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ORIGINAL RESEARCH

2 kDa Hyaluronic Acid Reduces Blemishes and Sagging in Human Facial Skin

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Objective: Collagen metabolism declines in aging skin, causing wrinkles. On the other hand, external factors such as UV exposure cause damage to DNA, resulting in skin problems such as spots and sagging. Hyaluronic acid (HA) is involved in many biological processes, including collagen metabolism, wound healing, and skin barrier function. This study aimed to evaluate the effect of 2 kDa HA oligosaccharides (HA2k) on skin spots and sagging.

Methods: In the human study, the effects of a HA2k lotion on spots and sagging were evaluated after 4 weeks of application to the skin. In vitro, the effects of HA2k on inflammatory cytokines, autophagy, and turnover of normal human epidermal keratinocytes (NHEK) were evaluated under two different stimulation conditions: injury with hydrogen peroxide or UVB.

Results: In the human study, HA2k treatment reduced facial spots and sagging skin after 4 weeks compared to an untreated group. The percentage reduction from baseline after 4 weeks was also higher in the HA2k-treated group. In vitro, HA2k inhibited hydrogen peroxide-induced NHEK inflammation. Furthermore, HA2k inhibited UVB-induced NHEK damage and promoted LC3-II production. In unstimulated NHEK, HA2k promoted involucrin production.

Conclusion: HA2k reduced facial spots and sagging. The effect is thought to be due to the promotion of autophagy and keratinocyte turnover. HA2k application is expected to improve skin condition.

Keywords: hyaluronic acid, autophagy, anti-aging, human study

Introduction

Human skin concerns include spots, dullness, redness, sagging, and visible pores,[1–4] mainly caused by aging and UV exposure to the skin.[3,5–7] Aging decreases epidermal cell turnover, making it reduce desquamation.[8–10] As a result, aging cells and melanin accumulate, resulting in skin spots and dryness. Furthermore, aging reportedly disrupts the balance of collagen metabolism in the dermis, which leads to a decrease in the amount of collagen in the dermis and deterioration of the quality of collagen fibers, producing wrinkles and sagging.

On the other hand, ultraviolet (UV) exposure has been reported to increase melanin production by melanocytes in the skin, leading to melanin accumulation and producing spots.[11,12] Direct cell membrane injuries, including cell death and cyclopyrimidine dimer formation due to DNA damage are transmitted to surrounding cells, releasing inflammation-inducing factors and triggering an inflammatory response responsible for skin redness.[11] Despite past efforts aiming at solving these skin problems, effective solutions have not been found so far.

Decreasing inflammation is a well-known countermeasure for skin problems. Activation of autophagy can also improve skin problems and can protect cells against UV exposure and melanosome degradation.[13–15] UV exposure damages the DNA in keratinocytes, but removing damaged cell organelles, proteins and genes via apoptosis minimizes this damage. However, excessive cell removal by apoptosis causes skin barrier dysfunction and inflammation. Given the increase in Caspase3, an indicator of apoptosis, observed in keratinocytes when autophagy was inhibited, autophagy may play an important role in protecting keratinocytes against UV exposure by suppressing excessive apoptosis.[13] Further, autophagy degrades melanosomes, and autophagy is expected to have effects on various skin problems.

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Hyaluronic acid (also known as hyaluronan, HA), which is also used in the cosmetics, aesthetic medicine, and other industries, can be an effective means of combating these skin concerns caused by aging and ultraviolet rays. HA is a polysaccharide present in the body and one of the components of the extracellular matrix. Its molecular weight ranges from 0.8 to 3000 kDa, depending on the type of tissue and biological state. Due to its high viscoelasticity and water retention properties, HA is used in cosmetics to help retain moisture in the skin and reduce the adverse effects of dryness on the skin. While generally HA has very low skin permeability, 2 kDa oligo HA penetrate the stratum corneum. In a human study, Abe et al reported that application of 2 kDa oligo HA activated collagen metabolism and improved wrinkles.[16] In vitro, HA has been reported to promote keratinocyte differentiation via CD44 phosphorylation, autophagy, and anti-inflammatory effects, and is expected to improve spots, etc.[17–19] However, its effects on human skin are unclear.

To confirm the effect of HA on human skin concerns related to aging and UV exposure, we conducted a human study with HA2k on healthy women aged 41–60 years. We performed instrumental measurements 4 weeks after application to assess skin condition, and investigated the effect of HA2k on the improvement of age spots and sagging in human skin. In addition, to examine the mechanism, we evaluated anti-inflammatory, autophagy, and keratinocyte turnover indices in vitro.

Materials and Methods

Human Studies

Evaluation of the Hyper-Pigmented Area, Translucency, Color (Lightness, Redness), Eyebags, Sagging and Skin Pores in Human Subjects

The human study was approved by the Institutional Review Board (approval numbers 1–220777-A-N-01-DICN23033) of DERMAPRO Ltd. and conducted after the participants were given an explanation of the study's purpose and informed consent was obtained. Twenty-one subjects were enrolled according to the following criteria: healthy women (age, 41–60 years) with facial pigmentation (melasma or sun lentigines) and sagging eyebrows, not pregnant or lactating, and having neither sensitive nor hypersensitive skin. During the study period, subjects did not use any cosmetics other than the test products. After washing their face, the sample products, provided as lotions, were applied twice daily (morning and evening). The lotion formulation is shown in Table 1. After cleansing, the HA2k lotion was applied to one side of the face and the control product to the other. Subjects were examined for hyperpigmentation areas, skin clarity, skin color (brightness, redness), eyelid sagging, and skin pores at baseline (week 0) and 4 weeks after treatment. Subjects were acclimatized for 20 min in a controlled atmosphere at 22°C ± 2°C and 50 ± 5% relative humidity before measurements.

