Pilot Comparative Study of the Topical Action of a Novel, Crosslinked Resilient Hyaluronic Acid on Skin Hydration and Barrier Function in a Dynamic, Three-Dimensional Human Explant Model

Hema Sundaram MD,a Nicolas Mackiewicz PhD,b Emeline Burton MSc,b Laurent Peno-Mazzarino BSc,c Elian Lati PhD,d and Stéphane Meunier PhDb
aDermatology, Cosmetic & Laser Surgery, Rockville, MD
bTeoxane SA, R&D Department, Geneva, Switzerland
cLaboratoire BIO-EC, Longjumeau, France

ABSTRACT

Background: Hyaluronic acid (HA) is a popular ingredient in topical formulations for cosmetic improvement of the skin. Most formulations contain linear, non-crosslinked HA oligomers, low molecular weight (LMW) HA, and/or high molecular weight (HMW) HA. Crosslinking of HA enhances its clinical longevity and mechanical characteristics. The objective of this study was to characterize the topical effects of a new, crosslinked resilient HA (RHA) that is also available as a cohesive, tissue-integrating injectable filler, compared with non-crosslinked HMW HA and LMW HA. Living human skin explants that preserve the 3-dimensional structure of in vivo skin were used to maximize clinical relevance.

Methods: Standardized doses of each HA product were applied daily for 9 days to human skin explant surfaces. Untreated explants served as controls. Water content of the stratum corneum and entire epidermis was analyzed by Raman spectroscopy. Transepidermal water loss (TEWL) was measured to assess skin barrier function. Explant morphology and microrelief were evaluated by optical and scanning electron microscopy.

Results: Crosslinked RHA achieved a significant increase in epidermal water content (76%) over the control. Spectral cartography confirmed a higher epidermal water content with RHA than with HMW HA or LMW HA. TEWL was reduced by 27.8% with RHA, and by 15.6% with HMW HA, but increased by 55.5% with LMW HA. Cutaneous microrelief improved with RHA. Corneocyte cohesion improved with RHA and HMW HA.

Conclusions: This comparative, multimodal study demonstrated greater benefits of topical crosslinked RHA over linear HMW HA or LMW HA in reducing TEWL, retaining and redistributing water within the epidermis, maintaining skin integrity, and improving skin barrier structure and function. RHA was a more efficacious humectant than LMW HA, and a more efficacious occlusive moisturizer than HMW HA. These integrative epidermal repair activities are of significant value for addressing primary deficits of aging skin, improving tolerance to retinoids and other topical agents, and optimizing procedural outcomes. A combination of topical and injectable HA provides an elegant model of synergistic, multi-level skin restoration.

Corneum results in depletion of the bilayer matrix and poorer water-retaining capacity. The worsening of skin barrier function manifests as an increase in TEWL. Therapeutic strategies to address this include cleansing protocols that minimize further impairment of barrier function, barrier creams to physically impede TEWL, and moisturizers to improve water content via occlusive, humectant and/or emollient activities. All moisturizers provide some form of temporary barrier, allowing time for damaged stratum corneum to be repaired. An ideal moisturizer would repair the skin barrier, maintain skin integrity and appearance, reduce TEWL, and restore the lipid barrier's ability to attract, hold, and redistribute water.

Classification of Moisturizers

Occlusive moisturizers are typically oily and immiscible with water. They maintain and restore skin barrier integrity and function by forming a hydrophobic film on the skin surface and within the superficial interstitium between corneocytes. This physically blocks water loss from the epidermis. Occlusive moisturizers often have a thick or greasy texture, and this can limit patient compliance, especially for facial application. Some occlusive moisturizers, such as lanolin or petroleum derivatives, have raised health and environmental concerns.

Humectant moisturizers draw water from the dermis to the epidermis. They are typically small, hygroscopic molecules such as glycerol, sorbitol, urea, or sugars. Humectants rarely draw water from the environment except when the ambient humidity exceeds 70%. Moisturizers that have only humectant activity actually increase TEWL when applied to skin with a defective barrier. A high concentration of some humectants, such as urea, glycerin, and propylene glycol, may be irritating and should be avoided in patients with sensitive skin.

