의학박사 학위논문

The Safety and Effectiveness of Ascorbyl Propyl Hyaluronate in Atopic Dermatitis

아토피 피부염 환자에 적용한

Ascorbyl Propyl Hyaluronate의

안전성 및 유효성에 관한 연구

2022년 8월

서울대학교 대학원

의학과 성형외과학 전공

김 지 훈

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이 논문을 의학박사 학위논문으로 제출함

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Abstract

The Safety and Effectiveness of Ascorbyl Propyl Hyaluronate in Atopic Dermatitis

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Atopic dermatitis (AD) is chronic relapsing inflammation of the skin associated with severe itching. It is results in structural and functional damage of skin. Recently, ascorbic acid (AA) was reported to plays an important role in AD. The AD symptom was alleviated, when the decreased AA was supplemented in the skin. Ascorbyl propyl hyaluronate (APH), a hyaluronate-ascorbic acid derivative, is a cosmetic ingredient improved stability and skin permeability. In this study, we aimed to assess the safety and efficacy of APH in AD patients. We demonstrated that APH is improved thermal stability compared with ascorbic acid. APH treatment did not affect cell morphology and viability of immortalized human keratinocytes (HaCaT) and human dermal fibroblasts (HDF). Moreover, APH had free-radical scavenging effect and reduced inflammatory cytokine such as TNF-α and IL-1α. Risk assessment of APH were evaluated by skin irritation and sensitization test. There was no adverse effect. We observed that topical application of APH improved EASI (eczema area and severity index), skin hydration, trans-epidermal water loss and pruritus of AD patients. These findings suggest that APH can be used as an active ingredient to alleviate AD symptoms.

Keywords: Atopic dermatitis, Ascorbic acid, Hyaluronic acid, Anti-oxidants, Anti-inflammation

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INTRODUCTION

Atopic dermatitis (AD) is a common chronic inflammatory skin disease characterized by recurrent severe pruritic eczematous lesions[1,2]. It affects all ages ranging from childhood (15−20%) to adulthood (1−3%) with an increasing prevalence worldwide[3,4]. Although the etiopathogenesis of AD is not fully understood yet, it is known to be complex and multifactorial involving interactions among genetic, environmental, and immunological aspects[5].

Skin barrier dysfunction and inflammatory reaction are the most important histopathologic findings for patients with AD[6]. An increase in trans−epidermal water loss(TEWL) is also observed in patients with AD[7]. Therefore, keys to successful management of AD should include skin hydration and skin barrier repair, topical anti−inflammatory medications (topical corticosteroids or calcineurin inhibitors), control of infection and elimination of exacerbating factors (including allergens, irritants and emotional triggers) that might exacerbate the scratch−itch cycle[8, 9].

Pruritus, the main symptom of AD, is known to be considerably alleviated by restoring functions of skin barrier and hydration[10,11]. Regulation of pruritus is considered to be critical in the treatment of AD. Thus, prescriptions for AD patients often include steroid ointments to decrease pruritus and inflammation with oral administration of antihistamine medicine for sedation[12]. However, side effects and the development of tolerance to steroid ointment are

perceived to be main problems of such AD treatment[13]. Thus, the development of a biocompatible therapeutic agent is urgently required[14-16].

Ascorbic acid (vitamin C) is an essential water-soluble nutrient that primarily exerts its effect on host defense mechanisms and immune homeostasis by being the most important physiological antioxidant[17,18]. Ascorbic acid can prompts anti-allergic reaction by controlling serum level of immunoglobulin E or preventing the secretion of histamine[19]. Furthermore, ascorbic acid is effective in suppressing PGE2, an inflammation marker, and controlling the increase of IL-6 and TNF-α cytokines during early stages of inflammation[20]. It can also stimulate collagen biosynthesis in the outer layer of keratinocytes and epithelial cells[21,22], thereby preventing a secondary infection caused by the loss of skin barrier by protecting damaged skin and hastening skin reproduction. Therefore, it is thought that ascorbic acid will be effective in treating skin diseases such as AD and psoriasis known to involve pruritus and inflammation. Recent studies have shown that ascorbic acid level in the skin of an AD patient is lower than that in a healthy person[23], demonstrating the importance of supplementing ascorbic acid in the treatment of AD. Despite its efficacy in biological activity, the instability and low skin absorption rate of AA[24] make it difficult to be actually applied as a remedy for AD.

Hyaluronic acid (HA) is an organic material created inside the body. It is a highly polymerized compound that consists of N-

acetylglucosamine and glucuronic acid joined alternately in the form of a chain. It is found in a considerable amount in the vitreous body of the eye, the umbilical cord, and the cockscomb of a chicken[25]. Its characteristics include its ability to absorb moisture several hundred times its weight, making it ideal for use in natural moisturizers. It is the main component of the skin and cartilaginous tissues[26]. Various derivatives of HA are effective in reproducing connective tissues. They are widely used for tissue regeneration[27,28]. For example, HA has been used as an anti-adhesion agent after surgery, prosthetics in plastic surgery, and arthritis treatment. One of the strategies to treat pruritus symptoms of AD is to minimize skin dehydration and supply moisture. Maintaining a clean skin is also important for suppressing the exacerbation of AD and preventing AD from becoming chronic as it can prevent skin dehydration and secondary skin infection. A desirable moisturizer should be able to prevent damage to the skin barrier and protect the skin from external microorganisms, pollutants, dust, and so on. AD patients are recommended to apply HA to the skin using moisturizes of cream or lotion type.

Ascorbyl propyl hyaluronate (APH) is a new material with a higher stability than AA by combining AA with HA to improve its biocompatibility. It can suppress oxidation-reduction reaction and radical reactions of AA. Using APH with its stabilized AA's antioxidant and anti-inflammation effects and HA's moisturization effect in the treatment of AD can help improve main symptoms of

immune response and dehydrated skin of AD patients. It is expected to have a higher effect than using AA or HA alone.

Thus, the aim of this study was to assess the stability of an ascorbic acid derivative APH and its anti-oxidant and anti-inflammatory effects in vitro. The safety and efficacy of APH in AD patients and its use in functional cosmetic products and medical supplies were also investigated.

MATERIALS AND METHODS

1. Reagents and antibodies

Reagents and antibodies. Dulbecco' s modified Eagle' s medium (DMEM) and fetal bovine serum (FBS) were obtained from Invitrogen-Gibco (Grand Island, NY. USA). Ascorbic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), lipoteichoic acid (LTA) and lipopolysaccharides (LPS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Oligo hyaluronic acid with an average molecular weight of 8,000 Da was obtained from SK bioland (Dongnam, Korea).

2. In-vitro thermal stability test

APH and AA were dissolved in water at concentrations of 1000ppm and 50ppm, respectively, and incubated at 50 ° C for 24 hours and 48 hours. Samples collected at 0h, 24 h and 48 h after incubation were analyzed by HPLC (Agilent technology 1200 series, Santa Clara, CA. USA). The area value of 0h sample as control was considered as 100%. Peak area values at 24 h and 48 h were calculated and compared with those of the control.

3. DPPH assay

Free radical scavenging activity can be estimated using the DPPH assay. We tested APH at eight different concentrations (10000, 5000, 1000, 500, 100, 50, 10, and 5 ppm). AA and HA as positive and

negative controls, respectively, were tested at same concentrations. Antioxidant–radical reactions were conducted for 10 min in the dark at ambient temperature. A decrease in absorbance at 517 nm was measured against a blank of pure ethanol to estimate the radical scavenging capacity of each sample.

4. *In vitro* skin permeation studies

Experiments were conducted using a Phoenix Robotic Diffusion Station (Hanson Research, USA) according to the Organization for Economic Co-operation and Development (OECD) guidelines. In this test, Strat-M® Membrane Filters were used to mimic the stratum corneum of the human skin. A dose of 150 μL of the formulation was applied to the donor compartment using a micropipette. Experiments were conducted at 32.5 ° C with a stirring speed of 600rpm. These three parameters allowed to maintain sink conditions. A volume of 450 μL of receptor fluid was collected at different time intervals up to 24 h and an equal volume of fresh temperature-equilibrated PBS solution was added to the receptor compartment. All samples were analyzed using a validated HPLC method.

5. Skin permeation analysis

Flux indicated the permeation rate, which could be calculated from the amount of permeation material per time and area, against the initial concentration applied to the upper skin area. Its unit is μg/h/cm2. Permeability coefficient (Kp) can be calculated from flux

value divided by the initial concentration of the test substance. Its unit is cm/h.

6. Cell line and culture conditions

Immortalized human keratinocytes (HaCaT) and neonatal human dermal fibroblasts (HDF, ATCC Catalog No. PCS-201-010) were purchased from ATCC (Manassas, VA, USA). These cells were maintained in DMEM supplemented with 10% (v/v) FBS and 1% penicillin/streptomycin at 37 ° C with 5% CO_2. The effect of APH on cell proliferation of HDF was evaluated using WST-1 assay. Briefly, 3 x 10^3 cells were suspended in 200 μl of assay medium, seeded onto a 96-well cell culture plate, and incubated at 37 ° C with 5% CO_2 for 24 h. Cells were treated with various concentrations of APH. An equivalent amount of AA was present in the APH as a control. After incubating at 37 ° C with 5% CO_2 for 72 h, WST-1 reagent was added to each well and the plate was incubated at 37 ° C in the tissue culture incubator for 1 h. The absorbance was measured at 450 nm using a microplate reader.

7. Fluorescent Immunohistochemistry

HaCaT cells were seeded and grown on NUNC Lab-Tek chamber slide system (Rochester, NY, USA) to 40%-50% confluence, serum-deprived overnight and then treated with APH for 24 h. At the end of experiment, cells were fixed with 3.7% formalin for 10 min

at room temperature and incubated with 100㎕ of blocking solution (2.5% bovine serum albumin in phosphate buffered saline) for 40 minutes. Zonula occludens-1 (ZO-1) in cultured HaCaT cells was stained with rabbit polyclonal antibodies (Molecular Probes, Waltham, MA. USA). After brief washing with PBS, cells were incubated with Alexa 488 anti-rabbit secondary antibodies (Molecular Probes, Waltham, MA. USA).

8. Enzyme-Linked Immunosorbent Assay (ELISA) and treatment

Anti-inflammation effects were measured by ELISA. PEG2, TNF-α, and IL-1α ELISA kits were obtained from R&D Systems (Minneapolis, MN, USA). All ELISAs were performed according to protocols provided by the manufacturer. For anti-inflammation assay, *Escherichia* coli LPS (150 ng/mL) was added to J774A.1 cells to induce the expression of PEG2 and TNF-α. These cells were then treated with the test compound either alone or in combination with LPS.

9. Statistical analysis for in-vitro experiments

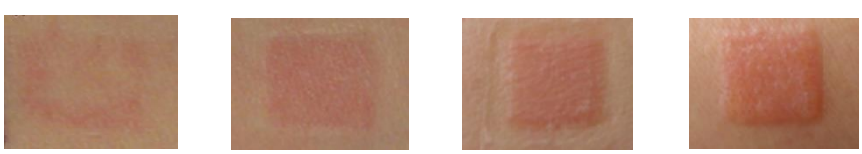
Each experiment was repeated at least three times. The results were presented as means±SEM, and statistical analysis were performed using Student's t-test or one-way ANOVA(Analysis of Variance), Bonferroni's Multiple Comparison Test. A p-values of <0.05 was

considered statistically significant.