Table 1 Skin Lotion Formulation Used in the Half-Side Comparison Study

Ingredient	Formulation (%)	
	Control	**HA2k**
Glycerin	3	3
Methylparaben	0.1	0.1
Phenoxyethanol	0.2	0.2
Alcohol	4.5	4.5
PEG-32	2	2
1, 3 butylene glycol	2	2
Citric acid	0.01	0.01
Sodium citrate	0.09	0.09
Disodium EDTA	0.1	0.1
Hydrolyzed sodium hyaluronate (HA2k)	—	0.1
Water	88.0	87.9
Total	100	

Abbreviations: PEG, polyethylene glycol;EDTA, ethylenediaminetetraacetic acid.

Measurement of Hyper-Pigmented Areas

Hyper-pigmentation areas were measured with ANTERA 3D® CS (Miravex Limited, Ireland), using two parameters (Hyperconcentration area, Affected area) at baseline and after 4 weeks of treatment.

Measurement of Skin Translucency

Skin translucency was measured on the cheeks with a Translucency Meter TLS850 (Dia-Stron, UK) and two parameters (K, Area) were analyzed at baseline and after 4 weeks of treatment. K is the amount of light reflected vertically from the inner skin and Area, the total amount of light reflected from the inner skin.

Measurement of Skin Color (Lightness, Redness)

Skin brightness was measured using VISIA® CR (Canfield, USA) and analyzed using Image-pro® plus (MediaCybernetics, USA). The mean lightness intensity was analyzed on the cheeks at baseline and after 4 weeks of treatment. Skin redness was measured with ANTERA 3D® CS (Miravex Limited, Ireland). The "hemoglobin mean" of skin redness was analyzed on the cheeks at baseline and after 4 weeks of treatment.

Measurement of Eyebag Sagging

Eyelid sagging was measured with PRIMOS® CR (GFMesstechnik GmbH, Germany). 3D image analysis of the same area at baseline and after 4 weeks of treatment was performed using the software Primos 5.8 version to analyze the ridge volume for eyelid sagging at baseline and after 4 weeks of treatment.

Measurement of Skin Pores

Pores were measured in the center of the cheeks using ANTERA 3D® CS (Miravex Limited, Ireland and three parameters (volume, affected area, and number) were analyzed at baseline and after 4 weeks of treatment.

In vitro Studies

Materials

Normal human epidermal keratinocytes (NHEK), cell culture medium (HuMedia-KG2), and cell culture supplement kit were purchased from KURABO Industries Ltd. (Osaka, Japan). HAbooster™, a cosmetic-grade HA2k reagent, was obtained from Kewpie Corp. (Tokyo, Japan). Cell Counting Kit-8 was purchased from Dojindo Molecular Technologies Inc. (Kumamoto, Japan). D-PBS (−), D-PBS (+) Preparation Reagent (Ca, Mg Solution), and RIPA buffer were purchased from Nacalai tesque Inc. (Kyoto, Japan). Hydrogen peroxide was purchased from Fujifilm WAKO Pure Chemical Corp. (Osaka, Japan). The autophagy Enzyme Linked-Immuno Sorbent Assay (ELISA) Kit (LC3-II Quantitation) was purchased from Cell BioLabs Inc. (San Diego, CA, USA) and the involucrin ELISA Kit from WUHAN HUAMEI BIOTECH Co., Ltd. (Wuhan, China). TaKaRa BCA Protein Assay Kit was purchased from Takara Bio Inc. (Shiga, Japan) and the Monarch Total RNA Miniprep Kit from BioLabs Inc. (NEB, USA). High-Capacity RNA to cDNA Kit, TaqMan™ Fast Advanced Master Mix, Human TNF-α qPCR Primer (Hs00174128_m1), Human IL-1β qPCR Primer (Hs01555410_m1), and GAPDH (4352934E) were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Cell Culture

The effects of HA2k on inflammatory, autophagy, cell proliferation, and turnover were evaluated in three different NHEK conditions: under hydrogen peroxide (H_2O_2) or UVB damage, and non-treated. In the pre-culture, cells were seeded at 2.5×10^3 cells/cm^2 in 75 cm^2 flasks and incubated at 37°C, 5% CO_2 incubator; the medium was changed the next day. When the cells reached confluency, they were used for further testing. For the H_2O_2- and UVB-induced tests, cells were seeded at a density of 5×10^3 cells/cm^2. Non-treated cells to evaluate turnover and determine the effect on cell proliferation were used at a density of 4×10^3 cells/cm^2.

Hydrogen Peroxide Damage

NHEK were seeded at a density of 5×10^3 cells/cm^2 in 24-well and 12-well plates and cultured for 24 h, then replaced with a test medium supplemented with HA2k for 48 h, followed by H_2O_2 treatment. Based on preliminary results, H_2O_2

induction was performed at 1 μM. To this end, 2 μM H_2O_2 /D-PBS (−) and a solution of the test substance at twice the final concentration were mixed immediately before stimulation and added to NHEK, which were incubated at 37°C for 10 min. After the end of stimulation, the H_2O_2 in the wells was removed, they were washed twice with D-PBS (−) and once again with culture medium, before adding the respective test medium and incubating at 37°C, 5% CO_2 for 24 h.