Emollient moisturizers have the ability to instill small droplets of oil into the cracks between desquamating corneocytes in dry skin, and consequently to improve softness, smoothness, and flexibility of the skin. They include a wide variety of fatty acids, such as stearic, linoleic, linolenic, oleic, and lauric acid. Omega-3 or omega-6 fatty acids can be enzymatically converted to eicosanoids, which function as signaling molecules within the skin to mediate inflammation and cell repair. While emollients are not usually occlusive, they may also function as a barrier to water loss when applied heavily.

HYALURONIC ACID: BACKGROUND AND PURPOSE OF THIS STUDY

Hyaluronic acid (HA) is a popular ingredient in many topical cosmetic formulations. From the perspective of safety and tolerability, it is reported to be non-toxic, non-immunogenic, and non-carcinogenic, and to have low sensitization potential. Because of its capacity to attract water and its excellent water solubility, HA has typically been classified as a humectant moisturizer. Diverse forms have been used, ranging from oligomers and low molecular weight (LMW) HA to high molecular weight (HMW) HA. To date, formulations of crosslinked HA that are specifically designed for topical application have been less common.

Crosslinking of HA confers longevity and can enhance its mechanical characteristics. The type of crosslinked HA that was evaluated in this study, resilient HA (RHA), was developed for use as an injectable soft tissue filler for aesthetic applications. A number of RHA filler products are approved for injectable use in Europe with CE marking, in Canada, and elsewhere in the world. RHA fillers are currently under review by the US Food and Drug Administration (FDA) for use in the United States. RHA has a structure that preserves the inherent molecular coiling of HA, with the objective of allowing it to adapt well to the skin's movements.

"A combination of topical and injectable hyaluronic acid provides an elegant model of synergistic, multi-level skin restoration."

With the rationale that these properties might also be of value in a topical formulation, the objective of this study was to evaluate the effects of crosslinked RHA vs linear, non-crosslinked HMW HA and LMW HA. Living skin explants were selected as the substrate for this study because they preserve the 3-dimensional structure of in vivo skin, and permit precise, multimodal analysis of each skin layer. The parameters that were assessed, including epidermal cell morphology, water content, transepidermal water loss (TEWL), and skin surface micrelief, provided an accurate analysis of skin hydration and barrier function.

MATERIALS AND METHODS

a) Preparation of Hyaluronic Acid Test Samples

Cross-linked RHA (Teoxane Laboratories, Geneva, Switzerland) was synthesized under sterile conditions using high molecular weight sodium hyaluronate, with 1,4-butanediol diglycidyl ether (BDDE) as a crosslinking agent. The final RHA concentration was 25 mg/mL. Samples of HMW (1.5 MDA) and LMW sodium hyaluronate (50KDa) were purchased from the manufacturer (HTL Biotechnology, Javené, France) and diluted with phosphate-buffered saline to a concentration of 25 mg/mL prior to thermal sterilization. All 3 samples were then diluted to a final concentration of 3% in an aqueous carboxymethyl cellulose gel, and kept at room temperature for the duration of the study.

b) Preparation of Human Skin Explants

A total of 19 human skin explants was prepared, each with a mean diameter of 1.1 cm. After informed consent in accordance with the institutional review board, the skin was harvested from healthy donors.
with the Declaration of Helsinki, full-thickness human skin punch biopsies were obtained from the abdomen of a 65-year-old Caucasian female donor who was undergoing elective plastic surgery. Punch biopsies were placed immediately after collection in a Minimum Essential Medium (Gibco MEM 1x, Thermo Fisher Scientific, Waltham, MA), and transported at room temperature to the testing laboratory (BIO-EC Laboratory, Longjumeau, France). The hypodermis was removed from each punch biopsy, and cylindrical skin specimens (1.1 cm mean diameter, 0.2 cm thickness, and 180 mg weight) were then excised using a sample punch. They were placed immediately, with the dermis face down, in a liquid–air interface in Bio-EC Explant Medium containing gentamicin and fungizone for 48 hours, before being transferred to antibiotic-free Dulbecco’s modified Eagle’s medium (DMEM 12-04; Lonza, Basel, Switzerland).

