical Study

Study Design

Fifty-nine healthy female subjects aged 42 to 60 were enrolled in a 12-week clinical study to evaluate the efficacy and tolerability of the facial serum (H.A. Intensifier, SkinCeuticals Inc., New York, NY). The subjects were predominantly Caucasian (66.1%) with Fitzpatrick skin type II (55.9%). The remainder of the subject population consisted of African American, Latino, Asian, and other multiracial ethnicities, with Fitzpatrick types I, III, IV, or V. Thirty of the subjects had self-perceived sensitive skin. The inclusion criteria for the study included a clinical evaluator score of 3 to 6 on a 10-point visual analog scale (0 to 9) for the following characteristics: mild to moderate facial sagging, loss of firmness, rough skin texture, and the presence of fine lines and wrinkles in the crow's foot area, nasolabial folds, and marionette lines.

At the screening visit, subjects who met all inclusion and exclusion criteria were enrolled in the study. Subjects were provided with a facial cleanser (Gentle Cleanser, SkinCeuticals, Inc.), and a broad spectrum sunscreen (Ultimate UV Defense SPF 30, SkinCeuticals, Inc.). They were instructed to wash their faces twice daily at home with the cleanser, followed by application of the sunscreen in the morning, prior to sun exposure.

Study subjects returned 7 days later for their baseline visit. They spent at least 15 minutes in an environmentally-controlled room prior to any clinical assessments of facial skin attributes, tolerability, or instrumentation measurements. Subjects were dispensed a pre-weighed unit of test product (H.A. Intensifier, SkinCeuticals Inc., New York, NY) after the baseline visit was complete, and instructed to apply it to a cleansed face and neck morning and evening.

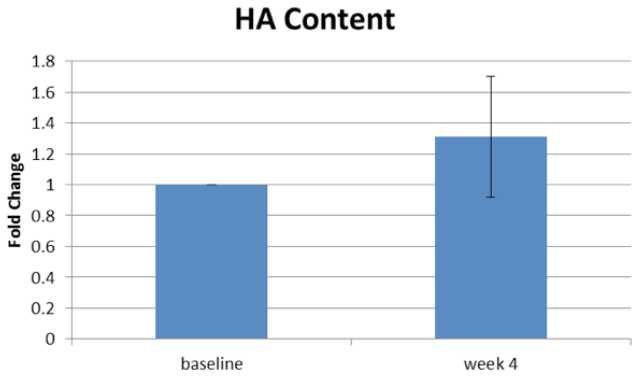
Assessment of Facial Skin Attributes

Facial skin attribute evaluations were conducted at pre-treatment baseline and weeks 4, 8, and 12. Facial skin attribute parameters were clinically graded on a modified Griffith's scale where 0 = none (best possible condition), 1-3 = mild, 4-6 = moderate, and 7-9 = severe (worst possible condition). Efficacy measures included visual evaluation and grading by the investigator of overall skin appearance/quality, radiance, skin density, elasticity, sagginess and plumpness, evenness of skin tone, fine lines/wrinkles in the crow's foot, nasolabial fold, and marionette line areas, and crepiness of the neck. Tactile evaluations included skin firmness on the face and skin texture on the face and neck.

Tolerability Assessment

Tolerability assessments were conducted at pre-treatment baseline and weeks 4, 8, and 12. Tolerability parameters were graded on a scale where 0 = none, 1 = mild, 2 = moderate, and 3 = severe. Cutaneous tolerability was evaluated by assessing subjective and objective irritation of the treatment area. Clinically-graded objective irritation parameters included erythema, dryness, scaling, and edema. In addition, subjects self-assessed burning, stinging, itching, tightness, and tingling.

FIGURE 1. Fold change in hyaluronic acid content in human forearm skin at baseline and week 4 of topical test product usage.



HA content of the Baseline and Week 4 biopsies were performed as described in the Methods section. The values from the 12 biopsies were quantified and statistical analysis was performed.

Bioinstrumentation

At pre-treatment baseline and week 12, each subject had measurements of transepidermal water loss (TEWL) and skin conductance on either the left or right side of the face, as determined by computer-generated randomization. TEWL measurements were taken in duplicate from the center of the cheek, using a Tewameter 300® (Courage-Khazaka, Köln, Germany). Conductance measurements, reflecting stratum corneum hydration, were taken in triplicate from the center of the cheek, using a Corneometer® CM 825 (Courage-Khazaka, Köln, Germany). To ensure standardization of sampling at baseline and week 12, the site of testing on the cheek was a constant distance in millimeters from the angle of the mouth. TEWL data were expressed in g/m^2/h and conductance data were expressed in arbitrary units (AU).