10. Skin irritation test

Skin irritation was assessed by visual scoring using Frosch & Kligman method[29] and the Cosmetic, Toiletry, and Fragrance Association (CTFA) safety testing guideline[30]. The test was conducted for 24 h or 48 h with an occlusive patch. After dropping 20 ul of the sample into an IQ chamber (Chemotechnique Diagnostics, Vellinge, Sweden), it was applied to the back as test site and then fixed with micropore tape (3M Health Care, MN, USA). The patched site was observed and graded for irritation at 30 min and 24 h after removal.

Symbol	Grade	Clinical Description
−	0	None
+	1	Slight erythema, either spotty or diffuse
++	2	Moderate uniform erythema
+++	3	Intense erythema with edema
++++	4	Intense erythema with edema & vesicle



Grade 1 Grade 2 Grade 3 Grade 4

11. Skin sensitization test

The test-product and control were applied to patch test filter paper

discs and then applied to the right or left back (scapular area) of study subjects. Applications were performed on Mondays, Wednesdays, and Fridays during three consecutive weeks. At 48 h after the application, the patch was removed by trained technicians. At approximately 30 minutes after the patch removal, the site was assessed in order to check the presence of possible clinical signs. After this period (induction), there was a minimum of 10 day−period when no patch was applied to study subjects' back (rest period). The challenge period then started. A single application of the patch test was performed, followed by readings at 48 h and 72 h. Study subjects were assessed by a dermatologist at the start and the end of the study. They were supervised throughout this study.

12. A four weeks open, noncomparative clinical study

This study was conducted for four weeks as an open, noncomparative clinical study. For this study, 24 patients (age: 4−39 years) who were diagnosed with AD based on the diagnostic criteria of Hanifin and Rajka[31] were enrolled by the dermatology research institute of Ellead Co., Seongnam, Korea. The present study was approved by the Institutional Review Board (IRB no. EL−IRB−150520219S071) of Ellead Co. Patients voluntarily agreed to participate in this study. Four participants dropped out of this study for personal reasons. Twenty participants completed this study.

12.1. Ingredients of product

Water, defined cell culture media-9, dipropylene glycol, 1,2-hexanediol, glycerin, niacinamide, butylene glycol, hydrogenated lecithin, Centella asiatica extract, Ficus carica (FIG) fruit extract, carbormer, Oenothera bennis (evening primrose) flower extract, Chamaecyparis obrusa water extract, Houttuynia cordata extract, adenosine, Amaranthus caudatus seed extract, Ulmus davidiana root extract, polusorbate 20, hydroxyethylcellulose, ceramide 3, ethylhexylglycerin, carpryloyl dipeptide-17, ascorbyl propyl hyaluronate, sodium hyaluronate, and copper tripepride-1 were ingredients of the product.

12.2. Study population

1) Inclusion criteria

- Children and adolescents (only those who were permitted by their guardians) and adults aged between 4 and 39 years
- Those who were diagnosed with AD according to Hanifin and Rajka's criteria
- Patients who were schedule visits during the research period and those who could follow research instructions
- Patients who understood details of the test and agreed to the informed consent

2) Exclusion criteria

- Patients who had taken drugs that might impact testing, such as antihistamines or steroids, for the treatment of atopic dermatitis within the last one month

- Patients who had skin scaling, laser, or skin care on the test portion during the last one month
- Patients who had used medicines that might disrupt testing in the recent two weeks, such as pharmaceuticals or cosmetics for treating local AD
- Patients who were allergic to cosmetics or medicines
- Patients who had severe AD requiring medication
- Patients who suffered from another skin disease other than AD
- Patients with skin problems in the test area other than AD
- Patients with severe dietary deficiencies
- Patients with drug addicts or alcoholics
- Patients who were exposed to intense UV rays in the test area
- Patients who were deemed by a dermatologist to be difficult to perform a clinical test
- Patients who had participated in similar or identical tests in the previous three months

3) Restrictions for test participants

- Until the end of the study, it was forbidden to use a sauna, towel, or any other things that could irritate the test area
- Until the end of the study, products other than test products (products that may affect the test, such as moisturizers and medicines) were forbidden from being used in the test area
- Excessive exposure to ultraviolet radiation on the test area was forbidden until the study was completed

4) Criteria for suspension of test and dropout

- Failure to comply to restrictions imposed on test participants
- If a test participant experienced serious adverse events or requests that the test be suspended due to adverse events
- When the evaluation of results was hindered due to excessive drinking, smoking, and so on
- If the test participant or the test participant's legal representative requested that the test should be suspended
- If the test participant broke the method of use or schedule for no explicable reason
- If the test product's compliance fell below 80% throughout the test period
- If the test participants withdrew their consent to participate in the study
- If tracking the test subjects was impossible
- When using medications that might affect the determination of research results without the direction of the research director during the study

5) Selection of test participants

The efficacy of the test product for AD skin improvement was tested with 24 participants who met the inclusion criteria but not the exclusion criteria.

12.3. Overview of the clinical study schedule and methods

Study participants visited four times during the study period [one week before (−1 week), before use (0 week), 2 weeks after use,

and 4 weeks after use]. Those who came to the lab one week before the usage of the test products (first visit) were registered as participants and guardians if the researcher assessed that they were suitable according to the criteria for inclusion/exclusion of participants. All participants and guardians prepared their personal information and clinical informed consent as well as a case report form (CRF) that included gender, age, and medical history after receiving sufficient explanation from the researcher about the purposes of the study and outline, the test method, and the risk and adverse reactions for participating in this study. A participant number was assigned to each registered participant. All participants were given a test product before the usage of the test product (second visit). The sample was allowed to be used for the lesion and dry areas for a total of four weeks.

* How to use the sample: Pump the product 2 to 4 times at usage. Use it more than twice a day or more frequently as needed, apply it to the lesion as well as dry regions such as the face, neck, hands, and so on. After showering, tap off the water with a soft towel and apply the product to the lesion within three minutes.

Participants were forbidden to use all therapies, cosmetics (moisturizers), and medicines intended to treat atopic skin that could affect evaluation results, medical therapy, and massage treatments. The test product was not used for 12 hours before the

lab visit. After cleaning the test portion with lukewarm water and stabilizing under constant temperature and humidity conditions for 30 minutes, the test was conducted. The test was performed at a constant temperature and humidity (20 to 24° C, relative humidity of 40 to 60%) without air movement or direct sunlight Measurements were taken in the order listed below.

1. Visual Evaluation using eczema area and severity index (EASI) Score
2. Evaluation of skin moisture using a Corneometer
3. Evaluation of trans-epidermal water loss using a Tewameter
4. Evaluation of pruritus
5. Subjective survey assessments
6. Taking a photo using a DSLR camera
7. Evaluation of adverse reactions

12.4. Clinical assessment and evaluation

Adverse effect evaluations were performed for all participants. The EASI score, skin hydration, TEWL, and pruritus were assessed at 0, 2, and 4 weeks. The severity of AD was evaluated by two dermatologists using the EASI score. Mean values were calculated. Skin hydration (arbitrary unit) was measured using a Corneometer CM 825 (Courage and Khazaka, Cologne, Germany). TEWL ($g/h/m^2$) was assesses with a Tewameter TM 300 (Courage and Khazaka, Cologne, Germany). The measurement was also performed three times within two minutes in each time to record the mean value. All

measurements were conducted in a room that was not climate controlled. The room's temperature and humidity were relatively stable (20℃ ~24℃ and 40%~60% relative humidity). Subjects were prohibited from washing for at least an hour before measurement. They took a break for over 30 minutes in the same room. Severity of pruritus was calculated with a scoring system proposed by Duo and modified by Mettang[32,33].

1) Visual Evaluation using EASI Score

A dermatologist assessed the severity of each participant's AD using the EASI score*. Each of the four body regains (head/neck (H), Upper Limbs (UL), Body (Trunk), and Lower Limbs (LL)) regions was assessed separately for key signs of erythema, induration/papulation, excoriations, and lichenification. The average degree of severity of each sign in each of the four body regions was assigned a score of 0 to 3[34].

Body region	< 8yr	>8yr
Head/Neck (H)	(E+I+Ex+L)* x area x 0.2	(E+I+Ex+L) x area x 0.1
Upper Limbs (UL)	(E+I+Ex+L) x area x 0.2	(E+I+Ex+L) x area x 0.2
Trunk (T)	(E+I+Ex+L) x area x 0.3	(E+I+Ex+L) x area x 0.3
Lower Limbs (LL)	(E+I+Ex+L) x area x 0.3	(E+I+Ex+L) x area x 0.4
EASI score	Sum of above 4 region scores	Sum of above 4 region scores

*E=Erythema, I=Induration/Papulation, Ex=Excoriation, L=Lichenification

Based on: (1) the Physician's Assessment of Individual Signs (E, I, X, L) scored 0 = none, 1 = mild, 2 = moderate, 3 = severe; and (2) the percent body surface area affected, scored 0 = 0%, 1 = 1 ~ 9%, 2 = 10 ~ 29%, 3 = 30 ~ 49%, 4 = 50 ~ 69%, 5 = 70 ~ 89%, 6 = 90 ~ 100%.

2) Evaluation of skin hydration using a Corneometer

With a Corneometer CM825 (Courage and Khazaka, Germany), the amount of moisture on the volar surface of the right forearm of the patient was measured three time, and the average result was obtained. The Corneometer CM825 works on the principle of measuring the capacitance of a current conducted through an electrode interval adhered to a skin surface. Because the moisture content of the skin and the electrostatic load are proportional, the higher the value, the higher the moisture level of the skin. The average value of measurement results of the test subject before use (0 week), 2 weeks after use, and 4 weeks after use was used to calculate the increase rate.

3) Evaluation of trans-epidermal water loss using a Tewameter

A Tewameter TM300 (Courage and Khazaka, Germany) was used to measure Trans-epidermal moisture loss on the volar surface of the right forearm of the patient. Trans-epidermal water loss (TEWL) is a significant aspect of the process of skin barrier damage and recovery. Its unit is g/h/m2. The Tewameter probe was fixed in the test section so that it would not move for 60 seconds. The probe measures the density gradient of the water evaporation from the skin by two pairs of sensors located at different heights, a temperature and humidity sensor. The average value for 50 seconds, excluding the first 10 seconds, was calculated. The average value of measurement results of the test subject before use (0 week), 2

weeks after use, and 4 weeks after use was used to calculate the decrease rate.

4) Evaluation of pruritus

A scale established by Duo and deformed by Mettang[32,33] was used to evaluate the pruritus. Before and after the test period, the severity of pruritus was assessed on a 5-point scale and the dispersion of pruritus was assessed with a 3-point scale. The value (a x b) was multiplied by the severity and dispersion to obtain a score. The worse the pruritus, the higher the score.

a) Severity of pruritus

How severe do you feel the itch? (Check V in the box)	
1: I feel itchy, but it is not itchy enough to scratch with my hands.	
2: Sometimes I scratch because it is itchy, but it is not to the point where the skin peels off.	
3: I scratch often because it itches.	
4: Because it itches, I scratch so much to the point that the skin peels off.	
5: I often become restless because of itch.	

b) Dispersion of pruritus

What part of your body itches? (Check V in the box)	
1: One part	
2: More than two parts	
3: Overall	

5) Subjective survey assessments by participants

At two or four weeks after the sample was used, test participants or their legal representatives were surveyed for general evaluation

(usability), efficacy evaluation, skin adverse reaction evaluation, and favorability for the test product.