UVB Damage

NHEK were seeded in 24-well plates at a density of 5×10^3 cells/cm^2 and cultured for 24 h, before replacing the media with a test medium supplemented with HA2k for 48 h, followed by UV-damage. To this end, a UVB lamp (G15T8E, Sankyo Denki Co., Ltd., Tokyo,Japan) was used as UVB light source, and the dose (peak wavelength 306 nm) was measured before damage using a digital UV intensity meter (UV-340A, Kenis Co., Ltd., Osaka, Japan). First, the test medium was removed and cells were washed with D-PBS (−), and then replaced with D-PBS (−) before UVB-induced (25 mJ/cm^2). After the end of stimulation, the D-PBS (−) in the well was removed, cells were washed twice with D-PBS (−) and once with culture medium, before adding the respective test medium and incubating at 37°C, 5% CO_2 for 24 h.

Evaluation of Cell Viability

We evaluated cell viability 24 h after induction of damage with H_2O_2 and UVB. In untreated cells, cell viability was evaluated 24 and 72 h after HA2k addition. Cell viability was assessed using a Cell Counting Kit-8 (Dojindo Molecular Technologies Inc). Measurements were performed according to manufacturer's instructions. Absorbance was measured at 450 nm using a Powerscan HT Multi-Detection Microplate Reader (BioTek Japan). The formula for cell viability was as follows:

$$Cell\,Viability\,(\%)\ \frac{OD\,treatment\,group - OD\,blank\,background}{OD\,non\,treatment\,group - OD\,blank\,background} \times 100$$

RNA Extraction and Quantitative Real-Time PCR

Cell Collection and RNA Extraction

NHEK were seeded in a 12-well plate at a density of 5×10^3 cells/cm^2 and cultured for 24 h, before replacing with the test medium supplemented with HA2k and incubated for 48 h (37°C, 5% CO_2).

The cells were induced with 1 μM H_2O_2 and washed with D-PBS (−) after removing H_2O_2. Thereafter, the medium was replaced by a medium containing HA2k and incubated for 24 h.

Monarch Total RNA Miniprep Kit was used according to the manufacturer's protocol. Test medium was removed from the 12-well plates and the cells were washed with D-PBS (−). Then, the lysis buffer from the Monarch Total RNA Miniprep Kit was directly added and pipetted without mixing, and the cell lysate was transferred to an RNase-free tube equipped with a DNA removal column and centrifuged at 16,000 g for 30s. An equal volume of ethanol (>95% ethanol) was added to the filtrate. Then, the RNA solution with ethanol was added to a new RNase-free tube containing an RNA purification column, centrifuged at 16,000 g for 30s, and the filtrate was discarded. After adding 500 μL of RNA Wash Buffer and spinning at 16,000 g for 30s, a mixture of 5 μL of DNase I and 75 μL of DNase I Reaction Buffer was added to the RNase-free tube and allowed to react for 15 min at room temperature. Then, 500 μL of RNA Priming Buffer was added and centrifuged at 16,000 g for 30s, followed by addition of 500 μL of RNA Wash Buffer and centrifugation at 16,000 g for 30s. The column was then transferred to a new RNase-free tube, 50–100 μL of Nuclease free water was added, and centrifuged at 16,000 g for 30s to extract the RNA. The extracted RNA was stored at −80°C until use.

cDNA Synthesis, qPCR, and Quantification of Gene Expression Level

Using the High-Capacity RNA to cDNA Kit, cDNA synthesis was performed on all isolated RNAs according to the manufacturer's protocol and subjected to real-time PCR. In real-time PCR, TNF-α and IL-1β expression levels were measured and normalized to those of GAPDH. The calculation was performed using the calibration curve method, and fluorescent labeling was performed using the FAM dye. qPCR was performed using the Real-Time QPCR System Mx3005P (Agilent Technologies) with TaqMan® fast advanced master mix according to the manufacturer's protocol. The reaction was performed for 60 cycles of initial denaturation (95°C, 20s, 1 cycle), annealing (95°C, 1 s), and

polymerization (60°C, 20s). Amplified PCR products were quantified by measuring each gene and GAPDH mRNA calculated cycle thresholds. The amount of specific mRNA in samples was calculated from the standard curve and normalized to the GAPDH mRNA. The results were expressed as n-fold differences relative to normal controls (relative expression levels).

LC3-II ELISA

NHEK were seeded at a density of 5×10^3 cells/cm^2 in a 24-well plate and cultured for 24 h, then replaced with test medium containing HA2k and cultured for 48 h (37°C, 5% CO_2). In the control group, the culture medium was used as the test medium. 48 h after the addition of the test medium, the medium was collected and replaced with D-PBS (-), and the lid of the microplate was removed and UVB irradiation was performed. After UVB irradiation, the D-PBS (-) was removed and replaced with the test medium, and incubated at 37°C, 5% CO_2 for 24 h. 24 h after UVB irradiation, the test medium was removed and the cells were washed with D-PBS (-). Diluted Cytosolic LC3 Removal reagent was then added to the wells and reacted at room temperature for 5 min while shaking. Next, the wells were washed three times with D-PBS (+) solution, and chilled x 1 RIPA buffer was added and reacted on ice for 10 min to lyse the cells. After 10 min of reaction, the cells were detached and collected in a microtube. The cells were then centrifuged at 4°C, 12,000 rpm (13,500 rcf) for 10 min, and the centrifugal supernatant was used as the sample for analysis. The protein level of the keratinocyte autophagy marker LC3-II was measured using the Autophagy ELISA Kit (CELL BIOLABS, INC). The protein level in keratinocytes was measured using the BCA Protein Assay Kit (TaKaRa. Bio. Inc). The amount of LC3-II produced was calculated using the following formula:

$$Amount\ of\ LC3-II = \frac{LC3-II\ concentration}{Protein\ concentration}$$

$$Relative\ Amount\ of\ LC3-II\ (\%) = \frac{Amount\ of\ LC3-II\ in\ treatment\ group}{Amount\ of\ LC3-II\ in\ Control\ group} \times 100$$