Skin samples were cultured under physiological conditions (temperature of 37°C, CO2 concentration of 5%). Half of the culture medium (1 mL) was refreshed every 2 days.

c) Application of Test Hyaluronic Acid Products to Skin Explants
The skin explants were divided into 5 batches:
1. Batch T0: Three reference, untreated explants that were analyzed on day 0 (baseline)
2. Batch T9: Four reference, untreated explants that were analyzed on day 9
3. Batch P1: Four explants that were treated with topical RHA and analyzed on day 9
4. Batch P2: Four explants that were treated with topical HMW HA and analyzed on day 9
5. Batch P3: Four explants that were treated with topical LMW HA and analyzed on day 9

On day 0 (pre-treatment), each explant, except those from the reference untreated batch T0, was stripped of the outer portion of the stratum corneum with adhesive-coated discs (D-Squame, Cuderm, Dallas, TX). 2μL of HA test product was applied topically to each explant on days 0, 1, 2, 3, 5, 6, 7, and 8, and spread using a small spatula. Half of the culture medium (1 mL) was refreshed on days 2, 5, and 7.

d) Analysis of Explant Morphology and Microrelief With Optical and Electron Microscopy
For morphological analysis, skin explants were fixed for 24 hours in buffered 1% formaldehyde/1.6% glutaraldehyde solution, then dehydrated and embedded in paraffin using automatic tissue processor systems (TP 1020 dehydration automat, EG 1160 embedding station Leica, Nanterre Cedex, France). 5μm sections were cut on a Minot-type microtome (RM 2125, Leica), mounted on specially-treated histological glass slides (Superfrost, Fisher Scientific, Pittsburgh, PA), and stained in accordance with the Masson–Goldner trichrome staining protocol. Stained sections were examined with a Leica DMLB or a BX43 Olympus microscope (Olympus France SAS, Rungis Cedex, France). Standardized digital images were obtained with an Olympus DP72 camera and Cell D data storing software.

For analysis of cutaneous microrelief, half-explants were dehydrated, placed on sample holders, and metalized. Electron microscopic examinations were realized with a MEB Quanta 200 scanning electron microscope under high vacuum pressure (50 Pa).

e) Raman Spectroscopic Measurement of Water Content
Molecular analysis of water content was performed on 10 μm thick frozen skin sections with a confocal Raman microscope (Xplora, Horiba, Palaiseau, France), using a frequency-doubled, diode-pumped 532 nm Nd:YAG laser. Point-by-point analysis was performed by Raman spectroscopic measurement at 60 distinct points on the stratum corneum and 60 distinct points on the underlying living epidermis of each explant. After correction of the baseline and smoothing of the Raman spectra, the data were normalized using the spectral region from 1300 cm⁻¹ to 1800 cm⁻¹. After normalization, water content was measured by the integrated intensity of the spectral band between 3200 cm⁻¹ and 3400 cm⁻¹, corresponding to the region of vibration of hydrogen-oxygen bonds within water molecules.

f) Measurement of Transepidermal Water Loss
TEWL measurements were performed on day 0 (pre-treatment) and day 2, prior to application of the test products. The explant surfaces were first wiped carefully with an absorbent tissue (Kim-Wipes, Kimberly-Clark, Irving, TX) to remove surface water.

FIGURE 1. Morphology of a skin explant before treatment on day 0 (Batch T0), and of untreated and treated explants on day 9 (Batches T9, P1, P2, and P3, respectively). Masson-Goldner Trichrome staining. Black scale bar on the images represents 50 μm.
expressed as g/m²/h, was then measured using an open-chamber probe (Tewameter TM300, Courage-Khazaka Electronic GmbH, Cologne, Germany). Forty consecutive measures (1 measure per second) were obtained for the same skin explant zone, the measured values were stabilized, and mean values were calculated.

**FIGURE 2.** Electron microscopic images of the skin surface by electronic microscopy on day 9 of (A) a reference untreated explant, and explants after treatment with (B) crosslinked resilient hyaluronic acid (RHA), (C) linear high molecular weight hyaluronic acid (HMW HA), and (D) linear low molecular weight hyaluronic acid (LMW HA). Magnification x 200. White scale bar on the images represents 200 µm.