12.5. Statistical analysis for clinical study

Statistical analysis were performed using Student's t-test and repeated-measures ANOVA. Results are shown as mean and standard deviation (mean ± SD). A p-value of less than 0.05 was determined to be significant after a statistical test. All statistical analysis were performed using IBM SPSS Statistics 21.0 (IBM Co., Chicago, IL, USA).

RESULTS

1. The structure of APH

According to results of NMR analysis, HA-AA conjugate was found to bind two molecules of AA (**Figure 1A**) per five monomers of HA (**Figure 1B**). The molecular weight of one unit of hyaluronic acid was 379 Da. The molecular weight of the linker, propyl ($CH_2CH_2CH_2$), was 42 Da and the molecular weight of ascorbic acid was 176 Da. The weight of AA in the HA-AA (**Figure 1C**) conjugate was about 15% of the weight of HA-AA conjugate when calculated based on the above binding ratio and the molecular weight of each unit. Thus, in this experiment, 15% of the HA-AA conjugate weight was used as an equivalente amount of AA contained in HA-AA conjugate.

2. APH has thermal stability and anti-oxidant effects

To compare thermal stabilities of AA and APH, their solutions dissolved in water were incubated at 50 ℃ for 24 h and 48 h, respectively, and then subjected to HPLC analysis. As a result, ascorbic acid was degraded within 24 h, whereas APH was maintained up to 80.4% of ascorbic acid at 24 h, and up to 70.6% by 48 h (**Figure 1D**). Antioxidant effect of APH was measured using DPPH assay. AA and APH showed free radical scavenging activities (**Figure 2**). APH at 1000 ppm is expected to contain 150 ppm or less of AA. For 100 ppm of AA, 86.7% of radical scavenging activity was

observed. For 1000 ppm of APH, 78.2% of radical scavenging activity was observed.

3. APH permeation

In vitro permeation profiles of APH using three independent Franz cells for 24 h are shown in **Figure 3**. As shown in the graph, the penetrated amount of APH in the receptor medium increased with increasing time. According to the definition of Marzulli[35], APH could be classified as a "fast" penetrant (**Table 1**).

4. Effects of APH on cell viability and morphology in HaCaT and HDF

To examine the effect of APH on skin cells, cytotoxicity of APH was determined using a WST assay. LTA (1 ppm) (**Figure 4A**) and LPA (0.1 ppm) (**Figure 4B**) suppressed cell viability of HaCaT cells to 78.8% and 74% of the control, respectively. However, such suppression was recovered by APH in a dose-dependent manner. Especially, APH at 250 ppm significantly increased viability of LTA (1 ppm) and LPA (0.1 ppm) treated HaCaT cells to 94.7% and 93% of control, respectively. AA alone restored HaCaT cell viability above the base line. However, HA did not affect the viability of HaCaT cells treated with LTA or LPS. Cell viability of HDF showed similar results to that of HaCaT cells (**Figure 4C**). APH increased cell proliferation of HDF. On the other hand, AA and HA did not affect the

viability of HDF cells.

To determine whether APH treatment could cause expression alteration of tight junction-associated proteins (ZO-1), HaCaT cells were treated with 250 ppm of APH. ZO-1 expression in HaCaT cells was examined by immunofluorescent staining. Results showed that exposure of cells to 250 ppm of APH for 24 h caused an increase in the expression of ZO-1(**Figure 4D**).

5. APH has anti-inflammatory effects

To investigate the anti-inflammatory effect of APH, HaCaT Cells were stimulated with LAT (1 ppm) for 48h. Amounts of Tumor Necrosis Factor α (TNF-α) and Interleukin (IL-1α) secreted into the culture medium were analyzed. Results showed that TNF-α and IL-1α secretion levels were dramatically increased in LTA-stimulated HaCaT cells than in control cells (616.9% and 32221.5% of the control, respectively). However, treatment with 10, 50, 100, or 250 ppm of APH inhibited LTA-induced TNF-α secretion (138.1%, 139.8%, 134.9%, or 125.9% of the control, respectively) (**Figure 5A**) and IL-1α production (4899.4%, 3543.6%, 3134.8%, or 1409.2% of the control, respectively) (**Figure 5C**) in HaCaT cells. Furthermore, LPS-stimulated upregulation of TNF-α and IL-1α production (537.0% and 5106.7% of control, respectively) were recovered by APH in a dose-dependent manner. Treatment with APH at 10, 50, 100, or 250 ppm suppressed LPS-induced TNF-α production (417.9%, 342.2%, 127.2%, or 118.8% of the control,

respectively) (Figure 5B) and IL-1α production (3495.8%, 2898.1%, 616.3%, or 421.0% of the control, respectively) (Figure 5D). The anti-inflammatory effect of APH was verified in J774a.2 cells (murine macrophages). LPS-stimulated J774a.2 cells displayed dramatically increased Prostaglandin E2 (PGE2) (2131.3 mg/ml) and TNF-α (973.3 mg/ml) known to, induce an inflammatory response by activating macrophages, whereas treatment with 100 or 200 ppm of APH significantly attenuated the expression of PGE2 (1701.3 mg/ml or 1625.0 mg/ml, respectively) (Figure 5E) and TNF-α (856.7 mg/ml or 832.7 mg/ml, respectively) (Figure 5F).

6. Skin irritation test

A total of 31 people participated in the skin irritation test. The average age of these subjects was 42.1 ± 6.2 years (range, 21-50 years). Results of examining their skin characteristics through a survey are shown in Table 2. During the trial period, no skin reactions were observed. Therefore, the test product was considered to be a non-irritant to the skin under experimental conditions of this study.

7. Skin sensitization test

A total of 70 people participated in the skin sensitization test (Figure 6). Their average age was 42.0 ± 15.2 years (range, 18-70 years). During the trial period, no subjects present skin clinical signs related to the test product. Thus, the test product was considered to be safe under the experimental conditions of this study.

8. Results of a four-week human clinical study

This clinical study registered 24 test participants. Of them, 20 completed the test. Test participants 1, 10, 19, and 21 dropped out from the study due to personal reasons.

8.1. Characteristics of patients of the clinical study

The mean age of patients was 13.80 ± 7.89 years. Their mean EASI score, skin hydration (A.U.), TEWL, and skin pruritus score at the beginning of the study were 2.57 ± 2.27, 20.63 ± 8.67, 39.27 ± 14.76, and 7.80 ± 2.67, respectively.

8.2. Eczema area and severity index (EASI) score

EASI scores during the study period are shown in **Table 3**. After applying the test product, EASI score decreased significantly by 25.48% and 35.22% at 2 weeks and 4 weeks respectively (both $p < 0.05$) (**Figure 7**). After using the test product for 4 weeks, 70% of users showed a decrease in EASI score. Detailed data on evaluation results are shown in **Supplementary Table 1**.

8.3. Skin hydration

Skin hydration values at baseline were compared with those over the course of this study. Results are shown in **Table 4**. After using the test product, skin hydration increased significantly by 44.31% and 70.17% at 2 weeks and 4 weeks, respectively (both $p < 0.05$)

(Figure 8). After using the test product for 4 weeks, 85% of users showed an increase in skin hydration. Detailed data on evaluation results are shown in Supplementary Table 2.

8.4. Trans-epidermal water loss (TEWL)

Table 5 shows changes in TEWL at 2 weeks and 4 weeks compared with TEWL value at baseline. After using the test product, TEWL decreased significantly by 20.07% and 30.82% at 2 weeks and 4 weeks, respectively (both $p < 0.05$) (Figure 9). After using the test product for 4 weeks, 90% of users showed a decrease in TEWL. Detailed data on evaluation results are shown in Supplementary Table 3.

8.5. Assessment of pruritus

Pruritus assessed during the study period are shown in Table 6. After applying the test product for 2 weeks and 4 weeks, pruritus value decreased significantly by 50.54% and 65.50%, respectively (both $p < 0.05$) (Figure 10). After using the test product for 2 weeks and 4 weeks, 85% and 95% of users showed a decrease in pruritus. Detailed data on evaluation results are shown in Supplementary Table 4.

8.6. Subjective survey assessment by test participants

According to self-evaluation of the test product, after using the test product for two weeks, 14 out of 20 (70.0%) participants were

satisfied with the test product based on their responses to a question regarding "Overall satisfaction" after using the sample for two weeks as shown in Table 7. When asked about "satisfaction with improvement on AD", 16 (80.0%) of 20 participants were satisfied (Table 8). When asked about "improvement on skin dryness", 13 (65.0%) of 20 were satisfied (Table 9). When asked about "improvement in pruritus", 13 (65.0%) of 20 were satisfied (Table 10). When asked about the "degree of smoothness of the skin," 15 (75.0%) of 20 were satisfied (Table 11). When asked about the "degree of effect compared to product recently used", 12 (60.0%) of were satisfied (Table 12). When asked about "willingness to recommend", 14 (70.0%) of 20 had said they would recommend (Table 13).

After using the sample for four weeks, 17 (85.0 %) of 20 were satisfied with the question about "Overall Satisfaction," according to the test product's self-evaluation (Table 7). When asked about "satisfaction with improvement on AD", 16 (80.0%) of 20 were satisfied (Table 8). When asked about "improvement on skin dryness", 13 (65.0%) of 20 were satisfied (Table 9). When asked about "improvement in pruritus", 15 (75.0%) of 20 were satisfied (Table 10). When asked about the "degree of smoothness of the skin", 10 (50.0%) of 20 were satisfied (Table 11). When asked about the "degree of effect compared to product recently used", 13 (65.0%) of 20 were satisfied (Table 12). When asked about "willingness to recommend", 18 (90.0%) of 20 said they would recommend (Table

13).

8.7. Safety Results

There were no reports of abnormalities in the participants. Table 14. Moreover, during this test period, there were no early terminations due to adverse drug reactions (ADR), particular skin adverse reactions (SAR), or significant adverse reactions (SAE).

DISCUSSION

As AD can becomes chronic (it improves and then recurs), the cost of treatment increases. Its social and economic burden can affect the quality of life of both the patient and the patient's family[36]. Although many studies have been conducted on the prevention and treatment of AD, currently there is no treatment that can cure AD completely. Therefore, preventing the disease and aggressive treatment of its symptoms at its early stages are critical. When managing and treating chronic AD, it is important to check genetic defects such as filaggrin[37]. Furthermore, it is important to understand that an immunological tissue disorder remains even though clinical symptoms might have improved. It has been reported that using emolients such as moisturizers and oils is effective in maintaining the skin barrier[38]. Therefore, there is a need to develop effective products that are economically sound and safe so that children can use them during early stages of AD.

The skin possesses endogenous and exogenous antioxidant protectors to prevent oxidative damage[39]. Among various skin antioxidants, AA is known to be the most effective antioxidant at the dermis. Its protective effect against oxidative stress has been demonstrated previously[21,40]. Among its various physiological functions, AA can enhance iron absorption[41], and prevent scurvy [42], melanin synthesis[43,44], and catecholamine synthesis[45]. It

can also help maintain and improve immune function[46], thereby making it an integral substance in the homeostasis of the body while having great ability to heal wounds[47].