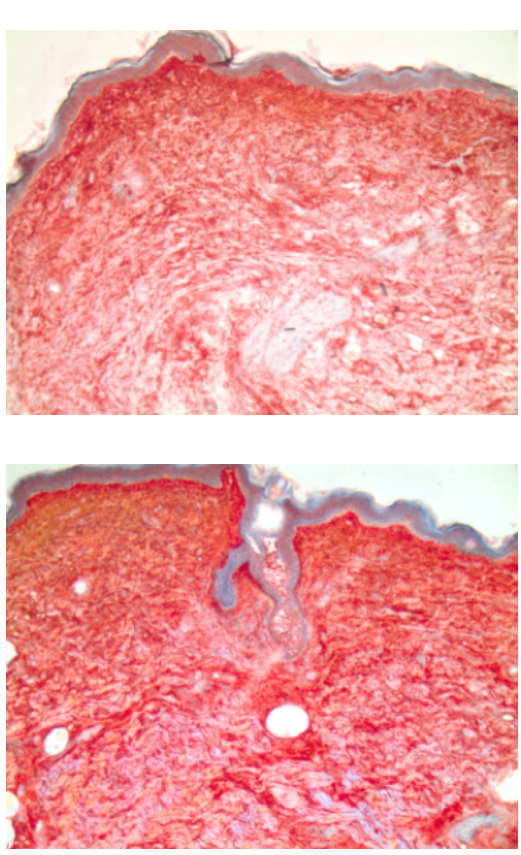
Ultrasound Measurement

At pre-treatment baseline and week 12 each subject had measurements of skin density on either the right or left side of the face according to a pre-determined randomization. A single ultrasound measurement was taken on the center of each subject's crow's foot area, using a 50 MHz ultrasonic transducer interfaced to a DUB 6100 OEM System (Taberna, Pro Medicum, Germany). Measurements were taken with the probe oriented perpendicular to the body axis while the subject lay supine on a padded patient table.

Standardized Digital Imaging Capture

Subjects had standardized digital photographs taken at the pre-treatment baseline, and at week 4, week 8, and week 12 time points using the VISIA-CR (Canfield Scientific, Fairfield, NJ) imaging system. Full frontal, left, and right profile views of the face were obtained, under visible (Standard 1, Standard 2, Standard 3), crossed-polarized, and parallel-polarized light.

FIGURE 2. Hyaluronic acid localization in human forearm skin biopsy samples at baseline (A) and week 4 (B) of topical test product usage.



Ten micron sections were stained using biotinylated HABP and streptavidin-conjugated horse radish peroxidase. Representative images of baseline and week 4 biopsy samples.

Raking Light Imaging Analysis

Raking light optical profilometry was applied directly to the standardized digital photographs, taken under raking light conditions, captured at pre-treatment baseline and week 12. The high-resolution digital images were analyzed using Image Pro software. A high-throughput method, Stephens Wrinkle Imaging using Raking Light (SWIRL), was used to identify wrinkles in the crow's feet area.[17]

Biopsy Analysis

In this study, twenty 3 mm facial punch biopsies were collected at baseline and week 12 following twice-daily application of the serum to the face. Ten biopsies were used for PCR analysis of HAS-1 and -2, hyaluronidase-1, CD44, and type I collagen, and 9 were used for semi-quantitative staining for HA content. For assessment of gene expression, total RNA was extracted from biopsies, and gene expression was measured using quantitative RT-PCR. Briefly, skin biopsies were immediately frozen in liquid nitrogen and stored at -80°C for further RNA extraction. Total RNA was extracted according to the manufacturer's instructions using

TABLE 1.

Mean Statistical Changes in Clinical Scores for Facial Skin Attributes from Baseline to Three Study Time Points

Skin Attribute	Mean Change from Baseline (±SD)		
	Week 4	Week 8	Week 12
Firmness	**-0.15±0.23**	**-0.34±0.27**	**-0.53±0.26**
Density	**-0.14±0.22**	**-0.34±0.24**	**-0.54±0.30**
Plumpness	**-0.48±0.32**	**-0.81±0.36**	**-1.12±0.41**
Sagginess	-0.07±0.17	**-0.29±0.25**	**-0.45±0.20**
Radiance	**-0.26±0.25**	**-0.40±0.22**	**-0.57±0.29**
Texture (tactile)	**-0.35±0.27**	**-0.64±0.31**	**-0.83±0.38**
Skin tone evenness	-0.10±0.24	**-0.21±0.30**	**-0.34±0.38**
Crow's feet wrinkles	**-0.21±0.27**	**-0.43±0.29**	**-0.64±0.37**
Nasolabial fold wrinkles	-0.05±0.15	**-0.25±0.25**	**-0.39±0.29**
Marionette line wrinkles	-0.08±0.18	**-0.28±0.27**	**-0.45±0.36**
Overall appearance of skin	**-0.14±0.23**	**-0.32±0.24**	**-0.53±0.31**

