 ORIGINAL ARTICLE

Rejuvenating Hydrator: Restoring Epidermal Hyaluronic Acid Homeostasis With Instant Benefits

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ABSTRACT

Skin aging is a combination of multifactorial mechanisms that are not fully understood. Intrinsic and extrinsic factors modulate skin aging, activating distinctive processes that share similar molecular pathways. One of the main characteristics of youthful skin is its large capacity to retain water, and this decreases significantly as we age. A key molecule involved in maintaining skin hydration is hyaluronic acid (HA). Concentration of HA in the skin is determined by the complex balance between its synthesis, deposition, association with cellular structures, and degradation. HA bio-equivalency and bio-compatibility have been fundamental in keeping this macromolecule as the favorite of the skincare industry for decades. Scientific evidence now shows that topically applied HA is unable to penetrate the skin and is rapidly degraded on the skin surface.

SkinMedica's HA[5] Rejuvenating Hydrator (SkinMedica Inc., an Allergan company, Irvine, CA) promotes restoration of endogenous epidermal HA homeostasis and provides instant smoothing and hydration of the skin. These dual benefits are accomplished through the combination of 2 breakthrough technologies: 1) a unique blend of actives powered by SkinMedica proprietary flower-derived stem cell extract that restores the endogenous production of HA; and 2) a proprietary mix of 5 HA forms that plump the skin, decreasing the appearance of fine lines/wrinkles.

Pre-clinical studies demonstrated that HA[5] induces expression of key epidermal differentiation and barrier markers as well as epidermal HA synthases. A decrease expression of hyaluronidases was also observed upon HA[5] application. Initial clinical studies showed that within 15 minutes of application, HA[5] instantly improves the appearance of fine lines/wrinkles and skin hydration. Subjects that continue using HA[5] (for 8 weeks) demonstrated significant improvements in fine lines/wrinkles, tactile roughness, and skin hydration. In summary, the blend of these 2 key technologies present in HA[5] promotes restoration of endogenous epidermal HA while delivering instant smoothing effects.

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INTRODUCTION

Karl Meyer and his colleague John Palmer isolated hyaluronic acid (HA) for the first time in 1934.[1] They were able to purify an unknown chemical substance from the vitreous body of cow eyes. The newly identified substance was named "hyaluronic acid" – a combination of the Greek world "hyalos" (glass) and uronic acid, one of the 2 sugar molecules they identified. It took another 20 years of research to fully resolve the chemical structure of this mucopolysaccharide,[2] which is now one of the most widely used natural macromolecules in medicine and skincare due to its bio-compatibility and safety, and the potential for increasing its HA-associated benefits via chemical modifications.

Interestingly, the first commercial use of HA was in neither of these areas but in the food industry as a substitute for egg white in bakery products. Since then, biological understanding of HA has evolved from a traditional space filler into a structural molecule, a key participant in processes such as lubrication and moisturization, a connective tissues supporter, and, more recently, a modulator of inflammation, wound healing, and cancer. Currently, HA-based products are dominating the areas of eye care, wound healing, drug-delivery, and skin care, to mention just a few.

The relatively simple structure of naturally occurring HA is well-conserved among various species and type of tissue origin. The

main variations detected are in the molecular weight (MW) of the polymers. In the body, most of the HA is in the form of salt, reaching high concentrations in connective tissues such as skin, synovial fluid, and vitreous humor. Adult skin accounts for approximately 50% of the total body HA. Most of the cutaneous HA is localized in the dermis, reaching concentrations of 0.5 mg/kg; while epidermal HA has been estimated to be around 0.1 mg/kg. Due to its rheological properties, cutaneous HA modulates the overall skin quality, hydration, permeability, and immune barrier function. In addition, its unique viscoelastic properties provide skin cells protection from mechanical damage, increasing cell survival (response to injury and wound healing) and promoting proliferation, migration, signal transduction, and immune surveillance.[3-6]

HYALURONIC ACID STRUCTURE AND PROPERTIES

Hyaluronic acid is a carbohydrate that is synthetized as a large linear polymer of alternating repeating disaccharide units composed of glucuronic acid and N-acetylglucosamine. These saccharides are linked together through alternating beta-1,4 and beta-1,3 glycosidic bonds (Figure 1a). The number of repetitions in a complete HA molecule can reach close to 10,000 or more with a molecular mass of about 4 million daltons (Da) with an average length of 1 nm that may reach 10 µm if stretched.

Hyaluronan – a term that encompasses the different forms of this carbohydrate such as acid (HA) and salts (hyaluronates) – forms particularly stable tertiary structures in aqueous solution with remarkable hydrodynamic properties, including non-Newtonian viscosity and water retention. HA solutions show very unusual rheological properties as well as high lubricious and hydrophilic properties. Structural studies have shown that in solution HA polymer chains form expanded random coils that, at lower concentrations, entangle with each other, trapping 1,000 times their weight in water during the process.[7] At elevated concentrations, HA solutions are sheer-thinning, which allows these gel-like solutions to flow easily when under pressure (ie, through a needle).