The anti-inflammatory effect of AA has been confirmed in the 2004 cytokine paper[48] by separating mononuclear cells of five volunteers. It was found that AA could effectively control increases of IL-6 and TNF-α, which are cytokines related to the first stages of inflammation. Our research showed that APH could also effectively control the increase of IL-1α and TNF-α. In addition to such anti-inflammatory effect of APH, recent studies have confirmed that AD patients have significantly lower intake of fruits than healthy controls[49].

Consequently, it can be concluded that supplement of AA can play a key role in healing the damaged skin barrier due to its anti-inflammatory and antioxidant effects.

Despite such benefits, rapid degradation of AA poses challenges for topical delivery of this molecule in cosmetic and pharmaceutical products. Moreover, physiochemical properties of AA such as melting points (190-192 °C), partition coefficient (log p(o/w) = -1.85), and dissociation constant (pKa = 4.25) are not optimal for transport across skin[24]. In other words, AA has four -OH groups, making it susceptible to oxidation. When such oxidation occurs through three stages, total browning of the substance causes it to lose its above-mentioned benefits. Therefore, numerous challenges exist in making it into a medication for skin application. Many

attempts have been made to develop derivatives such as EA(3-O-ethyl-l-ascorbic acid) and AA2G(Ascorbyl glucoside) that are stable while maintaining the effect of AA[24,50]. However, these derivatives have limitations in being used for external application on the skin.

APH is composed of cross-linked hyaluronic acid nanoparticles made by irradiating an acidic(pH 2.0) hyaluronic acid aqueous solution with an electron beam(100 kGy). APH uses ester bond and a linker made of carbo-chains and hydroxyls of no. 3 carbon, which is the most responsive among the four hydroxyls of AA to bind AA and HA together. An alkylation condition (1-chloro-3-iodopropane, NaHCO3, DMSO) capable of selectively binding to a hydroxyl group at position 3 of ascorbic acid protected in an acetal form was found and a protective group was removed to synthesize an ascorbic acid derivative in a form capable of binding to hyaluronic acid[51]. This can increase the stability by suppressing oxidation-reduction and radical response of AA. Thus, it can be absorbed into the skin with HA. The compound is then disconnected by the abundance of esterase in the skin fluid, thereby releasing AA to exert its effects. APH has shown superior thermal stability to AA. It does not succumb to browning even when it is stored at room temperature for months.

HA is the main component of the extracellular matrix in connective, epithelial, and neural tissues. It is known to play an important role in

tissue hydration and water transport mainly due to its enormously high water binding capacity[52]. The skin is the largest reservoir of HA in our body since it accounts for 50% of the total body HA content. Under ideal conditions, HA is found in all layers of the epidermis and dermis[53]. In the epidermis, HA is the most prominent in upper spinous and granular layers, where most HAs are extracellular. The basal layer of the skin also contains HA. However, it is predominantly intracellular. It is already known that HA taken orally does not show any benefit for the skin, because skin cells cannot extract HA from the bloodstream. Therefore, it must be applied topically in order to supplement the skin with HA.

The permeation and penetration of the skin by HA are limited by their molecular size and other physiochemical properties. According to the 500 Da rule, most of the chemical compounds and drugs with a molecular weight higher than 500 Da cannot easily penetrate the skin[54]. However, despite the high molecular weight and hydrophilicity of HA, it is known to be permeated through the human skin. The mechanism for trans-epidermal transport of HA has not been clearly verified yet[55]. As a macromolecular substance of more than 500 kDa, HA is not recommended because its large size prevents it from being absorbed into deep layers of the skin[56]. Instead of being absorbed, this macromolecular HA can be made into a film form to act as a barrier for preventing the loss of moisture.

An experiment has examined the absorption of full thickness porcine ear skin using Franz diffusion cells at 37 ° C for 48 h. It demonstrated

that HA of 357 kDa could be sufficiently absorbed by the epidermis. This result is likely to be similar in the human body. However, it was not applied to the dermis layer[56]. One study using Raman spectroscopy has demonstrated the skin permeability of a low molecular weight HA (20−300 kDa) and the impermeability of a high molecular weight HA (1000−1400 kDa)[57]. Another study has found that a low molecular weight HA of approximately 50 kDa can penetrate the skin better than larger sized HA[58]. APH is a HA−AA conjugate with an estimated size of about 10 kDa. We wondered whether APH could pass through the epithelial layer of the skin. In this study, we observed that APH could penetrate an artificial epithelial membrane (Strat−M®) in a time−dependent manner. In addition, we expected that It may provide greater permeation in AD patients because of their impaired skin barrier. Therefore, we thought that symptoms of AD could be alleviated by APH absorption in the strata cornea of atopic patients.

As a water−soluble product, HA can be easily incorporated with the water phase of cosmetic formulas, thus playing a role in the reduction of dermatological aging, reduction of depths of lines and wrinkles, decrease of skin fragility, amelioration of skin atrophy, improvement of skin firmness and texture, decrease in pore size, restoration of skin brightening, and improvement of skin barrier function[59].

Immunofluorescence microscopy results of APH−treated HaCaT cells revealed that ZO−1, a tight junction protein−1 that plays an

important role in cellular barrier function, showed increased levels and became dense, all of which suggested an improvement in barrier function of APH-treated keratinocyte[60,61].

The range of variation of skin hydration degree was between 0 and 130 arbitrary units (AU). In standard working conditions (temperature ° = 20-22 °C, humidity = 40-60%), variations of skin hydration degrees for the middle area of the front side of the forearm are shown in the following: under 30 AU, very dry, between 30 and 45 AU, dry, and 45 AU, sufficiently hydrated[62]. The average value for the skin hydration degree (29.99 AU) after 4 weeks did not appear to be suitably hydrated according to skin hydration results evaluated with Corneometer CM 825 in this investigation. However, such values could vary due to effects of exponential, exogenous, environmental, and instrument measurement/instrument-related factors. As such, results for a given anatomical position are recommended to be reported and compared as a relative (or percent) change in skin hydration value rather than an absolute value[63].

APH has added stability and effects of AA to HA. It can help improve the skin barrier function of AD patients with antioxidant and anti-inflammatory features. It has been demonstrated to be a non-irritant with significant effects in improving symptoms of the skin of AD patients. Furthermore, subjective evaluation of AD patients using

APH showed favorable results in all evaluation areas. Therefore, APH can be considered as an externally applied medicine for AD patients. It can also be used in anti-aging cosmetic products or wound healing medicine. Further research to verify this is needed in the future.

Figure 1. Chemical structure and thermal stability of APH.

Structures of ascorbic acid (AA) (A), hyaluronic acid (HA) (B), and APH (C) are shown. Thermal stability of AA was compared with that of APH (D). Values are presented as mean ± SEM of three independent experiments.

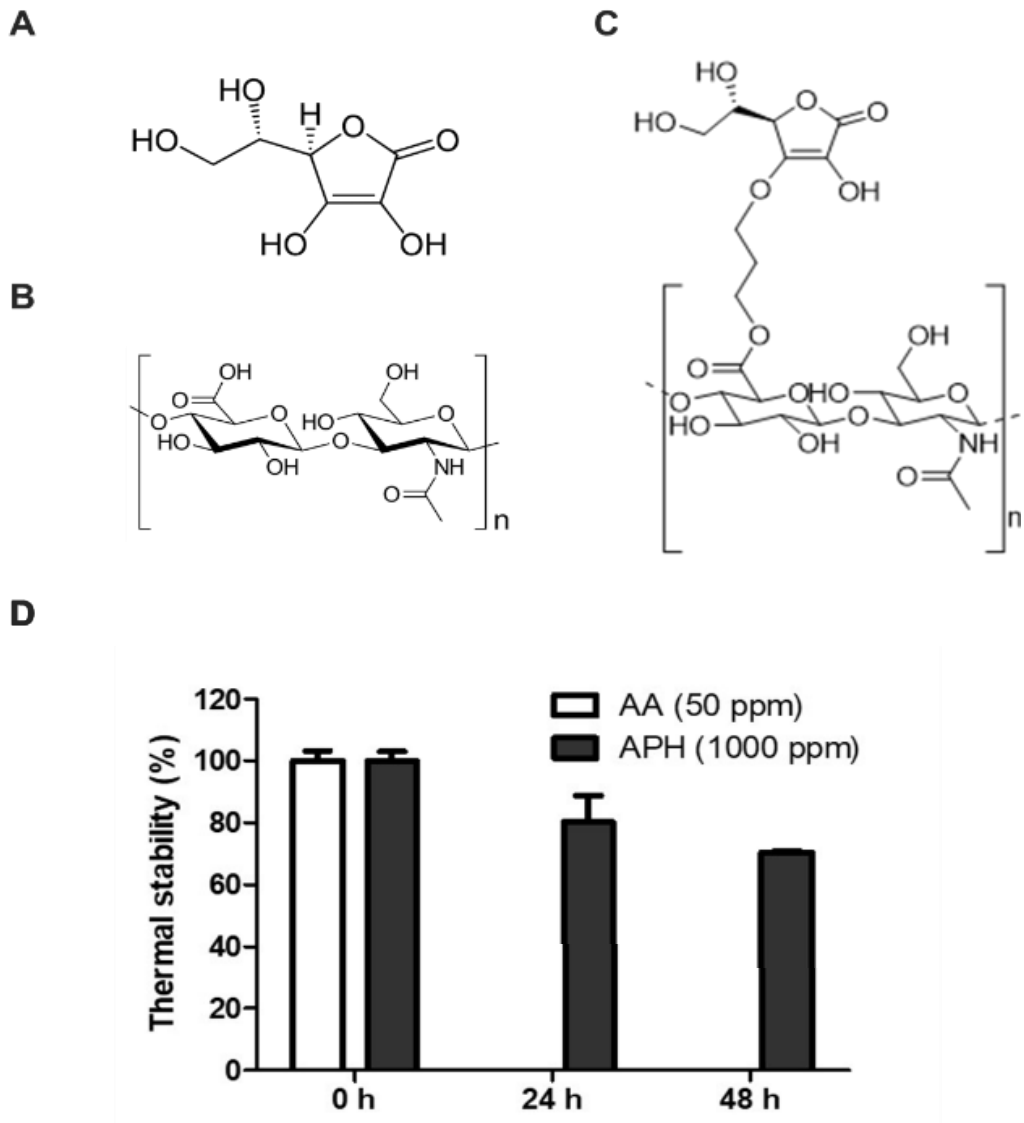
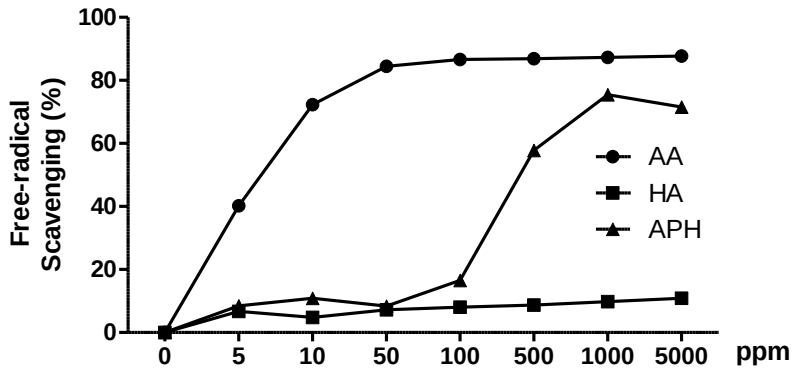


Figure 2. Free radical scavenging activity of APH.

