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"Evaluation of the skin physiology improving function of a novel cosmetic active ingredient poacic acid and its application to cosmetics"

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1. Introduction

The skin, that is an organ that exists in the outermost, protects the water loss from body and acts as a barrier to prevent the invasion of external substances. The protective role of skin can be maintained the function when stratum corneum consists of normally formed keratinocytes only. When the stratum corneum is damaged, moisture evaporates easily and the skin becomes dry and weak against external substances. Corneous also occurs at the same time. To enhance skin barrier and moisturizing, many cosmetic products have been developed. But chemical components contained in general cosmetic products caused skin side effects. Therefore our study was carried out using natural product to enhancing skin barrier, increasing moisturizing, and preventing wrinkle formation.

Poacic acid (diferulate, decarboxylated product from 8–5-diferulic acid, **PA**) was identified as a novel natural cell-wall-targeted antifungal agent by screening a collection of diferulates found in lignocellulosic hydrolysates using Saccharomyces cerevisiae as a discovery platform [1]. The postulated cellular target for **PA** is the β-1,3-glucan cell wall network. The interaction of **PA** with the cell wall leads to growth inhibition of several fungal species, such as the phytopathogenic fungi Sclerotinia sclerotiorum and Alternaria solani, as well as *S. cerevisiae*. It has been suggested that direct binding of **PA** to β-1,3-glucan interferes with polysaccharide maturation, leading to a weaker cell wall, but the mechanism of action is unknown. It is also uncertain whether **PA** can bind to other cell-wall components or polysaccharides because only qualitative evidence was provided.

Furthermore, the effect of **PA** on skin cells and its applicability in formulations other than as an antifungal agent are unknown. Therefore, we expected that **PA** would have an effective effect on skin cells and could be used as an active ingredient in cosmetic formulations.

In this study, we evaluated the proliferation and mRNA expression of epidermal and dermal function-related genes and production of skin tissue constituent proteins of **PA** on normal human epidermal keratinocytes (NHEKs) and normal human dermal fibroblasts (NHDFs).

Furthermore, we discovered the above gene expression and protein production were improved by O/W cream containing **PA** (**CRPA**) on reconstructed human skin model.

2. Materials and Methods

2-1. Preparation of poacic acid (PA)

Poacic acid (**PA**) was synthesized with former method (**Figure 1**) [2]. Starting from commercially available ferulic acid, a three-step synthetic procedure has been developed for the production of poacic acid. First, ferulic acid was esterified to produce ethyl ferulate. Second, peroxidase-catalyzed free radical dehydrodimerization of ethyl ferulate produced crude diferulates, mainly 8–5-diferulate. Finally, crystalline **PA** was obtained via alkaline hydrolysis of the crude diferulates after purification by flash-column chromatography.

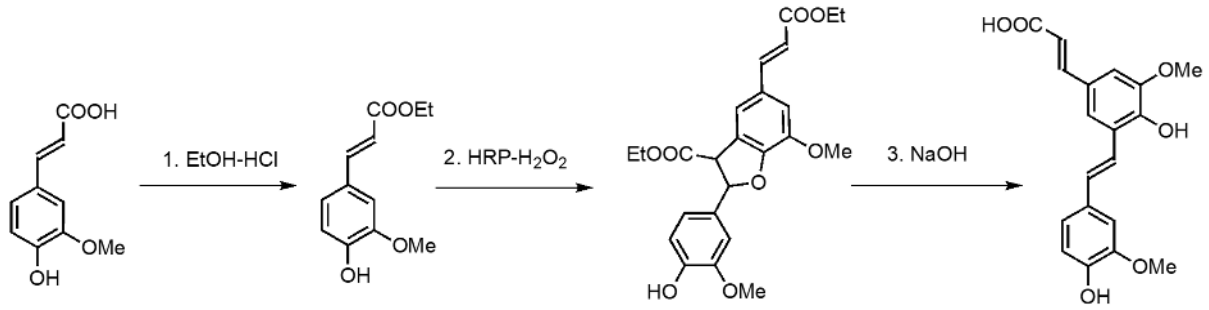

Figure 1. Synthesis scheme of poacic acid (**PA**)

2-2. Cells culture

Normal human epidermal keratinocytes (NHEKs; Kurabo Co., Osaka, Japan) were maintained in Humedia-KG2 (Kurabo Co. , Osaka, Japan) supplemented with insulin, human recombinant epidermal growth factor (EGF), hydrocortisone, gentamicin, amphotericin B and bovine pituitary extract (BPE), as instructed by the supplier.