HYALURONIC ACID SYNTHESIS AND DEGRADATION: KEEPING THE BALANCE

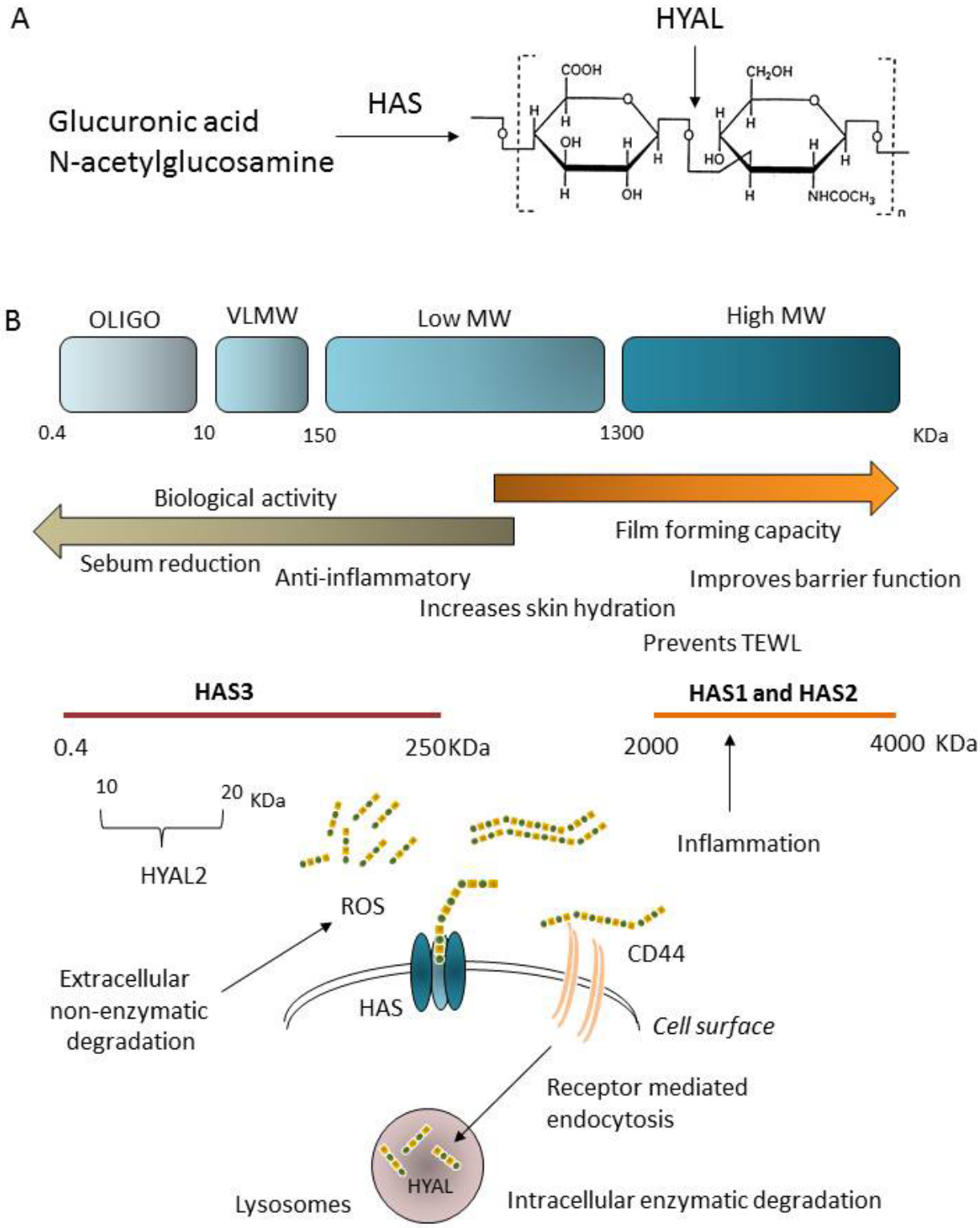
Hyaluronan can bind and hold large amounts of moisture (approximately 6 liters of water per gram of HA); therefore, young or youthful skin, which is well hydrated, contains large amounts of HA. Endogenous HA in skin exhibits a high turnover rate. It has been estimated that HA half-life in the dermis is < 1 day,[8,9] while in the epidermis is only about 2 to 3 hours.[10] The net amount of HA in the skin is regulated at different levels: synthesis, deposit, association with hyaladherin, or other components of the extracellular matrix (ECM), and degradation. While most of the glycosaminoglycans (GAGs) are synthetized in the Golgi apparatus, HA is mainly synthetized at the cell surface by a set of enzymes named hyaluronic acid synthases (HAS), a class of membrane-integrated glycosyltransferases (Figure 1).

Although it has been suggested that HA-synthesis also occurs in the cytosol, the role and significance of this unique pathway is yet to be determined.[11] The extension of the HA polymer occurs while extruding through the plasma membrane. Recent data suggest the dimerization in parallel orientation of HAS on the cell surface[12] and/or the potential formation of multi-enzyme constructs that may form pores for the extraction of the HA molecules.[10] There are 3 HAS isoforms (HAS1, HAS2, and HAS3), and they have different tissue and cell-specific expression patterns and Km values.[13] HASs incorporate uridine diphosphate (UDP) sugars into the non-reducing end of the growing sugar chains, forming different sizes of polysaccharide chains (Figure 1). HAS1 and HAS2 produce chains of 2-4 x 10^6 Da, whereas HAS3 synthesizes shorter chains (0.4-2.5 x 10^5 Da). HAS enzymes activities depend on post-transcriptional modifications such as ubiquitination, phosphorylation, and N-glycosylation.[14] Transcription of *HAS* genes is stimulated by growth factors including epidermal growth factor, platelet-derived growth factors, and transforming growth factors-beta (TGF-β). *In vitro* studies suggest that HAS2 is the main HA synthase in dermal fibroblasts and responsible for the bulk of dermal HA production. HAS1, the activity of which depends on elevated intracellular levels of UDP, is the least active of these enzymes under physiological conditions, but plays a key role in HA synthesis during inflammation and glycemic stress[15,16] as well as after TGF-β stimulation.[17] HAS2 and HAS3 show comparable levels of activity in keratinocytes *in vitro*.

"HA^5 application results in instant improvements in skin quality parameters and, within time, retrains the skin to enhance endogenous epidermal HA production."

Degradation of extracellular HA is initiated by the release of HA from its interaction with ECM component or hyaladherins, which strongly links the decrease in skin HA levels to aging and photoaging. Enzymatic catabolism of HA is achieved by a family of enzymes called hyaluronidases (HYAL). For a long time the HYAL family received little attention due mainly to the technical difficulties in its isolation, purification, and stabilization. Seven HYAL have been described, although differential enzymatic activity has not been determined for all of them. HYAL mechanism of action is associated with the hydrolysis of the hexosaminidic β (1-4) linkages between N-acetyl-D-glucosamine and D-glucuronic acid residues in HA (Figure 1). HYAL1 and HYAL2 are considered the more active members of the HYAL family, generating tetrasaccharides and HA fragments of 10-20 x 10^3 Da, respectively. HYAL1 is a lysosomal (acidic) active enzyme, while HYAL2 is a GPI-anchored plasma membrane protein that can be also localized in the lysosomes.[18,19] The role of HYAL-3 in HA degradation

FIGURE 1. Summary of hyaluronic acid synthesis, degradation, and size-related functions in the skin. (A) Schematic representation of HA synthesis by HAS and site of action of HYAL. (B) Different biological functions linked to HA depends on the size of this polymer as well as its interaction with its receptor, CD44.



HA, hyaluronic acid; HAS, hyaluronic acid synthases; HYAL, hyaluronidases; MW, molecular weight; ROS, reactive oxygen species; TEWL, transepidermal water loss.

TABLE 1.