Involucrin ELISA

The amount of involucrin protein, a differentiation marker for keratinocytes, was measured using the Involucrin ELISA Kit (WUHAN HUAMEI BIOTECH Co., Ltd). NHEK were seeded in a 24-well plate at a density of 4×10^3 cells/cm^2, cultured for 24 h, then medium was replaced with test medium supplemented with 100 µg/mL HA2k, and cultured for 24 and 48 h (37°C, 5% CO_2). In the control group, the culture medium was used as test medium. Then, the test medium was removed from the 24-well plate and the cells were washed twice with D-PBS(+), before adding a RIPA buffer and gently shaking for 5 min. After a 5 min reaction, cells were detached and collected using a disposable scraper and a micropipette. The collected cell lysate was centrifuged at 13,500 × g for 10 min at 4°C, and the centrifuged supernatant was used as analysis sample. The amount of protein in the supernatant was measured using TaKaRa BCA Protein Assay Kit (Takara Bio Inc), and the amount of involucrin produced calculated as follows:

$$Amount\ of\ Involucrin = \frac{Involucrin\ concentration}{Protein\ concentration}$$

$$Relative\ Amount\ of\ Involucrin\ (\%) = \frac{Amount\ of\ Involucrin\ in\ treatment\ group}{Amount\ of\ Involucrin\ in\ Control\ group} \times 100$$

Data Analysis

Test results are presented as mean ± standard error. Statistical analysis was performed using IBM SPSS Statistics 20 (IBM Corporation). In the human experiment, the Shapiro–Wilkes test was used to determine if the variables followed a normal distribution. For comparisons between time points, a paired *t*-test was used as parametric test and a Wilcoxon signed rank test for nonparametric variables. For comparisons between groups, paired t-tests for homogeneity tests, RM ANOVA or ANCOVA were used. Statistical significance is indicated in the Figure with * and # for $p < 0.05$. The percent change from baseline (%) was defined as: (baseline - after treatment)/baseline × 100. For in vitro experiments, a one-way

ANOVA was performed with a 95% confidence interval, and a two-tailed Dunnett test was used as a post-test. Statistical significance is indicated with *** for $p < 0.001$, ** for $p < 0.01$, and * for $p < 0.05$. For the evaluation of gene expression levels, the no-treatment group was compared with the control group using the Student's *t*-test.

Results

Human Studies

Effect of HA2k on Pigmented Area

Compared to baseline, the hyperconcentration and affected area of the treated site significantly decreased at 4 weeks ($p < 0.05$), with a decrease of −0.22 A.U. (9%) and −4.1 mm^2 (11%), respectively (Figure 1A and B). In comparison between groups, the hyperconcentration and affected area in the 0.1% HA2k-lotion site were significantly lower than in the control site ($p < 0.05$). This indicates that the application of HA2k-containing lotion improved the pigmented area of the skin.

Effect of HA2k on Skin Translucency

The K and area of skin clarity at the 0.1% HA2k lotion application site increased significantly after 4 weeks compared to baseline ($p < 0.05$), with increases of 44 A.U. (2%) and 2718 A.U. (4%), respectively (Figure 1C and D). Between-group comparison showed that the K and area of the 0.1% HA2k lotion site were significantly higher than in the control site ($p < 0.05$). This result indicates that the application of HA2k-containing lotion improved skin clarity.

Effect of HA2k on Skin Color (Lightness, Redness)

Regarding lightness, there was no significant difference in the mean intensity of skin at the site of 0.1% HA2k lotion application after 4 weeks compared to baseline (Figure 1E), nor was there a significant difference between groups.

Regarding redness, the mean hemoglobin level, which measures skin redness, at the 0.1% HA2k lotion application site was lower but not significantly different at 4 weeks compared to baseline (Figure 1F). In the between-group comparison, the average hemoglobin level in areas treated with 0.1% HA2k lotion was significantly lower than in the control site ($p < 0.05$). These results indicate that application of HA2k lotion improved skin redness.