Normal human dermal fibroblasts (NHDFs; Kurabo Co., Osaka, Japan) were maintained in FibroLife® basal medium (BM; Lifeline® Cell Technology, Maryland, USA) supplemented with L-glutamine, hFGF-β, insulin, ascorbic acid, hydrocortisone, and 2% fetal bovine serum (FBS), as instructed by the supplier.

2-3. Assay of cell proliferation

NHEKs and NHDFs were inoculated into 96-well microplates (3.0×10^5 cells/well and 1.0×10^6 cells/well) and cultivated for 24 h. After culture, the medium was changed to serum-free medium containing **PA**, and cultivated for 24 h. Control cells were cultivated without samples. After 24 h incubation, fibroblasts were used for the MTT assay, based on the Mosmann method.

2-4. Stimulation assay of the mRNA expression on NHEKs and NHDFs

After treatment of NHEKs (3.0×10^5 cells/well) or NHDFs (1.0×10^6 cells/well) with **PA**, cells were incubated RNAcell protect reagent (QIAGEN, Valencia, CA, USA). Total RNAs of NHEKs were extracted using NucleoSpin® RNA Plus (Machery-Nagel GmbH & Co. KG, Germany) and reverse-transcribed to cDNA with Oligo dT Primers and Random 6 mers using a PrimeScript® RT reagent Kit (TaKaRa Bio Inc., Japan). Real-time PCR was performed with SYBR® Premix Ex TaqTM II (TaKaRa Bio Inc.) and the products were analyzed using a Thermal Cycler Dice® Real Time System TP800 (TaKaRa Bio Inc.). Primers used for quantitative PCR are provided in Table 1.

Table 1. Primers used for quantitative PCR

	Sense	Anti-sense
AQP-3	5'-CCACAGCTTAGGTTTGGACC-3'	5'-TCCCGGATCCCTAAGACTGT-3'
Claudin1	5'-GGTCAGGCTCTCTTCACTGG-3'	5'-GCCTTGGTGTTGGGTAAGAG-3'
Filaggrin	5'-TCAAACAGGAGGGACAGACC-3'	5'-TATCCTCCCTGACCACTTGC-3'
Involcrin	5'-CCAGTGAAAGGAGACGTGCT-3'	5'-TATGGGTGAGTAGGCCAGCT-3'
Loricrin	5'-GTGGAAAGACCTCTGGTGGA-3'	5'-TGGAACCACCACCTCCATAGGAA-3'
Nrf 2	5'-TCTTGGAGTAAGTCGAGAAGTGT-3'	5'-GTTGAAACTGAGCGAAAAAGGC-3'
GCLc	5'-GGGGTGACGAGGTGGAGTA-3'	5'-GTTGGGGTTTGTCCTCTCCC-3'
GCLm	5'-AGGAGCTTCGGGACTGATCC-3'	5'-GGGACATGGTGCATTCCAAAA-3'
GR	5'-GTGAGCCGACTGAACACCATCTAT-3'	5'-CTTCTTCCCGTTGACCTCTACTG-3'
HO-1	5'-AAGCCGAGAATGCTGAGTTCA-3'	5'-GCCGTGTAGATATGGTACAAGGA-3'
COL1A1	5'-GTGGCCTGCCTGGTGAG-3'	5'-GCACCATCATTTCCACGAGC-3'
COL1A2	5'-ATGCCGTGACTTGAGACTC-3'	5'-CCTTGGTGGTAACTCCTTCC-3'
FBN-1	5'-CTTCCACCTAACAGGCCATTAACA-3'	5'-CTATCACATGGTTCCATAGGTGCAG-3'
MFAP-4	5'-CCTATGCCAAGTACGCTGACTTCT-3'	5'-AACTTCTGGCCACTGTGGTAGGAC-3'
EMILIN-1	5'-CCTTCLACAGAGTCCTGCTCAA-3'	5'-CGCTCAGCAAGTAGCGTCCA-3'
LTBP-4	5'-TTCACLACTGTCAGCTGTGCTC-3'	5'-TTTCAAAGCCGGTTGGACAAG-3'