Main Epidermal Functions in Which Hyaluronic Acid Plays a Role

Function	Markers	Ingredient in HA[5]
Proliferation Differentiation Migration	Ki-67, Involucrin, loricrin, filaggrin, keratin 1, 5, 10 and 14	*VitisenSCE technology*
Barrier formation and hydration (restoring endogenous HA)	Formation of tight junctions (TJs): Claudins, occludin and ZO (1,2, and 3), MUPP-1 and symplekin. Transglutaminase (TGM)	VitisenSCE technology *Hydration:* crosslinked HA, time release encapsulated HA, high, medium and low MW HA. Plankton extract and natural biopolymers.
Anti-oxidant (oxidative stress protection)	UVB-induced SBC formation Lipid peroxidation Antioxidant enzymes (SOD, CAT and GPX) Intracellular levels of Glutathione	*VitisenSCE technology*
Anti-inflammatory	Pro-inflammatory mediators: TNF-α, IL-1β, PGs, LTs, etc	*VitisenSCE technology*
Cell survival (wound healing and stress response)	Collagen3, TGF-β, α-smooth muscle actin. Immune system activation	*VitisenSCE technology*

VitisenSCE technology is a proprietary blend of active ingredients from SkinMedica.

is not clear but it may enhance HYAL1 degradation activity. Activities of HYAL1 and HYAL2 depend on HA-CD44 association, which strongly suggests that HA-degradation is a receptor mediated process.[19] The role of extracellular HYALs in HA degradation is still controversial as HA-degradation products are rarely detected outside the cells *in vivo*.[20-22] The final destination of HA catabolic processes is different in the epidermis and dermis. In the epidermis, HA is mainly degraded intracellularly after endocytic internalization, while in the dermis HA-fragments are predominantly drained by afferent lymphatic vessels or uptaken via a receptor-mediated process by the endothelium.[23] Non-enzymatic depolymerization of extracellular HA is accomplished by reactive oxygen species (ROS) in the presence of reducing agents such as ascorbic acid, thiols, ferrous, or cuprous ions, and by Maillard products (that are also involved in the formation of advanced glycation endproducts).[24]

CD44: THE MAIN HYALURONIC ACID RECEPTOR

Hyaluronic acid binds to an ubiquitous, abundant, and functionally important family of cell surface receptors called CD44. CD44 is encoded by a single gene in which alternative splicing determines variations within different cell types. Binding of HA to CD44 requires the activation of the latter by removal of the sialic acid,[25] which suggests a very tight regulation in the activation of HA-CD44 signaling. HA-CD44 interactions activate unique signal transduction pathways that initiate a concomitant onset of multiple cellular functions. Two major intracellular signaling pathways (RhoA-ROK vs Rac-PKN) are partially responsible for the selective control of a variety of HA-linked functions such as proliferation, survival, migration, cell-cell adhesion, and barrier formation. Recent evidence suggests that the size of HA that binds to CD44 is a key factor in triggering a specific intracellular signal transduction pathway under physiological or pathological conditions such as skin atrophy, psoriasis, atopic dermatitis, actinic keratosis, and chronic non-healing wounds.[26]

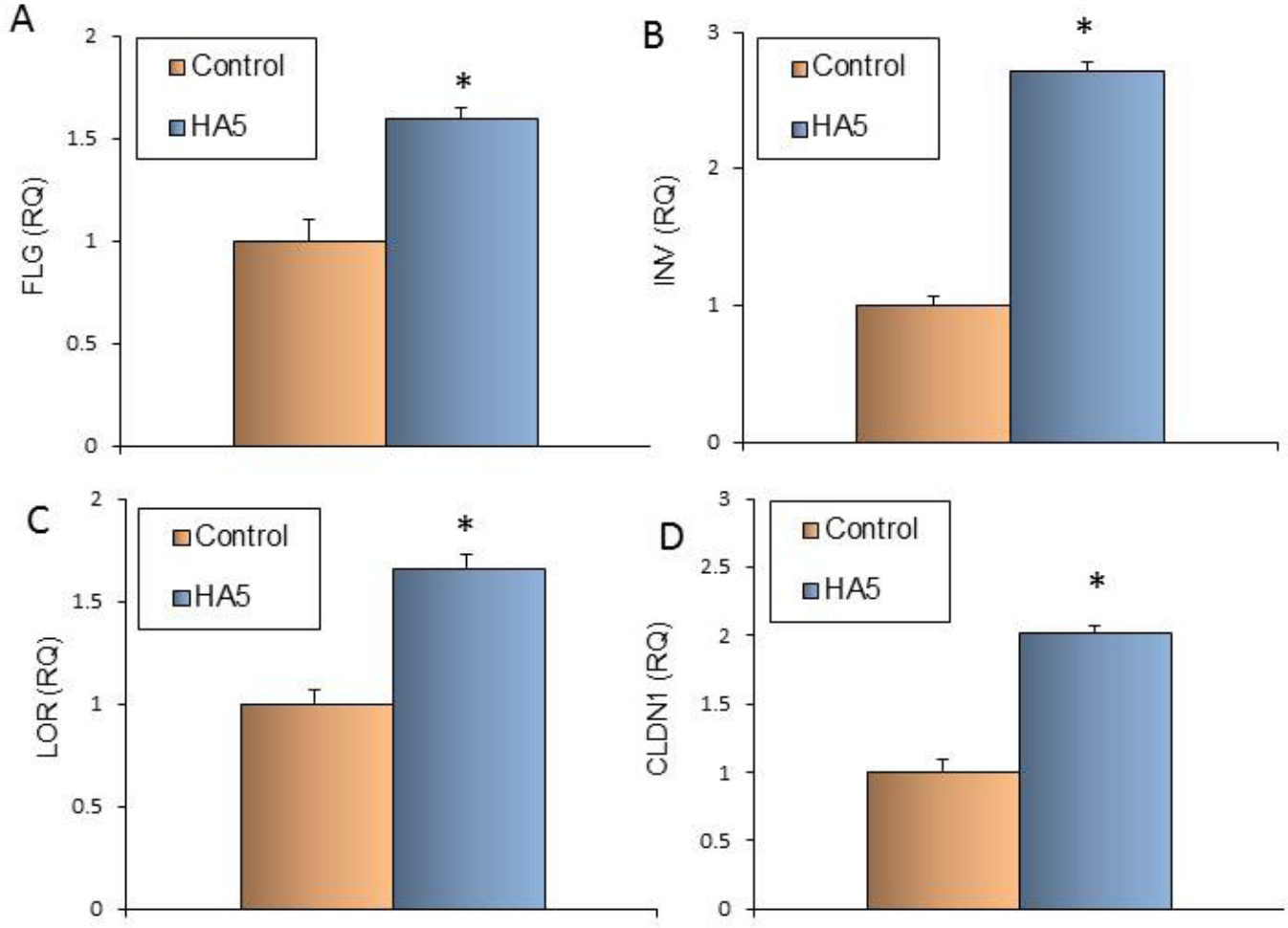
In addition, a close relationship exists between CD44 expression and HA levels in the skin. For example, it has been reported that a significant reduction of HA levels is observed

in transgenic mice expressing an antisense CD44 construct.[27] Furthermore, a significant decrease in the expression of several keratinocyte differential markers (ie, involucrin and filaggrin) is linked to the CD44 knock-out mice model, suggesting that both HA and CD44 play a key role in maintaining normal epidermal physiology and keratinocyte differentiation. In addition, epidermal hyperplasia induced by topical retinoids is accompanied by an enhanced expression of CD44 and HAS, which results in a net increase in the skin HA levels in both the dermis and epidermis.[28] The effects of retinoids on CD44 levels also explain their capacity to revert ultraviolet (UV)-induced lowering expression of this receptor.[29] In addition to CD44, HA can also bind to a less characterized receptor called RHAMM receptor and to hyaladherins (ie, versican, aggrecan, neurocan, brevican, fibrinogen, trypsin inhibitor), a family of molecules that promotes the stabilization of HA by preventing the degradation HA.