DPPH radical scavenging activities of AA, HA, and APH were determined. Values are presented as mean ± SEM of three independent experiments.



*AA=Ascorbic acid, HA=Hyaluronic acid, VITA-HA=Ascorbyl propyl hyaluronate

Figure 3. Permeability of APH through artificial membranes.

Permeated cumulative amounts in artificial membranes were determined. Values are presented as mean ± SEM (n=4) of three independent experiments.

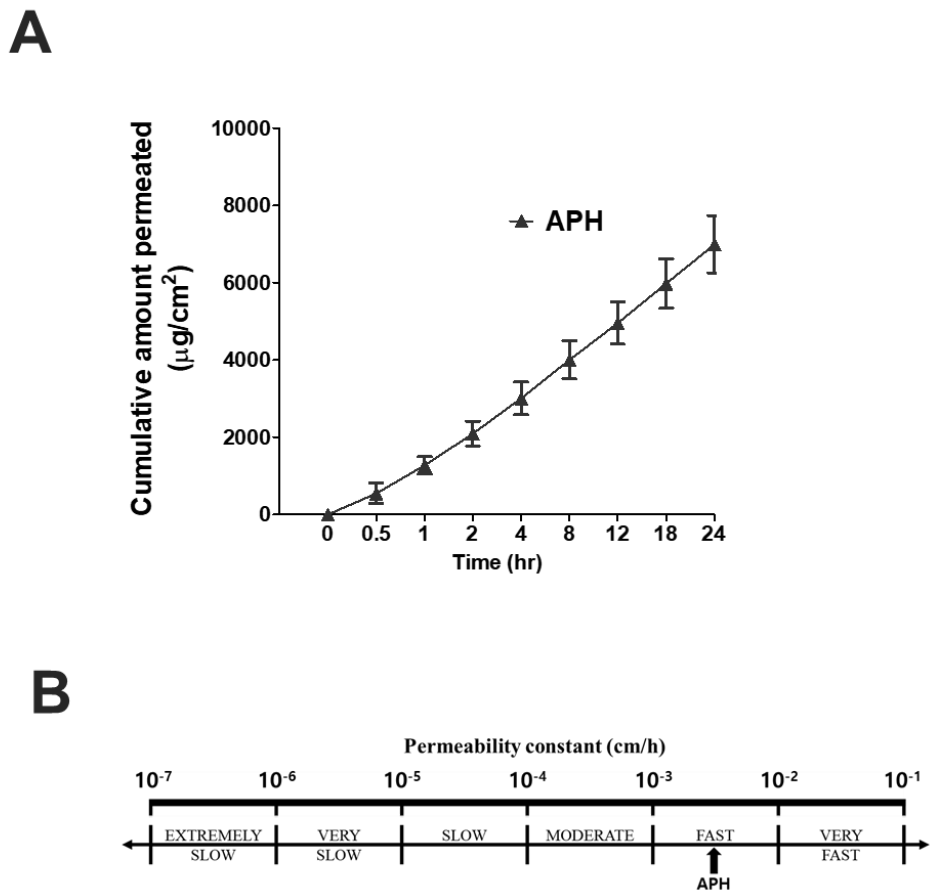
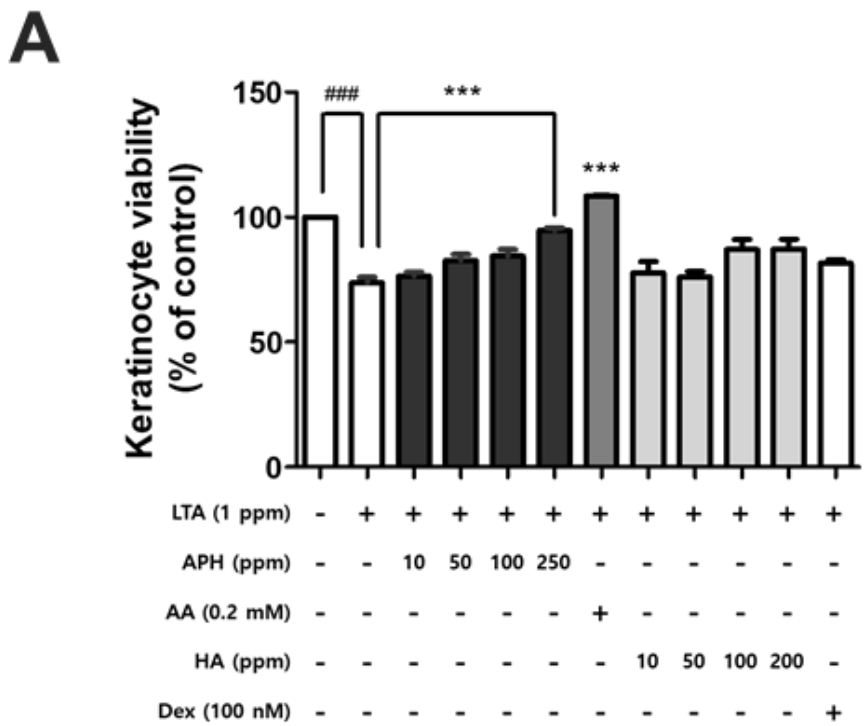
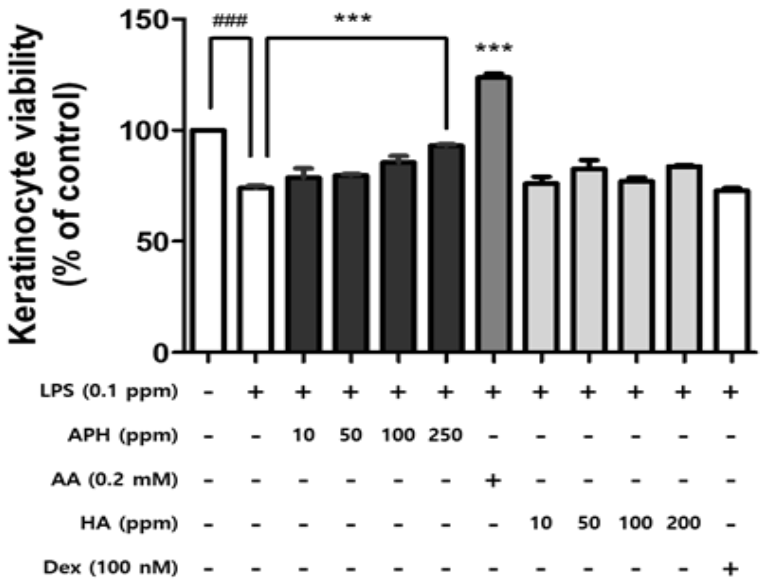


Figure 4. Effect of APH on cell viability and morphology.

Viabilities of HaCaT cells (A, B) and HDFs (C) were examined using WST-1 assay. Tight junction were observed by immunocytochemistry staining (D). ZO-1 (tight junction protein) was visualized in green in HaCaT cells, respectively. Values are presented as mean ± SEM of three independent experiments. ###p<0.001 compared with the non-irradiated control group. ***p<0.001 compared with the control (LTA or LPA treated group). Scale bar indicates 100 μm.



B


Keratinocyte viability
(% of control)
150
100
50
0
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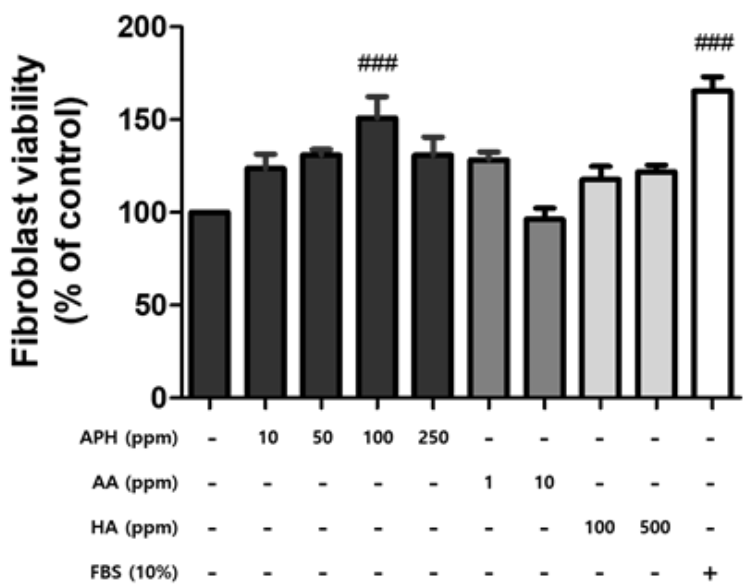
***
LPS (0.1 ppm) - + + + + + + + + + + +
APH (ppm) - - 10 50 100 250 - - - - - -
AA (0.2 mM) - - - - - - + - - - - -
HA (ppm) - - - - - - - 10 50 100 200 -
Dex (100 nM) - - - - - - - - - - - +

C


Fibroblast viability
(% of control)
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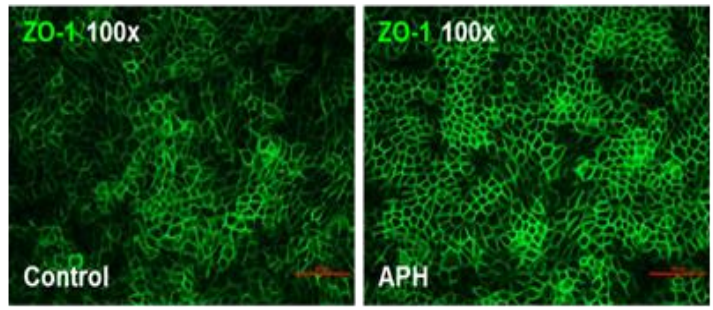
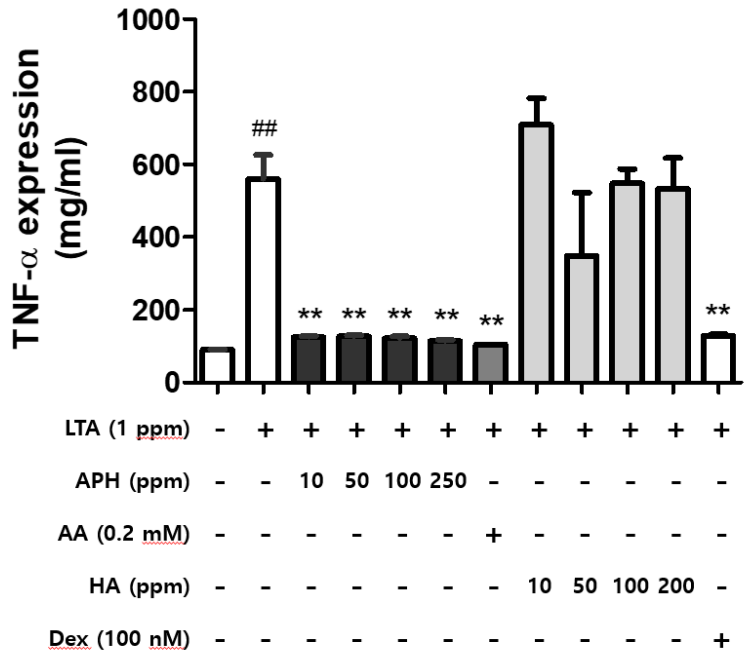


Figure 5. Anti-inflammatory effect of APH.