SD, standard deviation.
A more negative mean change indicates a greater improvement. All values are statistically significant with $P<0.05$. Values in boldface are statistically significant with $P<0.001$.

RNeasy fibrous tissue kit (Qiagen). RNA concentration was determined using Nanodrop 2000 spectrophotometer. cDNA was generated according to manufacturer's instructions using iScript cDNA synthesis kit (BioRad). Gene expression was studied using Taqman quantitative real time PCR, using 7300 RealTime System (Applied Biosystems). Data were normalized into the expression of the GAPDH house keeping gene. The analyses were carried out using SPSS 17.0 and SAS 9.2 statistical softwares.

HA staining was performed on 10 micron sections of skin biopsies, using biotinylated HABP and FITC-conjugated horseradish peroxidase. Following staining, sections were digitally photographed and the fluorescent stain was visually quantified on a scale of 1 (lowest) to 4 (highest).

Statistical Analysis

All subjects who completed the facial or forearm skin studies were included in the statistical analysis. Data were tested for normal distribution with the Shapiro-Wilk test, using a cutoff value of $P<0.01$ for efficacy and tolerability data. Efficacy and tolerability of the test formulation were evaluated by comparing the post-treatment data with the pre-treatment baseline data for each attribute. The significance of changes was determined using either a paired t-test for normally distributed data, or a Wilcoxon signed rank test for non-normal data. The cutoff value for significance was $P<0.05$. Instrumental data were evaluated similarly. The mean percent changes from pre-treatment baseline were reported at each time point for each attribute.

RESULTS

Forearm Study

Biopsy Analysis

The 4-week clinical study on 12 subjects on the forearm demonstrated a significant increase of HA content ($P<0.05$) in the skin after treatment compared with baseline. A mean increase in HA of 31% was observed (Figure 1). Figure 2 shows a representative image of HA staining in skin sections before and after 4 weeks of treatment. The visual increase in HA staining correlated with the 31% increase in HA content demonstrated in Figure 1 using the ELISA method.

Facial Clinical Study

Skin Attribute Evaluation

All clinically-graded skin attribute parameters showed a statistically significant decrease (improvement) in scores at weeks 4, 8, and 12 compared with baseline (Table 1). Significant improvements were observed in skin firmness (tactile), radiance, skin density, skin plumpness, skin sagginess, skin texture, skin tone

TABLE 2.

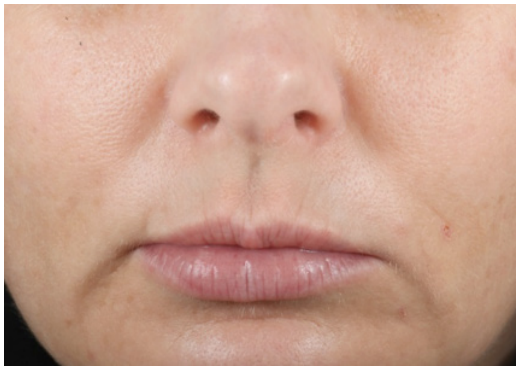
Bio Instrumentation Measures of Skin Parameters

Instrument	Baseline	Week 12	Mean Change	Mean Change (%)	*P*-Value
Corneometer	48.1±13	57.8±12	9.65	20	<0.001 (s)
Tewameter	17.9±5.5	16.4±4.2	-1.42	-7.9	0.017 (s)
Density (ultrasound)	44.5±8.8	46.1±8.4	1.54	3.5	0.012 (s)
Wrinkle length	90.9	83.1	-7.72	-8.5	0.108 (ns)
Wrinkle area	44.5	40.5	-3.9	-8.8	0.024 (s)
Wrinkle depth	521	507	-13.8	-2.6	0.813 (ns)

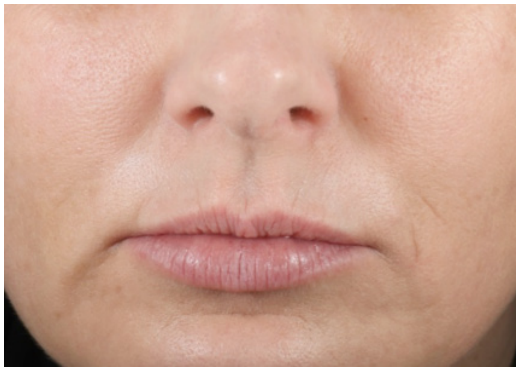
(s), statistical significance.
Lower tewameter readings and higher corneometer readings indicate greater improvements in skin moisturization and skin hydration, respectively.
Higher ultrasound readings indicate greater improvements in skin density.
Lower raking light image readings indicate an improvement in crow's feet wrinkles.