BIOLOGICAL FUNCTIONS: IT IS ALL ABOUT LOCATION AND SIZE

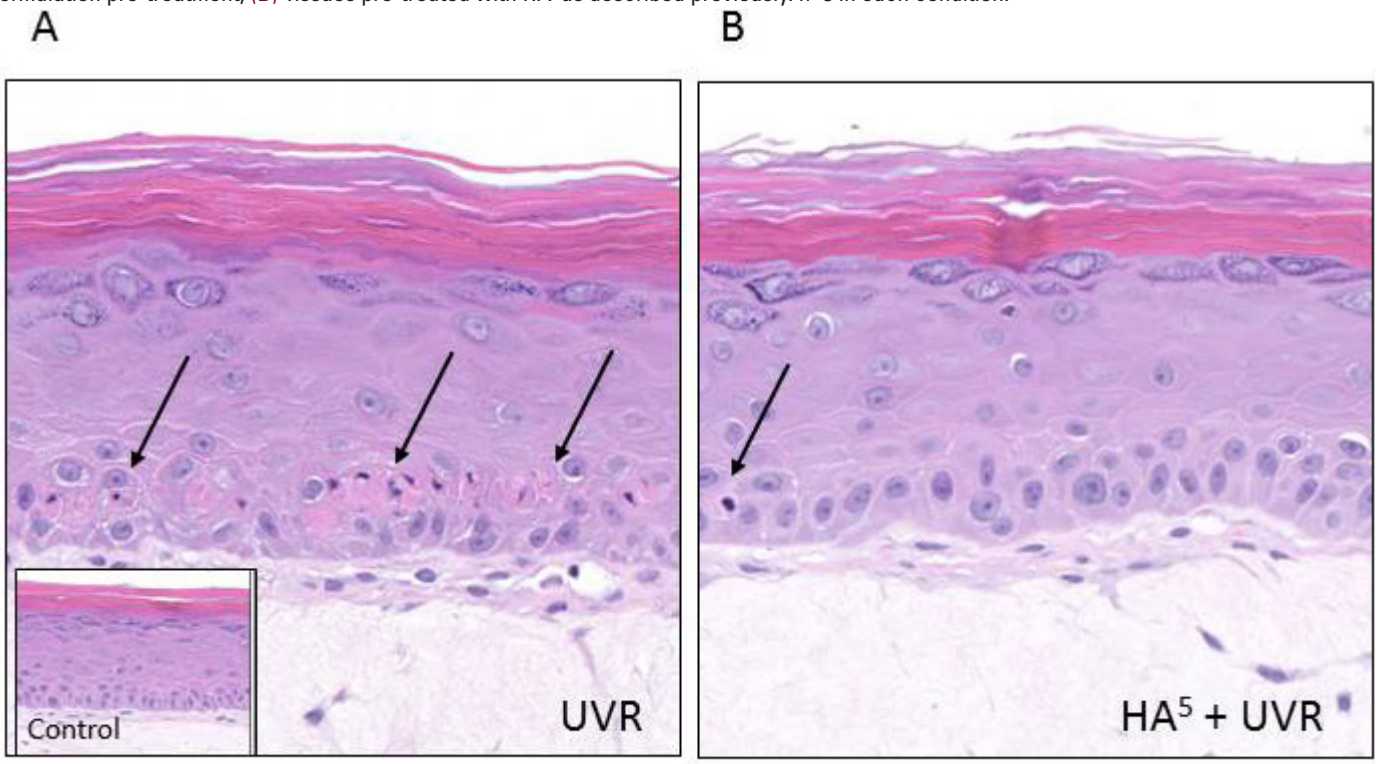
Although the HA primary structure is simple, its biological functions are complexly regulated by cellular localization and the structure and the size of HA-polymer. HA localization is divided into 3 categories: free extracellular HA, extracellular and cell-associated (pericellular) HA, and intracellular HA. Endogenous extracellular HA requires cross-linking to proteoglycans such as versican or aggrecan in an attempt to stabilize this molecule.[30,31] Further interactions with other matrix proteins such as collagen networks result in the formation of complex super-molecular structures that are key for the mechanical support and integrity of the tissues, as well as to confer resistance from shear forces.[32] Pericellular HA is connected to cell surface receptors such as CD44 and membrane-associated HA synthesizing enzymes (ie, HAS2 and HAS3).[33,34] The interaction of pericellular HA and its receptors is believed to play a role in the anti-adhesive properties

FIGURE 2. Treatment with HA[5] resulted in increased expression of epidermal differentiation markers and barrier formation. In brief, EpiDerm Full Thickness (EFT)-400 tissues were treated with 25 µl of HA[5] and allowed to recover for 24 hours. The epidermis and dermis were physically separated, and gene expression was assayed using commercially available TaqMan labeled primers for (A) fillagrin; (B) involucrin; (C) loricrin; and (D) claudin-1. n=6 in each experimental group. *$P<.01$ with respect to control (untreated) group.



CLDN1, claudin-1; FLG, fillagrin; INV, involucrin; LOR, loricrin.

FIGURE 3. Prevention of sunburn cell formation by pre-treatment with HA[5] formulation (representative histological results). In brief, EpiDerm Full Thickness (EFT)-400 tissues were treated or not with 25 µl of HA[5] 10 minutes before ultraviolet radiation and allowed to recover for 24 hours. The insert shows the result from control (untreated) tissues in which no SBC was detected. (A) Tissues subjected to UVB-radiation in absence of HA[5] formulation pre-treatment; (B) Tissues pre-treated with HA[5] as described previously. n=3 in each condition.



UVR, ultraviolet radiation.

of HA.[35,36] Association of HA with CD44, and to a lesser extent interaction with lymphatic vessel endothelial hyaluronan receptor-1 and hyaluronan-mediated motility receptor (RHAMM), also determines cell proliferation and differentiation during wound healing and inflammation.[37-40] The role of intracellular HA is less understood, but its presence has been linked to hyperglycemic conditions and emergency-room stress.[41-44] A potential role in cell division has also been proposed as intracellular HA and its receptor (RHAMM) co-localized with different structures of the spindle apparatus during mitosis, which may regulate nucleolar function or chromosomal rearrangements.[12,45]