#### g) Statistical Analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA). For all analyses, the desired level of statistical significance was preset to less than or equal to 0.05. Mean values for the samples were
corneum in the untreated explants and explants treated with LMW HA showed mild parakeratosis of the non-treated explants. The explants treated with LMW HA showed mild epidermal acanthosis, with 5 to 6 epidermal cell layers compared to 4 to 5 in the day 0 explants.

The relief of the dermal-epidermal junction was moderately good in explants treated with crosslinked RHA and HMW HA. The relief was poorer for the untreated explants on day 9, and more pronounced for explants treated with LMW HA. The density of collagen fibers in the papillary dermis decreased slightly in all explants on day 9. The morphology of dermal cells was rated as good in all explants, and unchanged compared with the untreated explants on day 0.

**RESULTS**

**Explant Skin Morphology**

Skin morphology was assessed by optical microscopy of the reference untreated explants on day 0, and of explants that had been either untreated or treated with one of the 3 HA formulations – crosslinked RHA, HMW HA, or LMW HA – on day 9 (Figure 1). General integrity of the skin explants was maintained throughout the study. Minor and relatively consistent evolutions in skin morphology from baseline to day 9 were observed in all explants.

On day 0, morphologic assessment of the reference, untreated explants (Batch T0) revealed good epidermal and dermal quality. The stratum corneum was of moderate thickness with slight lamination. The epidermis showed 4 to 5 cellular layers with good morphology, there was moderate relief of the dermal-epidermal junction, the papillary dermis showed a dense collagen fiber network, and dermal cell morphology was good.

On day 9, general epidermal morphology was good for all explants. The stratum corneum of the explants treated with crosslinked RHA was more laminated than in the untreated explants and in the explants treated with HMW HA or LMW HA. There was a moderate increase in thickness of the stratum corneum in the untreated explants and in the explants treated with LMW HA, and mild parakeratosis of the non-treated explants. The explants treated with LMW HA showed mild epidermal acanthosis, with 5 to 6 epidermal cell layers compared to 4 to 5 in the day 0 explants.

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**Cutaneous Microrelief**

Electron microscopy was used to visualize the cutaneous microrelief of explants from each treatment batch. Three images were obtained of each explant from different angles. Representative images at low magnification (x 200) are shown in Figure 2. High magnification (x 400) examination was also used to assess corneocyte cohesion.

On day 9, the microrelief of the untreated reference skin explant showed numerous deep primary lines and corneocyte cohesion was assessed as fair. The microrelief of explants treated with crosslinked RHA showed a significant decrease in the depth of the primary lines. Corneocyte cohesion was assessed as good
for the explants treated with crosslinked RHA or linear HMW HA. The cutaneous microrelief on day 9 of the explants treated with linear HMW HA or LMW HA was similar to that of the untreated reference explants. Corneocyte cohesion was perturbed in the explants treated with linear LMW HA, and prominent scaling was present.

Analysis of Water Content by Raman Spectroscopy

The water content of the stratum corneum and underlying epidermis of each explant was evaluated on day 9 according to the protocol described above. Based on integrated intensity of the spectral band associated with water (3200 cm⁻¹ to 3400 cm⁻¹), Table 1 shows the water content of the untreated, reference explant, compared with the explants treated with crosslinked RHA, linear HMW HA, or LMW HA.

On day 9, compared with the reference, untreated explants there was a statistically significant increase in the epidermal water content of explants treated with crosslinked RHA (+7.6%, P<.01). There was a statistically significant decrease in the water content of the stratum corneum of the explants treated with crosslinked RHA or linear HMW HA (-10.7%, P<.1, and -9.1%, P<.05, respectively).

The specific effects of treatment on the epidermis were further elucidated by spectral cartography of explants at day 9. A 1500 µm region (30 µm x 50 µm) on the epidermis of each explant was randomly selected and divided into an array of 54 individual squares. Three Raman spectroscopic analyses were obtained for each square and a mean value was calculated. From these mean values, relative water content was calculated and visualized by spectral cartography. The cartographic images were superimposed on video microscopic images of each analyzed zone (Figure 3). The color spectrum ranged from bright green, indicating high water content, to deep blue, indicating zero water content. In the upper panel, the cartographic images have been superimposed on video microscopic images of the analyzed epidermal zones.