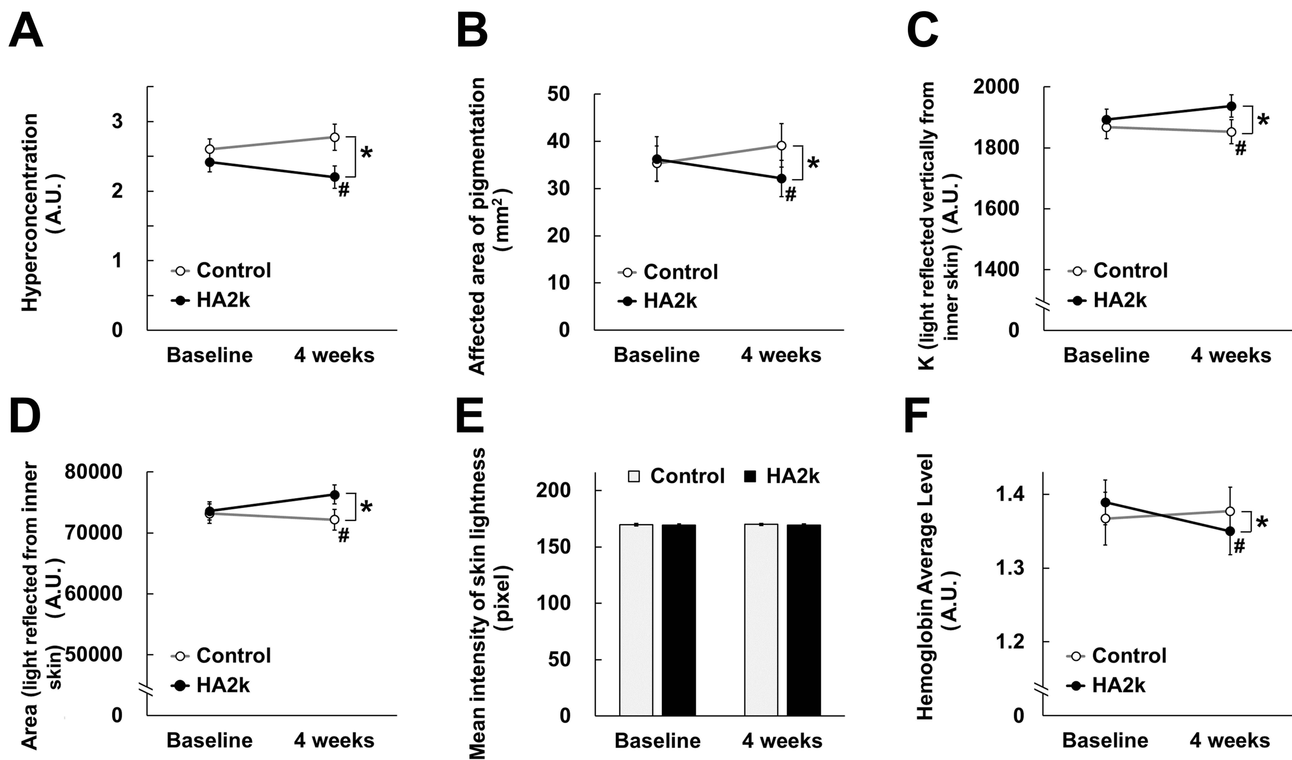

Figure 1 Continued.

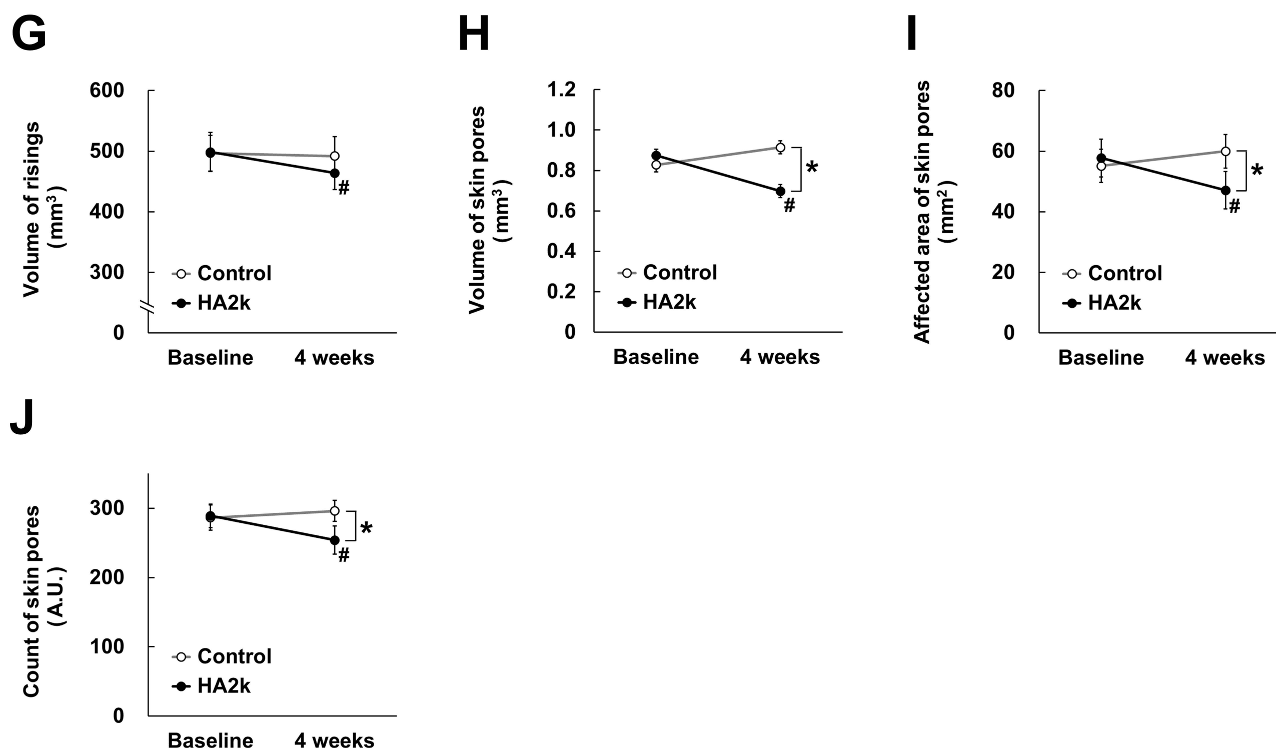

Figure 1 We investigated the effect of 0.1% HA2k lotion on pigmented areas, the effect on clarity, the effect on skin color (brightness, redness), the effect on sagging eyes, and the effect on skin pores (volume, affected area, count). The pigmented areas were measured with ANTERA 3D ® CS for two parameters (**A**) and (**B**). (**A**) Hyperconcentration pigmentation area intensity, (**B**) Affected area of pigmentation. The skin translucency was measured with Translucency Meter TLS850 for two parameters (**C**) and (**D**). (**C**) K (amount of light reflected vertically from the inner skin), (**D**) Area (total amount of light reflected from the inner skin). The skin brightness was measured with VISIA ® CR and analyzed using Image-pro ® plus. (**E**) Mean intensity of skin lightness. The skin redness was measured using ANTERA 3D ® CS. (**F**) Hemoglobin Average Level (Average hemoglobin level of skin redness). The eyebag sagging was measured with PRIMOS ® CR and 3D image analysis. (**G**) Volume of risings (Risings volume for eyelid sagging).The skin pores (volume, affected area, count) in the center of the cheek were analyzed with ANTERA 3D ® CS for three parameters (**H**), (**I**) and (**J**). (**H**) Volume of skin pores volume, (**I**) affected area of skin pores, and (**J**) Count of skin pores. Control: lotion without HA2k, HA2k: lotion with 0.1% HA2k. Mean ± SE. n = 21. *:Significantly different compared with group at p <0.05, #:significantly different compared with baseline at p < 0.05.