Under physiological conditions, most of the HA in the skin is high MW, though exposure to UV radiations as well as tissue injury may trigger HA fragmentation. Fragmented HA activates signal transduction pathways that control migration, survival, and re-differentiation in dermal fibroblasts and keratinocyte.[46-49] The differential functions observed between native and fragmented HA (Figure 1) can be attributed to selective interactions with specific receptors, which are controlled by the size of the polymer.[4] One of the most important non-hydrating functions of high-MW HA is its capacity to prevent uncontrolled inflammation by inhibiting macrophages proliferation and cytokine production during early response to injury, promoting wound healing.[6,50,51] HA also increases proteoglycan synthesis, reduces production of and inactivates pro-inflammatory mediators, blocks activation of nicotinamide adenine dinucleotide phosphate oxidase by antigen stimulation and metalloproteinase expression, alters the function of immune cells, and acts as a free radical scavenger.[52-55] The latter is accomplished by the capacity of the double bond in the D-glucoronic acid unit to complex with reactive molecules.[56] In response to cellular stress, inflammation, and viral infections, HA also forms leukocytes pro-adhesive cable-like structures when cross-linked to versican, heavy-chain molecules originated from inter-α-trypsin inhibitor and the complex TSG-6 and pentaxin-3.[57-59]

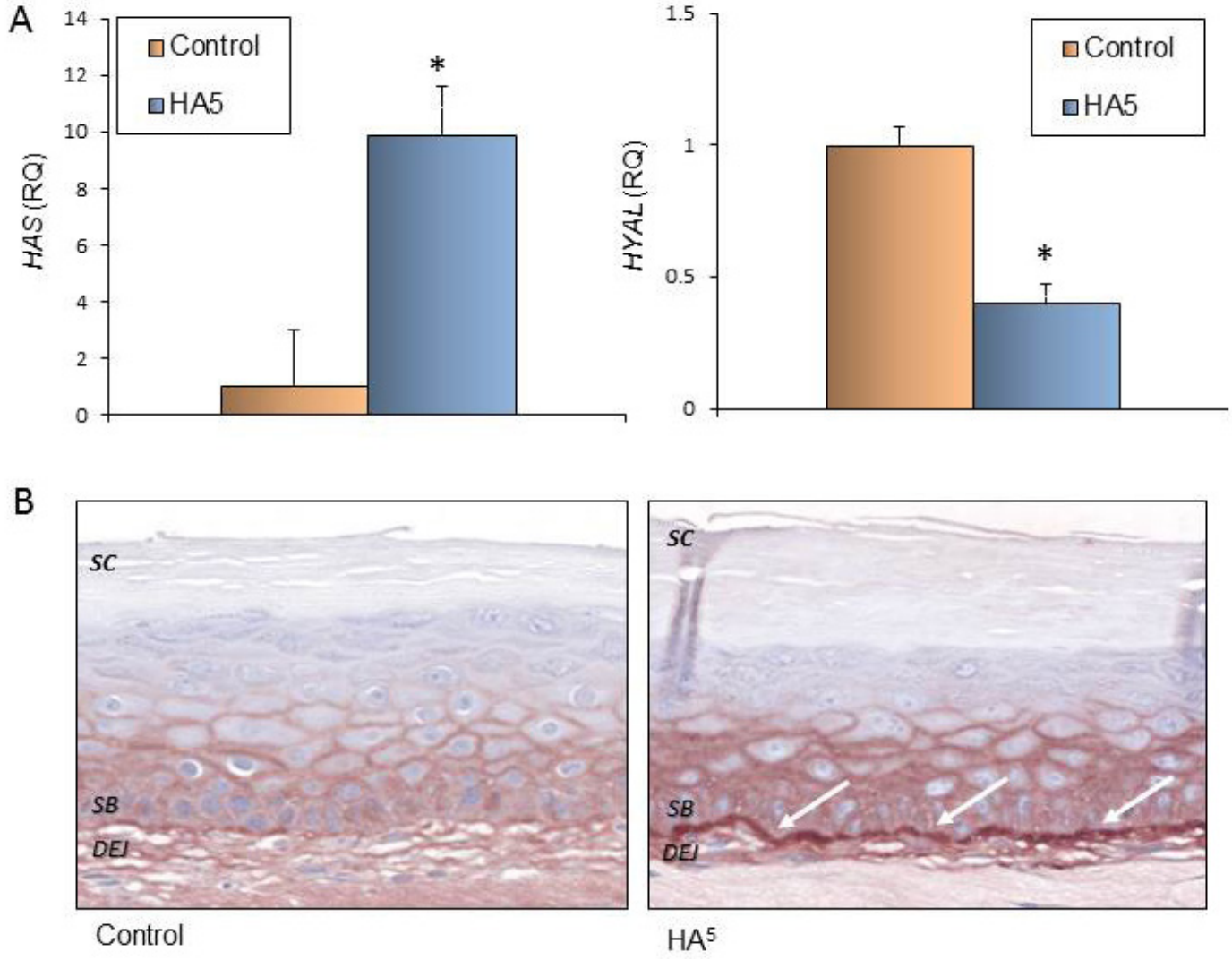
Interactions between CD44 and HA are also responsible for the modulation of T-cell function, though this interaction does not occur constitutively (resting T-cells do not bind HA). The formation of CD44-HA complexes is observed only in activated T-cells, which stimulate their extravasation into inflammatory sites[60] and trigger the suppressor activity of $CD4^+CD25^+$ regulatory T-cells.[61] The mechanisms that regulate this very selective interactions are not fully understood, but it is likely to be mediated by post-transcriptional modifications of CD44 (ie, glycosylation, chondroitin sulfate addition, sulfation, or removal of sialic acid motifs). In recent years, it has been shown that cholesterol

levels on the plasma membrane play a key role in regulating the CD44 binding capacity to HA by sequestering this receptor within specialized membrane domains called lipid rafts.[62] Finally, interactions between HA and CD44 are also partially responsible for the anti-allergic effects linked to HA.[63]

EPIDERMAL AND DERMAL HYALURONIC ACID IN GOOD AND BAD TIMES

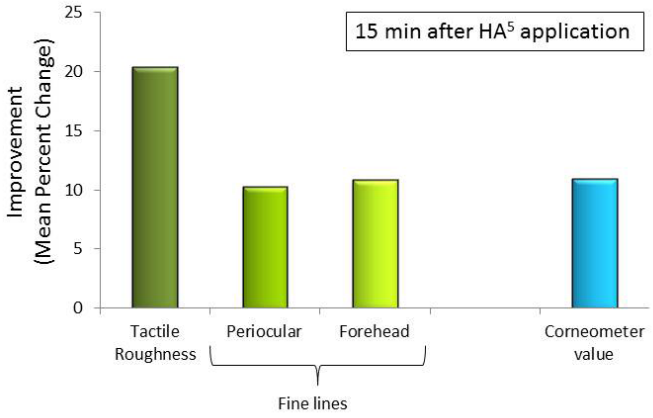
Highest HA concentrations are detected in developing skin.[64,65] Adult skin HA accounts for approximately 50% of the total body HA. Most of the cutaneous HA is localized in the dermis reaching concentrations of 0.5 mg/kg, while epidermal HA has been estimated to be around 0.1 mg/kg. Dermal HA plays a critical role in skin hydration by sequestering water as well as maintaining water balance with the aqueous component of the internal milieu (water associated with HA comprises a separate non-circulating aqueous compartment within the skin). Changes in HA amounts, level of fragmentation, and organization, as well as decreased expression of CD44, have been reported in both intrinsic and extrinsic aging. Disruptions of HA-homeostasis is linked to loss of skin moisture as well as impaired age-related wound healing and the delayed resolution of a variety of skin diseases.[66-68] In addition, non-fragmented HA fails to organize into normal structures such as the pericellular coats in photoaged skin.[69]