**TABLE 1.**

| Raman Spectroscopic Data on Day 9 Indicating Water Content of the Stratum Corneum and Underlying Epidermis of Explants After Treatment With Crosslinked RHA, Linear HMW HA and Linear LMW HA Compared With a Reference, Untreated Explant |
|----------------|----------------|----------------|
|                | Cross-Linked RHA | HMW HA          | LMW HA          |
| Stratum Corneum| -10.7%<sup>a</sup> | -9.1%<sup>b</sup> | -2.3%<sup>NS</sup> |
| Epidermis      | +7.6%<sup>a</sup> | -3.2%<sup>NS</sup> | +4.1%<sup>NS</sup> |

Calculated probability (P) values are from statistical analysis based on comparison with the water content of control, untreated explants on day 9.

RHA, Resilient Hyaluronic Acid; HMW HA, high molecular weight hyaluronic acid; LMW HA, low molecular weight hyaluronic acid

<sup>a</sup>Significant with P<.1; <sup>b</sup>Significant with P<.05; <sup>NS</sup>Significant with P<.01; NS, non-significant

**FIGURE 4.** Transepidermal water loss measurements on day 0 and day 2 of the living epidermis of reference untreated skin explants and explants treated with crosslinked resilient hyaluronic acid, linear high molecular weight hyaluronic acid, or linear low molecular weight hyaluronic acid. Black vertical bars show values for standard error of the mean.

Spectral cartography visually confirmed the higher water content on day 9 of the living epidermis of the explants treated with crosslinked RHA, compared with the reference untreated explants and the explants treated with linear HMW HA or LMW HA.

**Transepidermal Water Loss (TEWL)**

The methodology employed for the measurement of TEWL was the same as is customarily performed in vivo on study subjects to provide an accurate assessment of skin barrier function. Since this measurement is very sensitive to variations in the skin surface and the environment, measurements were obtained only for the first 2 days after application of the different HA formulations to living skin explants (Figure 4). On day 2, TEWL was diminished by 27.8% for the explants treated with crosslinked RHA, and by 15.6% for the explants treated with HMW HA, compared with the untreated reference explants. TEWL was increased by 55.5% for the explant treated with LMW HA.

**DISCUSSION**

This pilot study represents the first comparative evaluation of a topical crosslinked HA formulation versus non-crosslinked HMW and LMW HA formulations. The study is unique in evaluating topical efficacy of the same crosslinked RHA that is already established for use as an injectable filler for aesthetic applications.

Multimodal observation of living, 3-dimensional skin explants was performed with state-of-the-art analytical techniques. The use of living skin explants as the substrate for topical HA treatments had the objective of more closely replicating in vivo conditions, thus achieving greater clinical relevance than with in vitro cell cultures. This is important from an evidence-based...
perspective because the structure and function of the stratum corneum are dependent not only on its cellular and extracellular constituents, but also on their continuous interactions. Living skin explants provide a dynamic model of the epidermis, reflecting the variable metabolic activity of the extracellular matrix and the transitions that it undergoes as it moves towards the skin surface. This can provide a more accurate assessment; it has been noted that “static models of the epidermis may not do justice to the extracellular matrix,” whose most crucial functions may occur within highly plastic, dynamic domains.4

There were striking quantitative and qualitative differences in the topical activity of crosslinked RHA compared with non-crosslinked HA. The efficacy of crosslinked RHA as both a humectant and an occlusive moisturizer was reflected in its significantly increasing water content of the epidermis (Table 1), and the trend toward its decreasing TEWL after only 2 days of treatment (Figure 4). Topical crosslinked RHA also improved the skin microrelief, while maintaining good cohesion of corneocytes.

In contrast to crosslinked RHA, LMW HA functioned only as a humectant when applied topically. LMW HA showed a trend towards increasing epidermal water content, although to a lesser extent than crosslinked RHA (4.1% vs 7.6% on day 9). This was associated with a significant increase in TEWL, whereas crosslinked RHA was able to decrease TEWL. The data for HMW HA showed occlusive rather than humectant activity. This was evidenced by its ability to decrease TEWL, albeit to a lesser extent than crosslinked RHA (15.6% vs 27.8% on day 2), but not to increase epidermal water content as crosslinked RHA could. (Table 1 and Figure 4).