FIGURE 3. Standardized digital images of a study subject at pre-treatment baseline (A) and week 12 (B).

(A)



(B)



Standardized digital images of study subjects at pre-treatment baseline and week 12, representative of average results for the overall study panel. Improvements in marionette line wrinkles and nasolabial fold were apparent.

evenness, and overall skin appearance on the global face, and crepiness and skin texture on the neck. In addition, significant improvements were observed in fine lines and wrinkles in the crow's feet, nasolabial fold, and marionette line areas of the face. Improvements were apparent as early as week 4 and were maintained through the study conclusion.

Tolerability

Local cutaneous tolerability was excellent (data not shown). Use of the facial serum did not produce any significant worsening of any subjective or objective measures of irritation on the face or neck at any time point between baseline and week 12. Dryness and erythema were reduced significantly at week 8. No scaling, edema, or burning were observed with application of the serum. Subjective evaluations of stinging and tightness decreased by week 4, and itching and burning were not experienced.

Bioinstrumentation

Results of bioinstrumental measurements also showed significant improvements compared with baseline (Table 2). A mean 20% increase in stratum corneum hydration was observed by corneometry at week 12 (P<0.001). Use of the facial serum resulted in a significant decrease (-7.9%) in TEWL at week 12 (P=0.017). There was a statistically-significant improvement (3.5%) in skin density at the center of the crow's foot area by ultrasound (P=0.012) at week 12.

Standardized Digital Imaging

Standardized digital images of a selected study subject at pre-treatment baseline (A) and week 12 (B) (Figure 3) demonstrate improvement in marionette line wrinkles and nasolabial fold wrinkles, which were representative of average results for the overall study panel.

Raking Light Imaging

Results of raking light image analysis showed a statistically significant improvement (P=0.024) in values for wrinkle area (square mm; Table 2). While there was also improvement in wrinkle length, depth, and width, this did not reach statistical significance.

Biopsy Analysis

qPCR data on skin RNA expression showed significant increases in HAS-2 and collagen 1a1 with a mean percent change from baseline of 70.5% (P<0.0008) and 50.1% (P<0.0245), respectively (Table 3). There was no significant change in HAS-1, hyaluronidase-1, or CD44 gene expression. HA staining results showed a semi-quantitative increase in HA content by week 12 compared with baseline (Table 4, Figure 4).

DISCUSSION

There is growing appreciation for the role of the extracellular matrix and its constituents—particularly hyaluronan—in the

TABLE 3.

qPCR Analysis of Gene Expression for Markers at Baseline (Pre-Treatment) and Week 12

Biomarker	Mean (±SD)		Mean Change from Baseline (±SD)	Mean Change (%) from Baseline	*P*-Value
	Baseline	Week 12			
HAS-2	0.92±0.38	1.56±1.28	0.65±1.04	70.50%	0.0008
Col1a1	0.61±0.35	0.92±0.88	0.31±0.79	50.10%	0.0245

SD, standard deviation.
qPCR was carried out as described in the Methods section.

TABLE 4.

Hyaluronic Acid Staining Data: Statistical Analysis of the Semiquantitative Data				
	Mean (±SD)		Mean Change from Baseline (±SD)	*P*-Value
	Baseline	Week 12		
Hyaluronic acid	2±0.86	2.77±0.97	0.77±0.25	<0.0001

SD, standard deviation.
Biopsies were stained using biotin-labeled HABP and fluorescein isothiocyanate (FITC) streptavidin conjugate. Green color indicates HABP conjugated to streptavidin and blue color indicates DAPI counterstained cellular nuclei. Following staining, sections were digitally photographed and the fluorescence stain was visually quantified on a scale of 1 (lowest) to 4 (highest) and statistical analysis of the semi-quantitative data was performed.