FIGURE 4. Treatment with HA[5] resulted in boosting of endogenous epidermal hyaluronic acid production. In brief, EpiDerm Full Thickness (EFT)-400 tissues were treated with 25 µl of HA[5] and allowed to recover for 16 hours. (A) The epidermis and dermis were physically separated, and gene expression was assayed using commercially available TaqMan labeled primers. (B) Histological visualization of epidermal hyaluronic acid (HA) was performed using biotinylated HA-binding protein, followed by streptavidin immunohistochemistry detection system. In brief, stabilized EFT-400 tissues were treated as described in (A) and harvested 24 hours later.



HAS, hyaluronic acid synthases; HYAL, hyaluronidases; SB, stratum basale; SC, stratum corneum; DEJ, dermal epidermal junction.

FIGURE 5. Investigator assessment of the instant effects after topical application of the HA[5]. Instant effects were evaluated 15 minutes after application of HA[5] (n=24). Statistically significant improvements (all $P \leq .04$) in mean scores for periocular and forehead fine lines/wrinkles and tactile roughness were observed compared with untreated skin (baseline). Corneometer values reflected an increase in skin moisture content immediately after HA[5] application (15 minutes after).



Skin aging is a universal and inevitable process that is characterized by alterations in keratinocytes activities, epidermal function, and dermal changes. Epidermal senescence is characterized by barrier impairment, xerosis, slower cell turnover, and atrophy. In the epidermis, ECM components form an integral part of the hemidesmosomes and mediate keratinocyte attachment to the underlying base membrane. Extracellular HA is the major GAG in the epidermal and dermal ECM of most mammalian tissues. Epidermal HA levels are tightly correlated to keratinocytes proliferation, maturation, and differentiation. Elevated levels of HA in the epidermis are linked to highly proliferative keratinocytes. Epidermal HA is localized mainly between cells in the basal layers, disappearing during keratinocytes differentiation and becoming undetectable at the granular layer of the stratum corneous. The relationship between elevated HA levels and actively proliferative keratinocytes is also seen in pathological conditions such as epidermal hyperplasia and psoriasis.[64,70]

Interestingly, histological findings show the presence of intracellular HA in these highly proliferative cells, which disappears as keratinocytes are differentiating. The presence of extracellular HA in the upper layers of the epidermis is thought to maintain diffusion and to open up spaces to facilitate cell migration. Epidermal HA distribution is characterized by compact pericellular coats that are primarily maintained by the presence of CD44.[64,71] HA concentrations in these pericellular areas reach levels as high as 2.5 mg/mL.[70,72,73] Epidermal HA is more stable (less susceptible to degradation) than dermal HA, probably due to its association with hyaladherin (HA-binding proteins) that protects it against internalization and the action of HYALs.[74,75]

HA content of the dermis is significantly higher than in the epidermis, with highest levels in the papillary dermis. Dermal HA is in continuity with the lymphatic and vascular systems, regulating water balance, osmotic pressure, and ion flow, and functioning as a sieve, excluding certain molecules, enhancing the extracellular domains of the cell surfaces and stabilizing skin structures by electrostatic interactions. The main producers of HA in the dermis are papillary dermal fibroblasts. Dermal HA has access to the lymphatic system and is thought to play a role in regulating water contain in the dermis.[10]

TOPICAL HYALURONIC ACID: TRUTHS AND HOPES

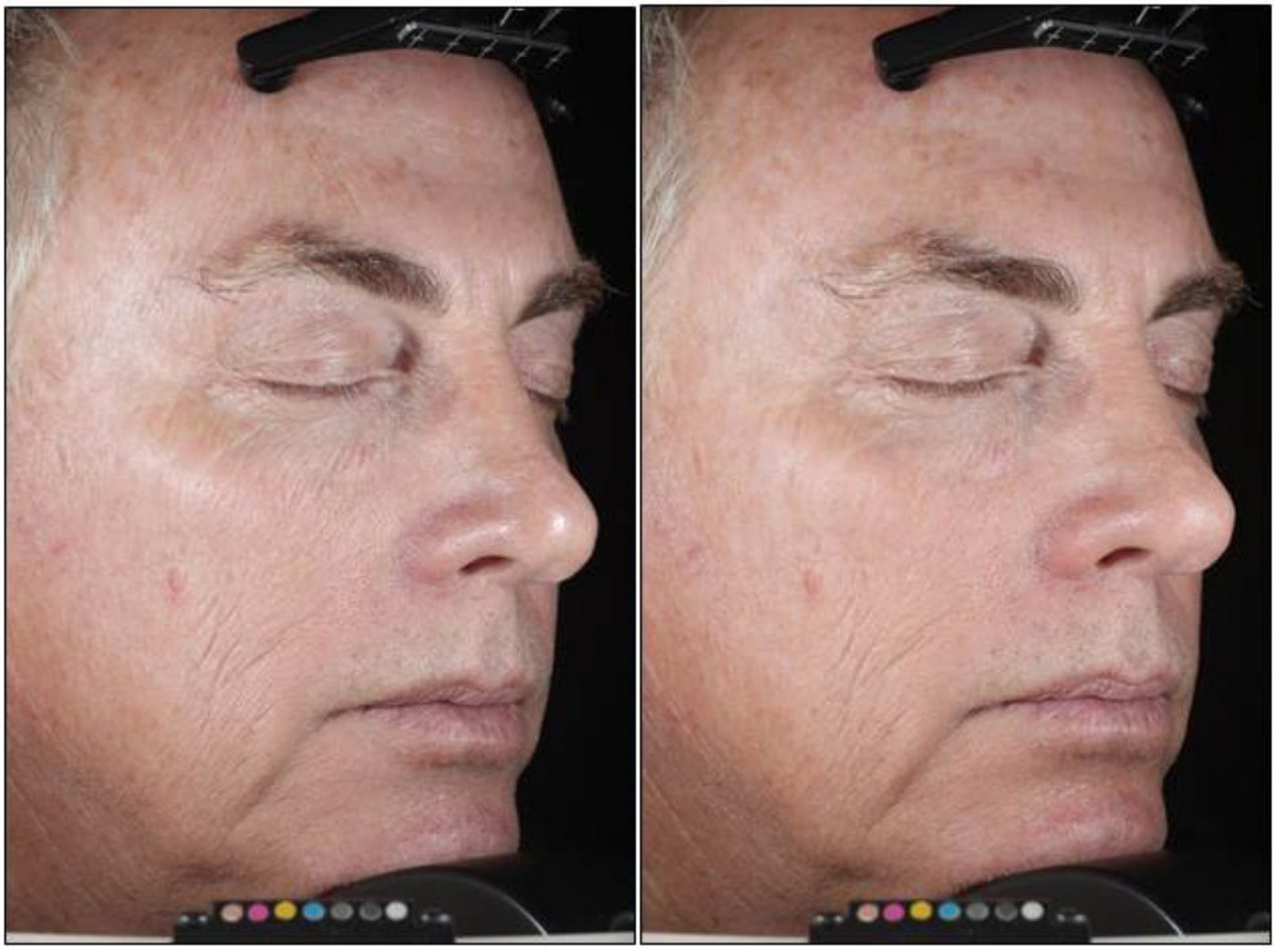
Skin hydration is highly correlated with the content and distribution of dermal GAGs, more specifically HA. One of the changes linked to photo-aged skin is a reduced level of HA and elevated levels of non-water binding chondroitin sulphate proteoglycans, resulting in a paradoxical increase in GAGs with decreased hydration.[76] In addition, the presence of solar elastosis in photo-aged skin further aggravates the decreased water binding capacity by displacing normal ECM structures with abnormally deposited elastotic material.[77] A higher level of fragmentation and aberrant deposition of HA is also linked to aged skin, diminishing even further the net decrease in this water trapping capacity.