Effect of HA2k on Eyebag Sagging

Sagging eyebags at the 0.1% HA2k lotion application site were significantly reduced after 4 weeks compared to baseline ($p < 0.05$), with a −34.7mm^3 (−7%) reduction (Figure 1G). No significant difference was observed between groups. These results indicate that the application of HA2k-containing lotion improved eyelid sagging.

Effect of HA2k on Skin Pores

Pore volume, affected area, and number (Count) at the 0.1% HA2k lotion application site were significantly reduced after 4 weeks compared to baseline ($p < 0.05$), with reductions of −0.18 mm^3 (−20%), −11 mm^2 (−18%), and −35 A.U. (−12%), respectively (Figure 1H–J). All parameters were significantly lower at the 0.1% HA2k-containing lotion application site than at the non-treated site ($p < 0.05$). This result indicates that the application of HA2k-containing lotion improved skin pore size.

In vitro Studies

Effect of HA2k on Cell Proliferation

There was no significant difference in cell viability after 24 h and 72 h between unstimulated NHEK cells treated with HA2k 0.1, 1, 10, and 100 µg/mL and the control group (Figure 2A and B).

Anti-Inflammatory Effect of HA2k

At 6 h after NHEK stimulation with 1 µM H_2O_2 for 10 min, the gene expression levels of inflammatory cytokines increased in the control group compared to the non-treatment group. The gene expression levels of TNF-α mRNA and

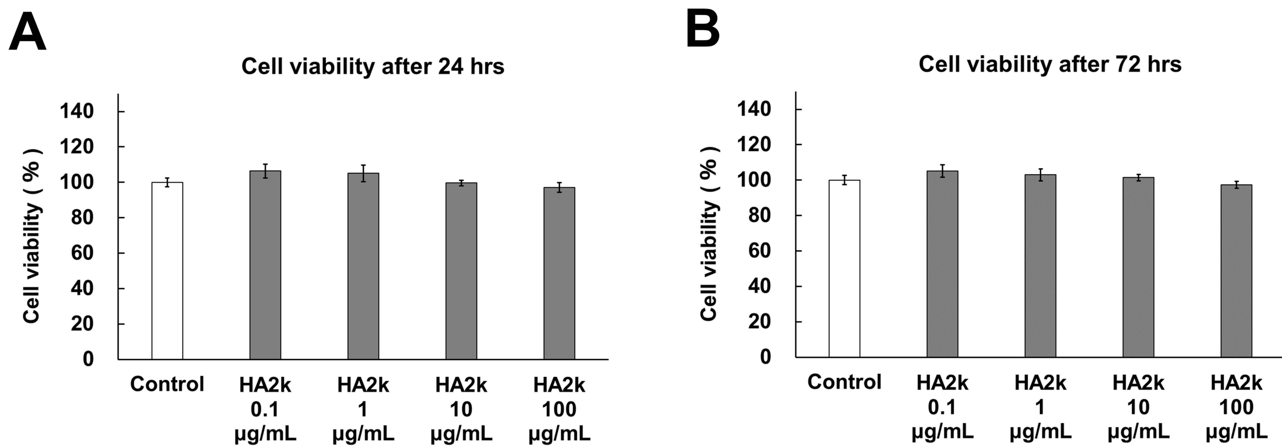

Figure 2 Effect of HA2k treatment on cell viability of NHEK cells. NHEK cells treated with HA2k at 0.1 μg/mL, 1 μg/mL, 10 μg/mL, and 100 μg/mL for 24 and 72 h. (**A**) Cell viability after 24 h, (**B**) Cell viability after 72 h. The ratio (%) to control at each time point is expressed as Mean ± SE. n=6. Results of two experiments. Dunnett test (vs control).

IL-1β mRNA in the control group were about 3.2-fold and 2.3-fold higher than in the no-treatment group, respectively. Conversely, the TNF-α mRNA and IL-β mRNA gene expression levels in the HA2k 10 μg/mL group were significantly lower than those in the control group (Figure 3A and B).

Effect of HA2k on Hydrogen Peroxide-Induced Keratinocyte Cell Death

After NHEK stimulation of with 1 μM H_2O_2 for 10 min, the cell viability of the control group at 24 h was 30% lower than that of the non-treated group. The 1 μg/mL, 10 μg/mL and 100 μg/mL HA2k groups showed significantly higher, and concentration dependent, cell viability than the control group (Figure 4).

Effect of HA2k on UVB-Induced Keratinocyte Cell Death

The cell viability at 24 h after NHEK stimulation with UVB 25 mJ/cm^2 was 17% lower in the control group than in the non-treated group, whereas in the HA2k 10 μg/mL and 100 μg/mL groups it was significantly higher than in the control group (Figure 5).