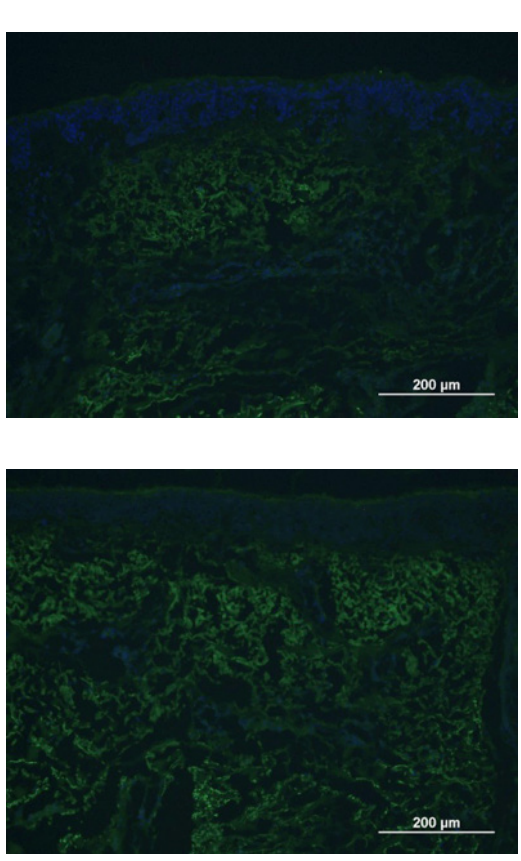
maintenance of the resilience, radiance, and hydration characteristic of youthful or undamaged skin. Although topically applied HA can provide surface moisturization, it has been widely suspected that available formulations do not effectively penetrate to the viable epidermis and dermis. In principle, the net HA content in skin can also be increased by adjusting the natural HA homeostasis. Accordingly, a serum was developed for the purpose of improving the skin's HA levels by stimulating endogenous HA synthesis, reducing HA breakdown, and providing surface hydration.

The components in the serum were demonstrated to have HA stimulation effects in previous in vitro studies. The composition of the serum contained a combination of fragmented, whole, and encapsulated HA, which has been demonstrated to have immediate and sustained hydration;[8] Proxylane, which has been shown to stimulate GAG synthesis and increase levels of the HA receptor CD44;[11-15] and purple rice extract and dipotassium glycyrrhizate, derived from licorice, which have been demonstrated to inhibit hyaluronidase activity.[10,16]

The clinical improvements observed in this study correlated with the biochemical observation that the serum increased the gene expression of HAS-2 and collagen-1 and demonstrated an overall increase in HA content of facial skin. A previous clinical study using HA fragments have demonstrated significant improvements in skin hydration, elasticity, and wrinkle reduction. The authors have speculated that the efficacy of the HA fragments may be due to increased penetration of the fragments.[8] However, this study did not evaluate or quantify cutaneous HA content after the topical application. There is also in vitro evidence that HA fragments may have other modulatory effects on skin, such as stimulating CD44, signaling pathways in keratinocytes,[9] and immunomodulatory action on macrophages in skin following skin injuries;[18] however, this has not been validated in vivo.

The beneficial role C-Xyloside plays in skin homeostasis/regeneration, keratinocyte GAG synthesis, and cell migration has also been demonstrated using a number of in vitro models.[11,14] In addition to these HA synthesis modulators, the tested serum also contained 2 ingredients known to inhibit cellular HA and GAG breakdown. Purple rice extract and glycyrrhizin have been demonstrated to be potent inhibitors of hyaluronidases.[10,16] The results from the 2 clinical studies demonstrate that a topical composition containing optimized amounts of these ingredients can increase the HA content of human skin. Biochemical data suggest that the clinical efficacy of the facial serum may be mediated by increased HA content.

FIGURE 4. Hyaluronic acid localization in human facial skin biopsy samples at baseline (A) and week 4 (B) of topical test product usage.



Biopsies were stained using biotin-labeled HABP and fluorescein isothiocyanate streptavidin conjugate. Green color indicates HABP conjugated to streptavidin and blue color indicates DAPI counterstained cellular nuclei.

The data presented here demonstrate the ability of the facial serum to address an important aspect of both photoaging and intrinsic aging—the loss of HA in aging skin. This improvement is mediated by biological mechanisms leading to an increase in HA synthase and collagen 1a1, and an actual increase in the HA content of facial skin.

DISCLOSURES

Susana Raab, Margarita Yatskayer and Stephen Lynch are employed by L'Oreal Research and Innovation. Christian Oresajo

was employed by L'Oreal Research and Innovation at the time of this work. Megan Manco is employed by SkinCeuticals.

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