One commonly used approach in the cosmetic industry is to improve water retention or to decelerate water loss with the use of moisturizers, which attempt to increase cutaneous water levels by using occlusive ingredients such as cocoa butter, lanolin, shea butter, and mineral oil. While these ingredients provide a smooth-feeling skin, only a temporary filling of the spaces between desquamating skin scales is accomplished. Due to the role of high MW HA in water retention, a logical step is to develop topical HA products that restore the natural hydration of dried or aged skin. Nonetheless, there are 2 major obstacles that prevent this logical solution from being the expected "holy grail" of skin hydration: *total lack of penetration and rapid degradation of externally applied HA*. In addition, the reduced capacity of aged epidermis to bind HA (due to decreased levels of CD44) further prevents topical HA from reaching the epidermis and restoring endogenous levels of this GAG.[78-81] In recent years, encapsulation and other targeted versions of HA-delivery have been attempted, but have proven to be unsuccessful in restoring epidermal HA levels.

HA[5] BREAKING THROUGH THE CLUTTER

Skin aging is linked to a decrease in the levels of HA in both dermis and epidermis. Restoration of lost dermal HA is successfully accomplished by injectable dermal fillers, which due to chemical modifications such as cross-linking are able to provide a long-lasting space-filling. However, as discussed in the previous section, topically applied HA preparations are unable

FIGURE 6. Instant smoothing of fine lines and wrinkles 15 minutes after HA[5] application. Subject 008–A (63 year old male) at baseline and 15 minutes after HA[5] application. Full-face fine lines/wrinkles visualization by parallel-polarized lighting.



to deliver any HA into the epidermis where this GAG controls key processes to maintain healthy skin such as keratinocytes proliferation and differentiation, among others.

The approach of SkinMedica (an Allergan company, Irvine, CA) to the epidermal HA dilemma led to the creation of HA[5] Rejuvenating Hydrator. Its goal was to combine 2 breakthrough technologies that retrain the skin to enhance endogenous epidermal HA production and prevent its degradation while providing an instant effect in smoothing fine lines and wrinkles and improving hydration. A breakthrough technology (VitisenSCE) was created for this product to stimulate the synthesis of endogenous HA while restoring epidermal homeostasis. VitisenSCE technology is powered by a flower stem cell extract in combination with marine micro-organism polysaccharides and peptides complex. Instant effects are delivered by combining 5 different forms of HA (labile cross-linked HA, time-release encapsulated HA, and HA with 3 different molecular weights) in combination with natural biopolymers and plankton extract. The rationale behind this mix was to provide an instant large water trapping capacity that can be sustained during extended periods of time (8-12 hours). Table 1 lists the 5 critical functions of epidermal HA and some of the biological markers that can be monitored to evaluate epidermal homeostasis.

Biological Testing

The ability of HA[5] to enhance epidermal homeostasis and promote endogenous production of epidermal HA was measured using EpiDerm Full Thickness, 3D human skin model (EFT-400, MatTek Corporation, Ashland MA, USA). In brief,

stabilized tissues were treated or not with 25 µl of HA[5] and allowed to recover for different lengths of time (16 or 24 hours). The epidermis and dermis were physically separated and independently processed for gene expression analysis using real-time quantitative reverse transcription PCR (qRT-PCR). Figure 2 shows the quantification of keratinocytes differentiation markers: fillagrin (FLG), involucrin (INV), and loricrin (LOR). It is important to note that the major function of the epidermis is to provide a barrier between the external environment and the internal organs of the body.[82] To fulfill this function, keratinocytes undergo a complex pathway of differentiation, which culminates in cornification and in the formation of extracellular, lipid-enriched lamellar membranes in the stratum corneum (SC).[82,83] This process is controlled by a cluster of genes present in chromosome 1q21, named epidermal differentiation complex (EDC). Alteration in the expression of EDC genes are linked to common skin disorders such as ichthyosis vulgaris, atopic dermatitis, and psoriasis. A decrease in the expression of these proteins is also associated with intrinsic and extrinsic aging. Our results demonstrated that treatment with HA[5] results in enhanced expression of FLG, INV, and LOR (Figure 2 A, B, and C).

Another important factor involved in preserving the functionality of the epidermal barrier and prevention of TEWL is the formation of tight junctions between keratinocytes, which occurs at the stratum granulosum level. Claudin-1 (CLDN1) and claudin-4 (CLDN4) are key factors in the formation of these structures, and deficiency in CLDN1 is linked to increased TEWL and abnormal SC formation.[84] We demonstrated that treatment with HA[5] triggers an increase in CLDN1 expression (Figure 2D) which, in addition to increased epidermal differentiation, may improve epidermal health and barrier formation/function.

An appropriated antioxidant capacity is a key factor involved in epidermal protection and homeostasis as this not only maintains the proper redox balance necessary for the normal metabolism but also neutralizes the deleterious effects of ROS. HA[5] contains a SkinMedica proprietary blend of antioxidants derived from flower stem cells, in addition to the blend of 5 different HAs, which may also act as free radicals scavengers. We evaluated the antioxidant capacity in HA[5] by radiating the tissues with UVB light. In brief, tissues were treated or not with 25 µl of HA[5] 10 minutes before UVB radiation (220 mJ/cm^2) and allowed to recover for 24 hours. As expected, this dose of UVB radiation (equivalent to 5 MED) triggered a significant increase in the number of sunburn cells (SBCs). However, pre-treatment with HA[5] significantly prevented formulation of SBCs (Figure 3, black arrows), indicating excellent antioxidant protection.