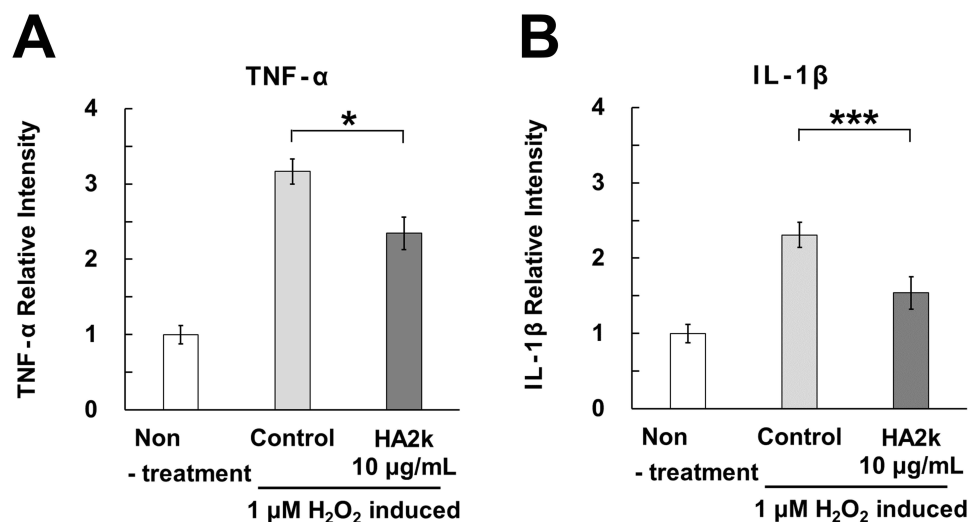

Figure 3 Effect of HA2k on inflammatory cytokine expression levels after H_2O_2 treatment. Cells cultured for 48 h in test medium supplemented with HA2k 10 μg/mL and stimulated with H_2O_2. (**A**) TNF-α mRNA gene expression level at 6 h after H_2O_2 stimulation. (**B**) IL-1β mRNA gene expression level at 6 h after H_2O_2 stimulation. Mean ± SE. n=6. Results of two experiments. *p < 0.05, ***p < 0.001, Student's *t*-test (vs control).

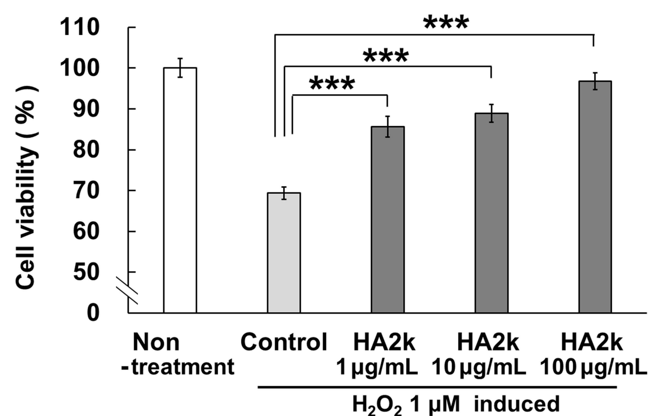

Figure 4 Effect of HA2k in cell viability after H_2O_2 treatment. Cells cultured for 48 h in test medium supplemented with HA2k and stimulated with H_2O_2. Cell viability at 24 h after H_2O_2 stimulation. Mean ± SE. n=12. Results of two experiments. ***p < 0.001, Dunnett test (vs control).

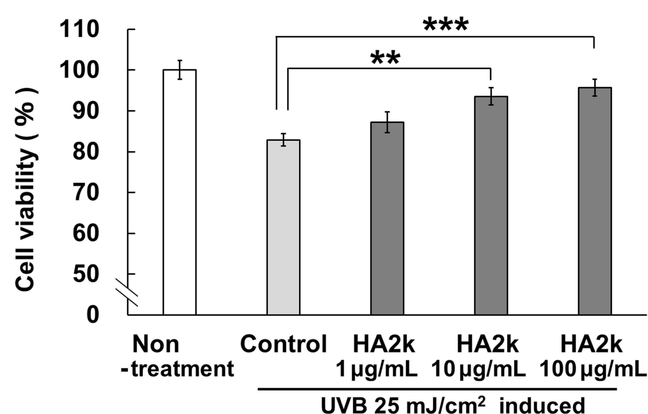

Figure 5 Effect of HA2k in cell viability after UVB treatment. Cells cultured for 48 h in test medium supplemented with HA2k and stimulated with UVB. Cell viability assessed at 24 h after UVB stimulation. Mean ± SE. n=13 for non-treatment and control groups, n=12 for each HA2k group. Results of two experiments. **p < 0.01,***p < 0.001, Dunnett test (vs control).

Effect of HA2k on Autophagy

The amount of LC3-II in (control) NHEK cells irradiated with at UVB 25 mJ/cm^2 at 24 h was about 2.3-fold higher than in the untreated group. Similarly, the HA2k 10 μg/mL and 100 μg/mL groups showed significantly higher values than the control group (Figure 6).

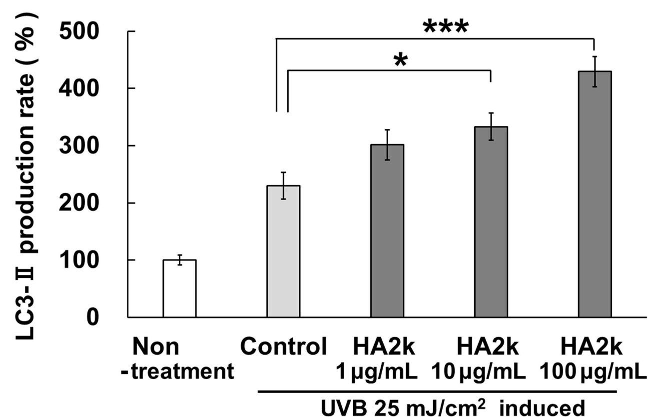

Figure 6 Effect of HA2k on LC3-II production in UVB-irradiated NHEK cells. Cells cultured for 48 h in test medium supplemented with HA2k and irradiated with UVB. Mean ± SE. n=12. Results of two experiments. *p < 0.05, ***p < 0.001, Dunnett test (vs control).