2-5. Filaggrin involucrin, loricrin,and glutathione synthesis assay

NHEKs were inoculated to 96-well microplate (3.0×10^5 cells/well) and cultivated for 24 h. After the cultivation with serum-free medium containing **PA** for 72 h, the cells were pelleted and sonicated in RIPA buffer. Amounts of filaggrin and involucrin, loricrin, glutathion were measured by using ELISA system and available quantitative kit.

2-6. Type I collagen and elastin synthesis assay

NHDFs was inoculated into 96-well microplates (1.0×10^6 cells/well) and cultivated for 24 h. After culture, the medium was changed to serum-free medium containing **PA**, and cultivated for 72 h. After culture, supernatants of each well were collected, and amount of type I collagen and elastin was by using ELISA system and available quantitative kit.

2-7. Improvement assay of O/W cream containing **PA** with human epidermal skin model

Cream (**CRPA**) containing **PA** was prepared by uniformly dispersing **PA** at several concentrations in a O/W cream formulation based on the Japanese Pharmacopoeia hydrophilic ointment. The improvement assay of **CRPA** was performed by using human epidermal skin model, LabCyte EPI-model (J-TEC Co., JAPAN). **CRPA** (25 µl) was added to the interior of skin model with 1.0 ml medium. After a fixed period of time, the collected cell layers and mediums were subjected to former assays.

2-8. Statistical analysis

The cell proliferation, filaggrrin content, loricrin content, involucrin content, glutathione content, collagen content, erastin content data were expressed as the mean ± standard error (S.E.), and subsequent inspection of means was evaluated by Student's t-test between two groups at significance levels of $p < 0.05$ (*).

3. Results

3-1. Cell proliferation of **PA**

PA showed the cell proliferation on NHEKs and NHDFs in dose-dependent manner (**Figure. 2**). PA showed 116% and 112% of the proliferation on NHEKs and NHDFs at a concentration of 5 μM.

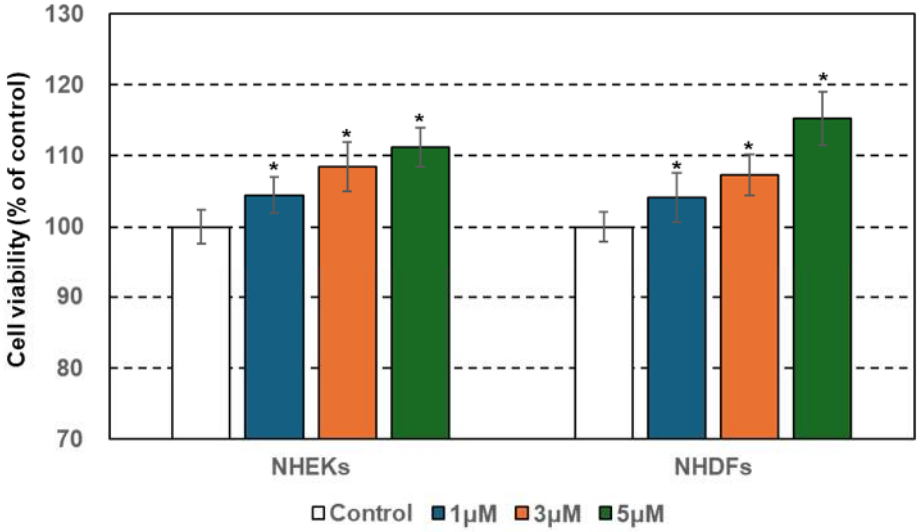

Figure.2. Cell proliferation of PA on NHEKs and NHDFs..