As discussed before, epidermal homeostasis is deeply linked to the presence of balanced levels of HA in this skin compartment. Adequate HA levels not only promote skin hydration but also play a critical role in controlling proliferation/differentiation/migration of keratinocytes, inflammation, and oxidative stress and promoting cell survival. Restoration of epidermal HA can only be accomplished by boosting endogenous HA production in the skin because topically applied HAs, including nano-sized hydrolyzed HA, are too large to penetrate the SC and/or are degraded rapidly. We observed that a single treatment with HA[5] significantly increased in the expression of epidermal HAS while decreasing HYAL expression (Figure 4A), suggesting a boosting of the endogenous epidermal production of HA. Furthermore, we observed an enhanced staining

FIGURE 7. Evaluation of the long-term effects of HA[5] on fine lines/wrinkles, tactile roughness. and hydration. Long-term effects at weeks 1, 4, and 8 were evaluated on the subjects' clean face (no product applied to the skin). In brief, subjects applied the product twice daily (morning and evening) to their facial skin immediately after cleansing, when the skin was still moist. Volunteers were also provided with a basic oil-free moisturizer (Ultra Sheer® moisturizer; applied twice daily after HA[5]) and sunscreen (Daily Physical Defense® SPF30+; applied after moisturizer in the mornings only). (A) Statistically significant improvements (all $P \leq .04$) in mean scores for periocular and forehead fine lines/wrinkles and tactile roughness were observed compared with untreated skin (baseline) at all follow-up visits (weeks 1, 4, and 8). (B) Corneometer instrument values reflected an increase in skin moisture content at week 1 that continued to improve over the 8 weeks of treatment. Twenty-three subjects completed the 8-week study.

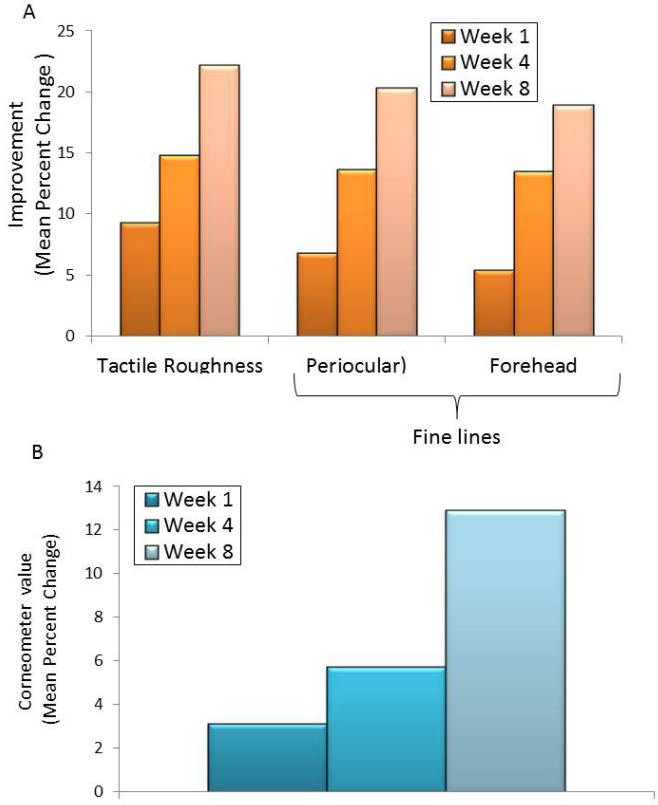


FIGURE 8. Improvements in skin texture at weeks 4 and 8 of treatment with HA[5]. Subjects from the open-label, single-center, clinical usage study as described in Figure 7. Top row: Subject 013-A (59 year-old female) at baseline and at week 4 of twice-daily use of HA[5]. Bottom row: Subject 015- A (54 year-old female) at baseline and at week 8 of twice-daily use.

for HA-binding protein (Figure 4B) upon treatment with HA^5. Taken together, these data strongly suggest a boost in endogenous epidermal HA synthesis and deposition in response to HA^5 treatment.

Clinical Testing

Instant and long-term effects after HA^5 treatment were assessed by an open-label, single-center clinical study.

Instant effects were evaluated at baseline visit after applying a thin layer of the HA^5 (nickel-sized amount) onto the entire face. We observed statistically significant improvements in mean scores for fine (periocular and forehead) lines/wrinkles and tactile roughness (Figure 5) within 15 minutes of application of HA^5 (all $P \leq .03$). In addition, HA^5 also increased intrinsic skin moisture content as measured by corneometer instrumentation (Figure 5). Figure 6 shows representative standardized digital photographs, with the visualization of the instant smoothing effects within 15 min of HA^5 application.

Long-term effects were assessed at weeks 1, 4, and 8 after treatment with HA^5 (twice daily). We observed a significant improvement in mean scores for fine (periocular and forehead) lines/wrinkles and tactile roughness (all $P \leq .04$) at all follow-up visits (Figure 7). Thus, treated skin showed a continuous increase in skin moisture content (hydration) at weeks 1, 4, and 8 compared with untreated (baseline) skin (Figure 8). Figure 8 shows representative standardized digital photographs of 2 subjects at weeks 4 and 8, respectively. Use of HA^5 produced high ratings in self-perceived efficacy from the subject questionnaires at all visits (long-term effects) as well as immediately after application (instant effects). For example, 96% of subjects noticed an improvement in the smoothness and softness of their skin after 1 week of use. Eighty-eight percent agreed that the HA^5 increased skin radiance and softened the look of lines and wrinkles at week 4, and by week 8, 87% of subjects agreed that their skin felt rejuvenated.

Overall, the HA^5 formulation was well-tolerated with mean tolerability scores remaining similar to baseline (untreated) scores. Three subjects withdrew from the study due to acne breakout (n=1) and under eye dryness (n=2).Thus, these data suggest that the temporary nature of the increased hydration is biologically significant and, if used regularly, HA^5 triggers a significant increase in endogenous HA because of enhanced biosynthesis as well as decreased degradation. On the other hand, the potential role in decreasing the inflammatory cascade has far-reaching implications for many conditions that individuals battle on a daily basis.

CONCLUSION

For decades HA has been considered a filler for empty spaces in the skin, though recent data have proven that HA is a specially active molecule in the skin and controls cell proliferation, differentiation, acute and chronic inflammation, and allergic reactions, in addition to trapping water and maintaining skin hydration. Thus, we developed the concept of *BIOHYDRATION* in which the benefits associated with the restoration of epidermal HA levels are not limited to increase water-binding capacity but also include promoting or restoring epidermal homeostasis as well as controlling inflammation. HA^5 application results in instant improvements in skin quality parameters and, within time, retrains the skin to enhance endogenous epidermal HA production.

DISCLOSURES

Virginia L. Vega, Rahul C. Mehta, Elizabeth T. Makino, and Kuniko Kadoya are employees of SkinMedica, an Allergan Company. The other authors have no relevant conflicts of interest to disclose.

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