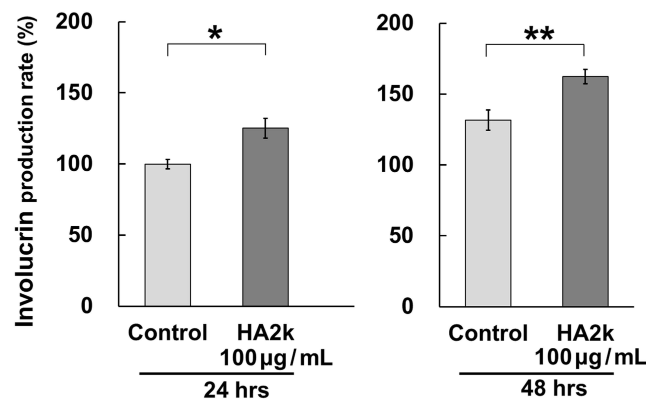

Figure 7 Effect of HA2k on involucrin production in non-stimulated NHEK cells. Mean ± SE. n=5. *p < 0.05, **p < 0.01, Student's *t*-test (vs control).

Effect of HA2k on Involucrin Production

The addition of 100 µg/mL of HA2k to non-stimulated NHEK cells resulted in significantly higher involucrin levels at 24 and 48 h compared to the control group (Figure 7).

Discussion

In this study, tests were conducted on humans applying HA2k for 4 weeks to evaluate pigmentation, transparency, brightness, redness, sagging under eyes, and pores. As a result, the effectiveness of HA2k was confirmed in all evaluation items except brightness. Although HA2k has been reported to have anti-wrinkle effects, this is the first human trial to clarify its effects on pigmentation and skin clarity.

In human study, pigmentation (intensity and size of spots) and transparency associated with dark spots significantly decreased with application of HA2k to the skin.

Dark spots are thought to be caused by abnormalities in aging keratinocytes. Keratinocytes contain melanosomes, which overgrow and accumulate in the skin, resulting in spots. Dullness is also caused by abnormalities in melanocytes. Melanocytes are responsible for protecting the skin by producing melanosome when stimulated by UVB, which are then passed to keratinocytes to create a melanin cap. However, with aging and UVB exposure, melanocytes become hyperactive and continue to produce melanin, which is considered dullness. For these reasons, in order to suppress dark spots and dullness, it is necessary to promote epithelial turnover as well as the degradation of melanosomes.

In vitro, the protein level of Involucrin, an indicator of turnover promotion, significantly increased with HA2k addition. HA-tetrasaccharide appears to induce keratinocyte differentiation through CD44 phosphorylation.[17] Since HA2k contains HA-tetrasaccharide it may increase involucrin through CD44 phosphorylation. In addition, the amount of LC3-II protein, an indicator of autophagy, and cell viability were examined under UV irradiation to confirm autophagy action. Activation of autophagy can protect cells against UV exposure.[13] Autophagy also degrades melanosomes in keratinocytes.[14] Since in vitro we observed increased production of involucrin and LC3-II, HA2k may act on keratinocytes to improve pigmentation and transparency via turnover promotion and autophagy activation. HA has been reported to exert various functions via TLRs and CD44.[17,18] The effects of turnover and autophagy are also thought to be mediated by TLRs and CD44, but the detailed mechanism needs to be confirmed in the future.

There was no significant difference in brightness compared to the control. Since the test period was short (4 weeks), partial pigmentation improved, but the overall face brightness did not. On the other hand, redness significantly decreased with HA2k application compared to untreated skin. Skin redness has been reported to be associated with inflammation. In vitro, gene expression levels of inflammatory cytokines such as TNF-α and IL-1β increased under H_2O_2, but were significantly suppressed by HA2k. These results suggest that inflammation was similarly suppressed and redness was reduced in the human study. Ultra-low HA (0.8 kDa) have been reported to inhibit TLR-mediated inflammation and UVB-induced keratinocyte inflammation.[18] Similary, HA2k is thought to act on TLRs in keratinocytes and suppress gene expression of TNF-α and IL-1β.

Sagging under the eyes significantly decreased with HA2k application. Since 2 kDa oligo HA has been reported to activate collagen remodeling,[16] the same mechanism may be involved in the improvement of eye sagging.

Volume, area, and pore number were significantly lower with HA2k application. Sagging skin, implicated in the condition of pores, was improved by HA via activation of collagen metabolism.[16] In addition, pores can be affected by clogged keratin plugs, and autophagy has been reported to play an important role in regulating adipogenesis in human sebaceous gland cells.[20,21] Thus, given the autophagy activation observed in NHEK, and the above evidence, the improvement of pore condition may be related to the activation of the collagen cycle and autophagy.

In the present study, no cytological evaluation was performed in human studies. Therefore, it is thought that HA2k penetrates the stratum corneum and acts via CD44 and other factors on epidermal cells, but the underlying mechanism remains unclear. In addition, whether the effects of autophagy are directly linked to the results in human studies needs to be investigated in future research through melanin degradation studies in sebaceous gland organoids.

Conclusion

When applied to human skin, HA2k suppressed blemishes and sagging. In vitro studies also suggested that it promoted keratinocyte autophagy and turnover. These findings indicate that HA2k may promote beneficial responses associated with improving the skin's barrier function, moisture retention and may be effective in treating skin diseases. This study suggests that HA2k may be useful as a cosmetic or pharmaceutical ingredient to restore damaged skin function.

Ethical Approval

This study was conducted in accordance with the Declaration of Helsinki and approved by the DERMAPRO Ltd. Institutional Review Board, with approval numbers 1-220777-A-N-01-DICN23033.

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Disclosure

The authors report no conflicts of interest in this work.

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