HaCaT (Human keratinocyte cell line) cells were treated with Lipoteichoic acid (LTA) or lipopolysaccharide (LPS). LPS-or LTA-pretreated cells were then treated with AA or APH. Cell supernatants were collected and levels of TNF-α (**A, B**) and IL-1α (**C, D**) were measured by ELISA. Murine macrophages (J774A.1) were treated with LPS in the absence or presence of AA or APH. PGE2 (**E**) and TNF-α (**F**) expression levels in supernatants of culture media at 24 hours after LPS treatment were then measured. Values are presented as mean ± SEM of three independent experiments. ##p<0.01, ###p<0.001 compared with the non-irradiated control group. *p<0.05, **p<0.01, ***p<0.001 compared with the control (LTA or LPA treated group).

A


TNF-α expression (mg/ml)
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600
400
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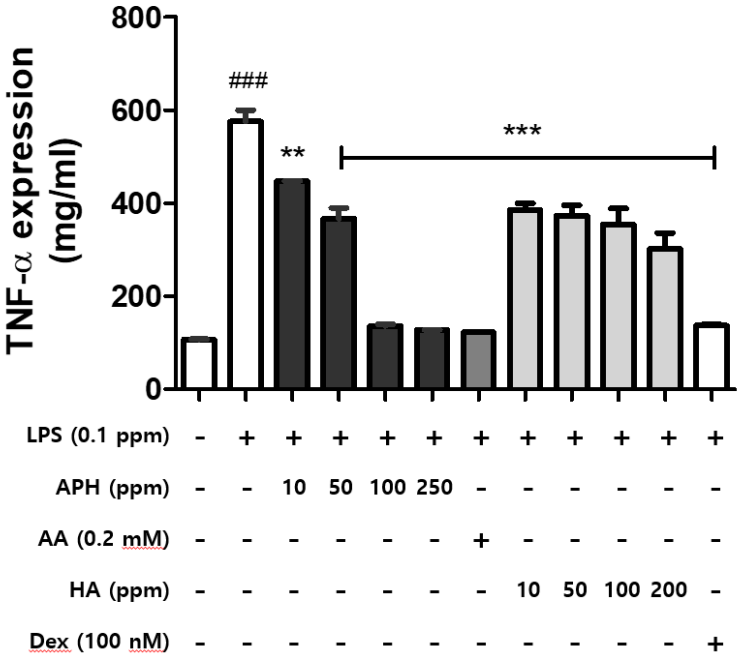
LTA (1 ppm) - + + + + + + + + + + +
APH (ppm) - - 10 50 100 250 - - - - - -
AA (0.2 mM) - - - - - - + - - - - -
HA (ppm) - - - - - - - 10 50 100 200 -
Dex (100 nM) - - - - - - - - - - - +

B


TNF-α expression (mg/ml)
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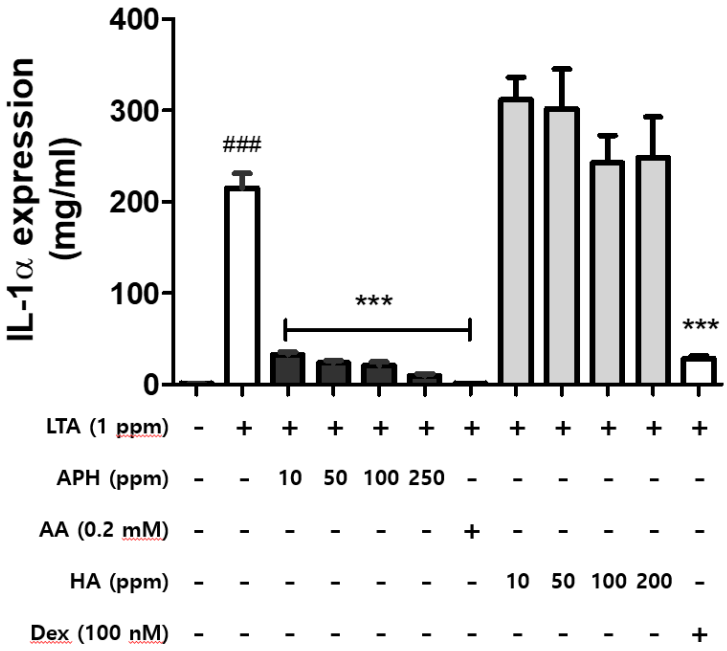
LPS (0.1 ppm) - + + + + + + + + + + +
APH (ppm) - - 10 50 100 250 - - - - - -
AA (0.2 mM) - - - - - - + - - - - -
HA (ppm) - - - - - - - 10 50 100 200 -
Dex (100 nM) - - - - - - - - - - - +

C


IL-1α expression (mg/ml)
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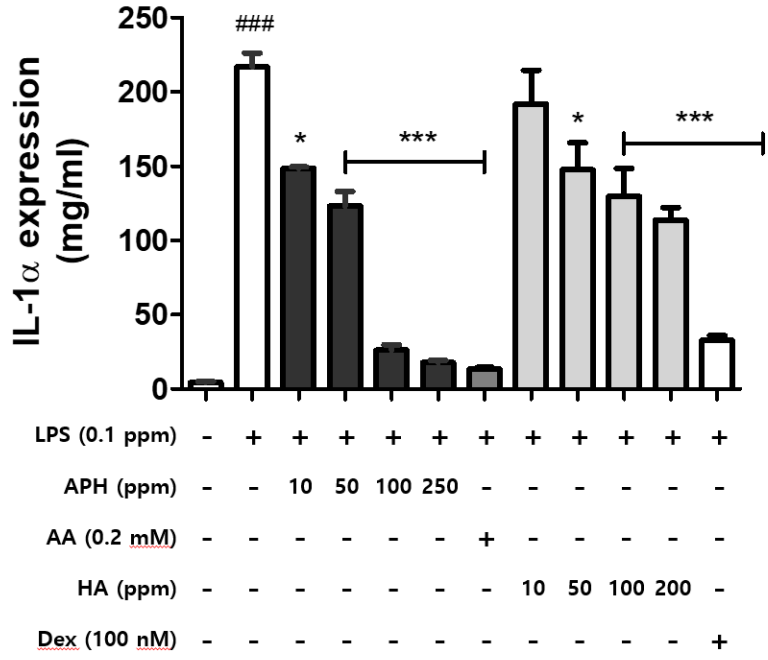
LTA (1 ppm) - + + + + + + + + + + +
APH (ppm) - - 10 50 100 250 - - - - - -
AA (0.2 mM) - - - - - - + - - - - -
HA (ppm) - - - - - - - 10 50 100 200 -
Dex (100 nM) - - - - - - - - - - - +

D


IL-1α expression (mg/ml)
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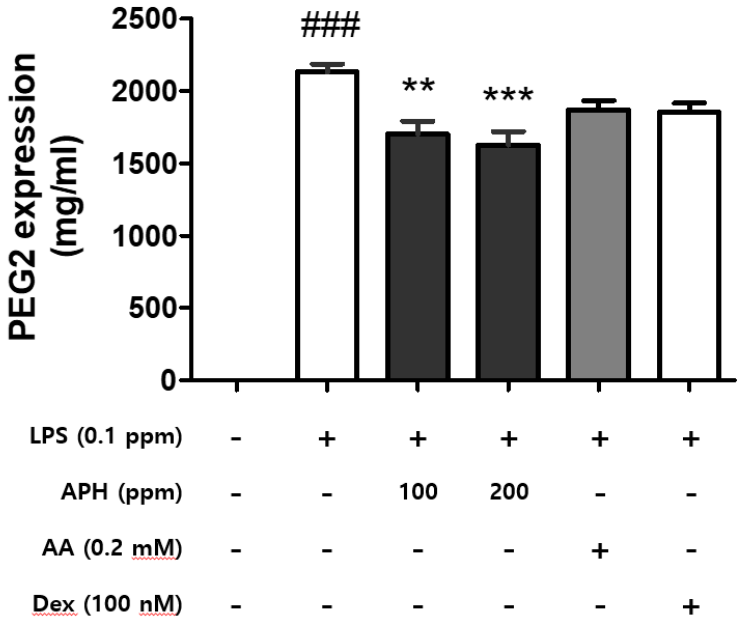
LPS (0.1 ppm) - + + + + + + + + + + +
APH (ppm) - - 10 50 100 250 - - - - - -
AA (0.2 mM) - - - - - - + - - - - -
HA (ppm) - - - - - - - 10 50 100 200 -
Dex (100 nM) - - - - - - - - - - - +

E


PEG2 expression (mg/ml)
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**
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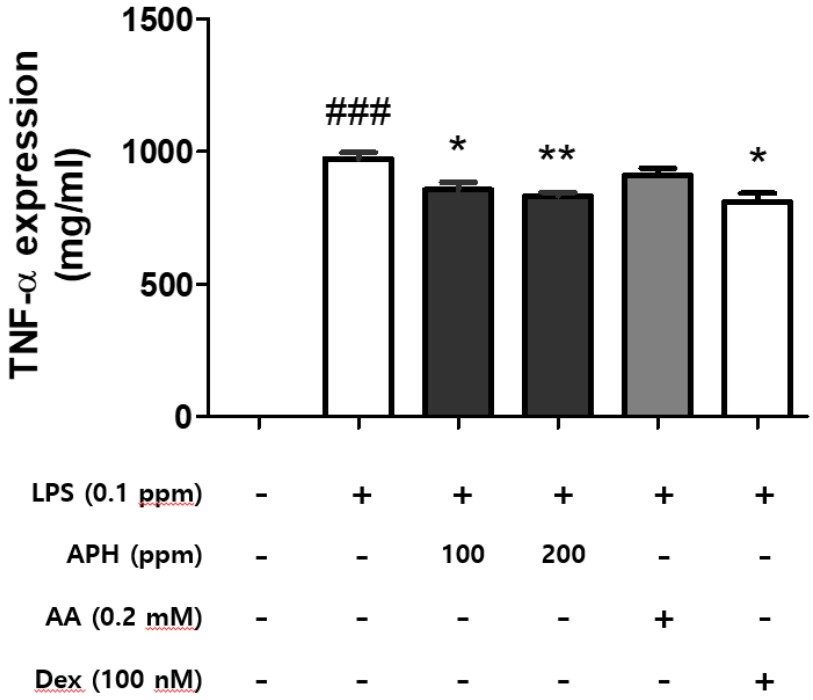
LPS (0.1 ppm) - + + + + +
APH (ppm) - - 100 200 - -
AA (0.2 mM) - - - - + -
Dex (100 nM) - - - - - +

F


TNF-α expression (mg/ml)
1500
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*
LPS (0.1 ppm) - + + + + +
APH (ppm) - - 100 200 - -
AA (0.2 mM) - - - - + -
Dex (100 nM) - - - - - +

Figure 6. Assessment schedule of the skin sensitization study.

			Stages				
			Sign Informed Consent	Clinical Assessment by the Dermatologist	Patch test Application	Patch test Removal	Assessments (Readings)
		Visit 1	X	X	-	-	-
Induction Period	Week 1	Visit 2	-	-	X	-	-
		Visit 3	-	-	X	X	X
		Visit 4	-	-	X	X	X
	Week 2	Visit 5	-	-	X	X	X
		Visit 6	-	-	X	X	X
		Visit 7	-	-	X	X	X
	Week 3	Visit 8	-	-	X	X	X
		Visit 9	-	-	X	X	X
		Visit 10	-	-	X	X	X
		Visit 11	-	-	-	X	X
Rest period - weeks 4 and 5 – no visits performed							
Challenge Period	Week 6	Visit 12	-	-	X	-	-
		Visit 13	-	-	-	X	X
		Visit 14	-	X	-	-	X

Figure 7. Eczema Area and Severity Index (EASI) score assessment results.