These data demonstrate the value of crosslinked RHA for effective protection and repair of the skin via multiple activities that are considered ideal to a therapeutic moisturizer. Crosslinked RHA achieved a reduction in TEWL, retained and redistributed water within the epidermis, maintained skin integrity, and improved skin barrier structure and function. The aphorism that “not all topical HA is alike” is underscored by the superiority of crosslinked RHA as a humectant compared with LMW HA, and as an occlusive agent compared with HMW HA.

Several studies of injectable HA fillers have highlighted how specific types of crosslinking confer different biophysical characteristics and resultant patterns of clinical behavior.17-19 The aphorism may hence be expanded and refined to the observation that “not all crosslinked HA is alike.” The concept that this specificity can extend to topical HA formulations is intriguing and has some support from currently available data. These indicate that crosslinked RHA may possess the capacity to form a robust occlusive film that protects the skin surface, while also generating some HA fragments that can penetrate the stratum corneum and act as a humectant. Further investigations are in process to define the role of the specific crosslinking and 3-dimensional structure of RHA in these dual activities, and its notable efficacy in repairing the skin barrier. As with the studies reported in this publication, the evidence level of these investigations is being addressed with study protocols of a high scientific level that have direct applicability to the living skin of patients.

CONCLUSION

Aging skin is characterized by impaired barrier function, increased TEWL, and decreased water content. Since these primary deficits have far-reaching structural, functional, and metabolic consequences, it is extremely important to address them by repairing the skin barrier, decreasing TEWL, and increasing water content. These actions may be considered prerequisites for optimal efficacy of all other interventions – both topical and procedural. From a practical perspective, a well-tolerated topical crosslinked HA formulation that can be applied as a cosmetically elegant film, rather than a thick, oily, or sticky barrier, promotes patient compliance and hence therapeutic efficacy. In the experience of one of the authors (HS), topical crosslinked RHA has achieved a uniformly high level of satisfaction in several hundred patients who consider themselves to have dry, oily, or combination skin types, with no reported episodes to date of skin irritation or sensitization. Significant improvements in skin lustre, tone, and texture have been noted after as little as a few days of use. Inclusion of topical crosslinked RHA in patients’ skincare regimens has increased their ability to tolerate other topical actives such as retinoids and alpha-hydroxy acids.

Given our emerging appreciation of skin aging as a multilevel and multifactorial process, the potential for synergy when combining topical crosslinked HA with injectable fillers and other procedures is obvious and logical. Integration of topical crosslinked RHA into treatment plans as a potent agent of epidermal repair creates a holistic skin rejuvenation strategy.

The discovery of CD44 receptors that bind HA within the epidermis highlights the need for further studies to fully understand the immense potential of topical HA. Recent publications on injectable crosslinked HA fillers have postulated that upregulation of collagen and elastin production occurs through interaction with intradermal CD44 receptors.20,21 In this context, the presence of intra-epidermal CD44 receptors is noteworthy. The distribution of CD44 receptors in the epidermis parallels the distribution of HA. Both CD44 and HA are found preferentially in the spinous cell and, to some extent, the basal cell layers, rather than in the stratum granulosum or stratum corneum.22 CD44 is present on plasma membrane domains that face extracellular spaces rich in HA.23 CD44 signaling, activated by HA in the extracellular matrix, has been shown to regulate keratinocyte activities and
normalize epidermal function.\textsuperscript{24} This opens up the fascinating prospect that topical HA, like injectable HA, may have opportunities to mediate long term restorative or regenerative effects in addition to its more immediate aesthetic benefits.

**DISCLOSURES**

Hema Sundaram MD serves as a clinical investigator and/or consultant for cosmeceuticals for Allergan/SkinMedica, Biopelle, Evolus/Strathspey Crown, SkinCeuticals, Suneva Medical, and Teoxane. Nicolas Mackiewicz PhD, Emeline Burton MSc, and Stéphane Meunier PhD are employees of the Research & Development Department of Teoxane SA, Geneva, Switzerland, Elian Lati PhD and Laurent Peno-Mazzarino BSc have no conflicts of interest to declare.

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