EASI score was assessed before and after treatment with APH. EASI score at 2 weeks and 4 weeks were significantly decreased compared the baseline value. Values are mean±SEM of twenty independent experiments. *p<0.05 compared with the basal.

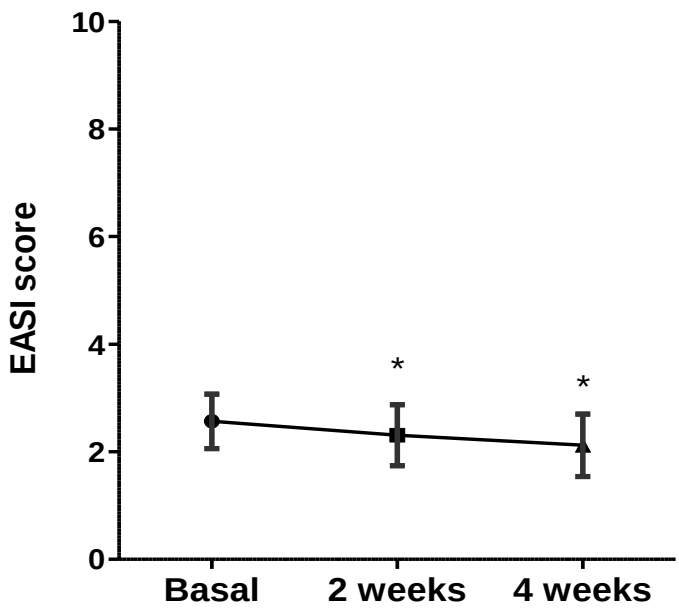

Figure 8. Skin hydration assessment results.

Skin hydration (A.U.) values of atopic dermatitis patients treated with APH were measured. Skin hydration was assessed before and after treatment. Values are presented as mean ± SEM of twenty independent experiments. ***p<0.001 compared with basal value.

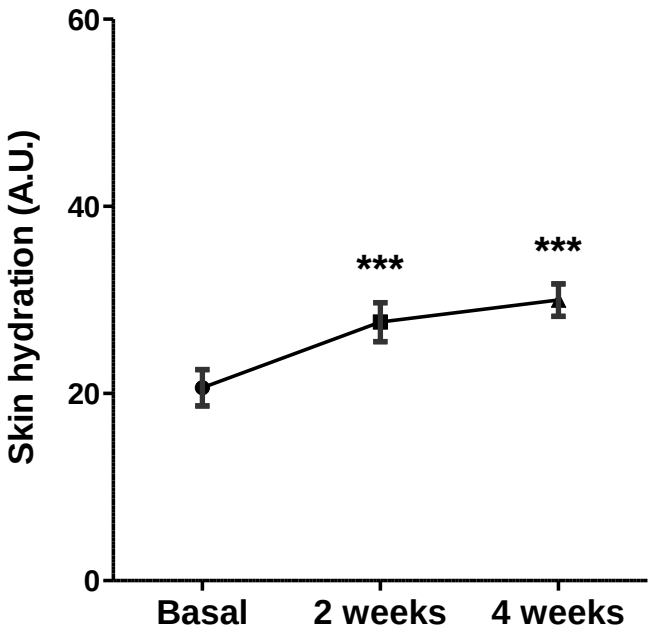

Figure 9. Trans-epidermal water loss (TEWL) assessment results.

TEWL(g/h/m^2) levels were measured in atopic dermatitis patients treated with APH. TEWL was assessed before and after treatment. Values are tresented as mean ± SEM of twenty independent experiments. **p<0.01 compared with the basal level.

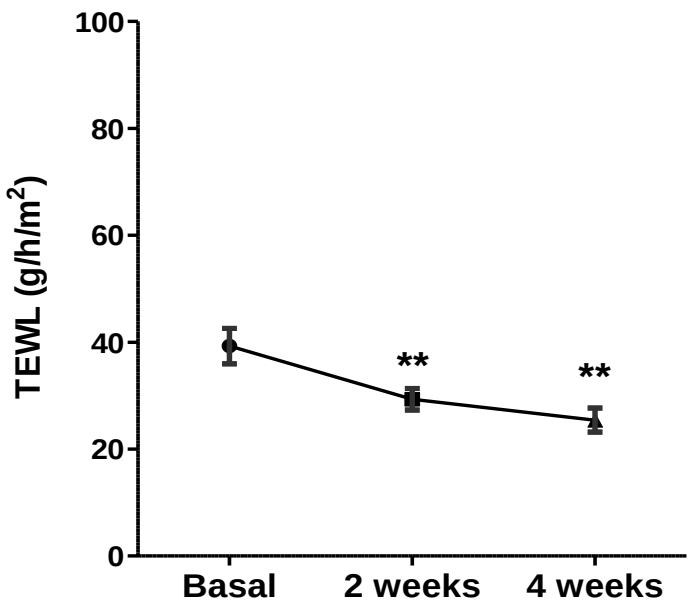

Figure 10. Pruritus assessment results.

Pruritus scores were measured for atopic dermatitis patients treated with APH. Pruritus was assessed before and after treatment. Values are presented as mean ± SEM of twenty independent experiments. ***p<0.001 compared with the basal score.

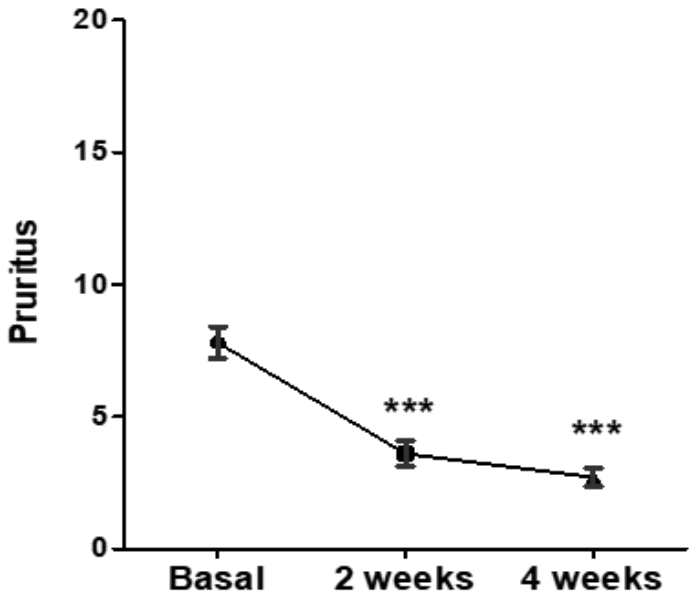

Figure 11. Clinical photographs.

Photos of the volar forearm at baseline, 2 weeks, and 4 weeks after APH application. Skin lesions showed significant improvement over time.

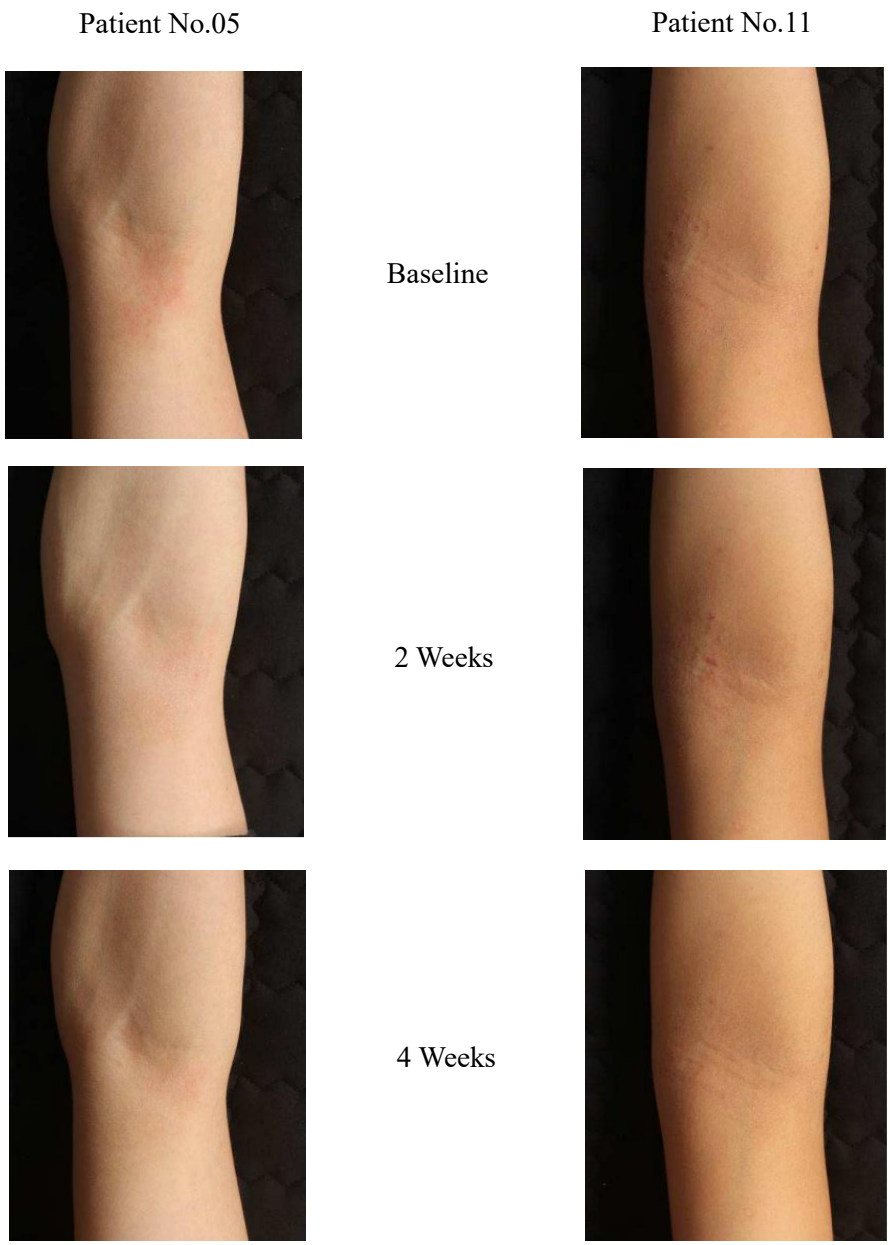

Table 1. Measurement of flux and permeability coefficient (Kp)

Concentration	Flux (μg/h*cm^2)	K_p (cm/h*10^{-4})
APH (200ppm)	734.44±211.52	621.74±179.07

Table 2. Skin characteristics of skin irritation subject (n=31)

Item	Classification	Frequency (n)	percentage (%)
Skin Type	Dry skin	8	25.81
	Normal skin	9	29.03
	Oily skin	4	12.90
	Dry to oily skin	10	32.26
	Problem skin	0	0.00
Irritability	Yes	0	0.00
	No	31	100.00
Stinging	Yes	1	3.23
	No	30	96.77
Side effects by cosmetics	Yes	0	0.00
	No	31	100.00
Allergy	Food allergy	0	0.00
	Metal allergy	2	6.45
	Photo allergy	1	3.23
	Extra allergy	0	0.00
	No	28	90.32
Skin diseases	Acne	1	3.23
	Atopy	0	0.00
	Hair loss	0	0.00
	Extra skin diseases	0	0.00
	No	30	96.77
Tight feeling	Yes	1	3.23
	No	30	96.77
Taking supplements	Taking korean herb medicines	0	0
	Taking nutrients	4	12.9
	Taking supplemenets	2	6.45
	No	25	80.65
Smoking	No	31	100
	Less than 10 pieces	0	0
	More than 10 pieces	0	0
Menstrual cycle	within 1 week before	7	22.58
	During menstruation	3	9.68
	Within 1 week after menstruation	9	29.03
	The others	12	38.71

Table 3. Change of Eczema area and severity index (EASI) score

Evaluation Parameter	Time point	Mean (SD)	Decreasing rate (%)	P-value
EASI Score	Baseline	2.57 ± 2.27	-	-
	2 weeks	2.31 ± 2.53	25.48%	0.043 *
	4 weeks	2.12 ± 2.59	35.22%	0.013 *

* Probability p (repeated measures ANOVA, significant: *p<0.05)

Table 4. Change of skin hydration

Evaluation Parameter	Time point	Mean (SD)	Increasing rate (%)	P-value
Skin hydration (A.U.)	Baseline	20.63 ± 8.67	-	-
	2 weeks	27.63 ± 9.32	44.31%	<0.001 ***
	4 weeks	29.99 ± 7.76	70.17%	<0.001 ***

* Probability p (repeated measures ANOVA, significant: ***p<0.001)

Table 5. Change of Trans-epidermal water loss (TEWL)

Evaluation Parameter	Time point	Mean (SD)	Decreasing rate (%)	P-value
TEWL (g/h/m2)	Baseline	39.27 ± 14.76	-	-
	2 weeks	29.34 ± 8.93	20.07%	0.005 **
	4 weeks	25.42 ± 10.08	30.82%	0.001 **

* Probability p (repeated measures ANOVA, significant: **p<0.01)

Table 6. Change of pruritus

Evaluation Parameter	Time point	Mean (SD)	Decreasing rate (%)	P-value
Pruritus	Baseline	7.80 ± 2.67	-	-
	2 weeks	3.60 ± 2.14	50.54%	<0.001 ***
	4 weeks	2.70 ± 1.59	65.50%	<0.001 ***

* Probability p (repeated measures ANOVA, significant: ***p<0.001)

Table 7. Q. 1) How satisfied are you with your product overall ?

Question No.	Scale	After 2 Weeks		After 4 Weeks	
		(n)	%	(n)	%
1	Not good at all	0	0.0	0	0.0
	Not so good	0	0.0	0	0.0
	Average	6	30.0	3	15.0
	Good	11	55.0	12	60.0
	Very good	3	15.0	5	25.0
	Total	20	100.0	20	100.0

Table 8. Q. 2-1) Are you satisfied with the overall AD improvement after use ?

Question No.	Scale	After 2 Weeks		After 4 Weeks	
		(n)	%	(n)	%
2-1	Not good at all	0	0.0	0	0.0
	Not so good	0	0.0	0	0.0
	Average	4	20.0	4	20.0
	Good	11	55.0	13	65.0
	Very good	5	25.0	3	15.0
	Total	20	100.0	20	100.0

Table 9. Q. 2-2) Have you noticed an improvement in your skin's dryness after using the test product ?

Question No.	Scale	After 2 Weeks		After 4 Weeks	
		(n)	%	(n)	%
2-2	Not good at all	0	0.0	0	0.0
	Not so good	1	5.0	0	0.0
	Average	6	30.0	7	35.0
	Good	8	40.0	10	50.0
	Very good	5	25.0	3	15.0
	Total	20	100.0	20	100.0

Table 10. Q. 2-3) Are you satisfied with the improvement in pruritus after using the test product ?

Question No.	Scale	After 2 Weeks		After 4 Weeks	
		(n)	%	(n)	%
2-3	Not good at all	0	0.0	0	0.0
	Not so good	1	5.0	0	0.0
	Average	6	30.0	5	25.0
	Good	7	35.0	8	40.0
	Very good	6	30.0	7	35.0
	Total	20	100.0	20	100.0

Table 11. Q. 2-4) Did your skin become smooth after using the test product ?

Question No.	Scale	After 2 Weeks		After 4 Weeks	
		(n)	%	(n)	%
2-4	Not good at all	0	0.0	0	0.0
	Not so good	1	5.0	0	0.0
	Average	4	20.0	10	50.0
	Good	9	45.0	4	20.0
	Very good	6	30.0	6	30.0
	Total	20	100.0	20	100.0

Table 12. Q. 2-5) Is the test product more effective than the one you used recently ?

Question No.	Scale	After 2 Weeks		After 4 Weeks	
		(n)	%	(n)	%
2-5	Not good at all	0	0.0	0	0.0
	Not so good	1	5.0	1	5.0
	Average	7	35.0	6	30.0
	Good	8	40.0	9	45.0
	Very good	4	20.0	4	20.0
	Total	20	100.0	20	100.0

Table 13. Q. 2-6) Will you recommend the test product to people around you ?

Question No.	Scale	After 2 Weeks		After 4 Weeks	
		(n)	%	(n)	%
2-6	Not good at all	0	0.0	0	0.0
	Not so good	0	0.0	0	0.0
	Average	6	30.0	2	10.0
	Good	9	45.0	11	55.0
	Very good	5	25.0	7	35.0
	Total	20	100.0	20	100.0

Table 14. Q. 3) Have you experienced any discomfort or adverse reactions while using the test product ?

Question No.	Scale	After 2 Weeks		After 4 Weeks	
		(n)	%	(n)	%
3	Yes	0	0.0	0	0.0
	No	20	100.0	20	100.0

Supplementary Table 1. Individual EASI score

Patient No.	Basal	After 2 weeks	After 4 weeks
2	1.2	1.4	1.2
3	1.0	0.8	0.2
4	0.6	0.4	0.2
5	1.4	0.8	0.4
6	0.8	0.6	0.2
7	1.2	0.0	0.0
8	3.4	3.0	3.2
9	5.6	4.6	3.8
11	5.8	6.0	6.6
12	2.6	1.8	1.0
13	0.2	0.0	0.2
14	8.0	8.4	9.0
15	6.0	6.8	5.9
16	0.6	0.4	0.2
17	2.2	1.8	2.4
18	1.9	1.6	1.0
20	1.8	1.0	1.9
22	1.2	0.4	0.0
23	0.8	0.8	0.6
24	5.0	5.6	4.4
Mean±SD	2.565±2.268	2.310±2.537	2.120±2.585
Mean±SEM	2.565±0.507	2.310±0.567	2.120±0.578

SD, standard deviation

SEM, standard error of the mean

Supplementary Table 2. Individual skin hydration (A.U.)

Patient No.	Basal	After 2 weeks	After 4 weeks
2	17.433	23.867	29.200
3	29.467	42.667	36.633
4	24.733	24.367	20.967
5	24.933	32.767	28.800
6	30.467	29.733	27.700
7	17.800	32.200	44.367
8	24.733	27.233	24.267
9	18.200	28.867	24.333
11	13.333	29.700	29.867
12	12.733	22.033	32.100
13	39.567	49.600	40.267
14	15.833	24.633	17.033
15	7.900	15.167	33.333
16	14.467	34.333	35.433
17	17.400	14.767	22.133
18	7.267	13.567	18.100
20	13.833	16.067	25.967
22	20.933	25.533	43.133
23	35.033	38.433	35.167
24	26.600	27.100	31.000
Mean±SD	20.633±8.665	27.631±9.319	29.990±7.755
Mean±SEM	20.633±1.938	27.631±2.084	29.990±1.734

SD, standard deviation

SEM, standard error of the mean

Supplementary Table 3. Individual trans-epidermal water loss (TEWL)

Patient No.	Basal	After 2 weeks	After 4 weeks
2	30.800	24.000	22.500
3	30.000	17.300	23.700
4	36.700	24.900	22.600
5	46.200	33.300	21.600
6	76.900	41.500	24.100
7	61.200	16.600	18.600
8	41.600	34.800	33.100
9	29.200	24.600	26.800
11	40.400	31.800	27.800
12	26.100	38.300	33.200
13	65.100	31.000	44.200
14	51.700	42.700	34.000
15	35.200	31.900	24.500
16	27.400	21.700	11.900
17	32.300	26.200	10.500
18	33.100	26.800	30.600
20	29.900	43.300	47.600
22	18.800	11.300	9.300
23	45.300	37.600	26.800
24	27.500	27.100	15.000
Mean±SD	39.270±14.758	29.335±8.925	25.420±10.081
Mean±SEM	39.270±3.300	29.335±1.996	25.420±2.254

SD, standard deviation

SEM, standard error of the mean

Supplementary Table 4. Individual pruritus score

Patient No.	Basal	After 2 weeks	After 4 weeks
2	8	2	1
3	8	6	4
4	6	4	2
5	6	4	2
6	6	4	2
7	6	1	1
8	8	1	3
9	12	6	4
11	12	6	4
12	9	1	1
13	6	1	1
14	8	8	2
15	15	4	6
16	6	4	2
17	6	6	6
18	6	4	2
20	9	1	4
22	6	1	2
23	9	4	4
24	4	4	1
Mean±SD	7.800±2.667	3.600±2.137	2.700±1.592
Mean±SEM	7.800±0.596	3.600±0.478	2.700±0.356

SD, standard deviation

SEM, standard error of the mean

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요약

아토피 피부염 환자에 적용한 Ascorbyl Propyl Hyaluronate의 안전성 및 유효성에 관한 연구

아토피 피부염(AD)은 심한 가려움증을 동반하는 만성 재발성 염증성 피부질환이다. 이는 피부의 구조적 및 기능적 손상을 초래한다. 최근 아스코르브산(AA)이 아토피 피부염에서 중요한 역할을 하는 것으로 알려졌다. 피부에서 감소된 AA를 보충하면 아토피 피부염 증상이 완화되었다. 히알루론산-아스코르빈산 유도체인 아스코르빌 프로필 히알루론산(APH)은 안정성과 피부 투과성을 개선한 화장품 성분이다. 본 연구에서는 아토피 피부염 환자에게서 APH의 안전성과 유효성을 평가하고자 하였다. 저자는 APH가 아스코르브산에 비해 열안정성이 향상된 것을 입증했다. APH 처리는 HaCaT 및 인간 진피 섬유아세포(HDF)의 세포 형태와 생존력에 영향을 미치지 않았다. 또한, APH는 자유-라디칼 제거 효과가 있었고 TNF-α 및 IL-1α와 같은 염증성 사이토카인을 감소시켰다. APH의 위해성 평가는 피부 자극 및 과민성 시험으로 평가하였으며, 부작용은 관찰되지 않았다. 저자는 APH의 국소 적용이 아토피 피부염 환자의 습진 면적 및 중증도 지수, 피부 보습, 경피 수분 손실 및 가려움증의 개선을 가져온다는 것을 관찰했다. 이러한 연구 결과는 APH가 아토피 피부염 증상을 완화시키는 활성 성분으로 사용될 수 있음을 시사한다.

주요어: 아토피 피부염, 아스코르브산, 히알루론산, 항산화제, 항염증

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