Each value reported represent means ± SD.

Values were significantly different from the control group, at $p < 0.05$ (*).

3-2. Stimulation of **PA** of the mRNA expression of epidermal function-related genes on NHEKs

Treatment of NHEKs with **PA** showed the significant upregulation of mRNA expression of epidermal function-related genes, Aquaporin-3 (AQP-3), Claudin1, Filaggrin, Involcrin, and Loricrin in a dose dependent manner (**Figure 3**). **PA** stimulated upregulation of mRNA expression of AQP-3, Claudin1, Filaggrin, Involcrin, and Loricrin at a concentration of 5 μM by 1.77, 1.74, 3.14, 3.98, 3.43 times. Especially, **PA** showed the higher up-regulation of mRNA expression of Filaggrin, Involcrin, and Loricrin, which involved in the formation of connified envelope.

3-3. Stimulation of **PA** of the mRNA expression of antioxidative function-related genes on NHEKs

Treatment of NHEKs with **PA** showed the significant upregulation of mRNA expression of antioxidative function-related genes, NF-E2-related factor 2 (Nrf2), Gultamate-cysteine ligase catalytic subnit (GCLc), Gultamate-cysteine ligase modifier subnit (GCLm) , Glucocorticoid receptor (GR), Heme Oxygenase 1 (HO-1) in a dose dependent manner (**Figure 4**). **PA** stimulated upregulation of mRNA expression of Nrf2, GCLc, GCLm, GR and HO-1 at a concentration of 5 μM by 1.41, 2.78, 2.98, 2.11, 4.16 times. Especially, **PA** showed the higher up-regulation of mRNA expression of HO-1, whose expression is induced by heat, oxidative stress, inflammatory cytokines.

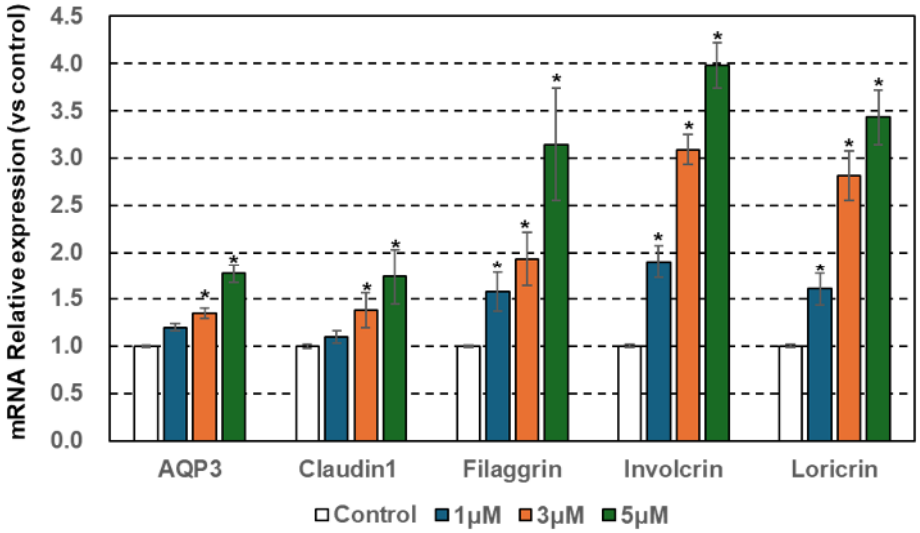

Figure 3. Up-regulation of PA on the mRNA expression of F mRNA expression of epidermal function-related genes (AQP3, Claudin1, Filaggrin, Involucrin, Loricrin) on NHEKs.

Each value reported represent means ± SD.

Values were significantly different from the control group, at $p < 0.05$ (*).

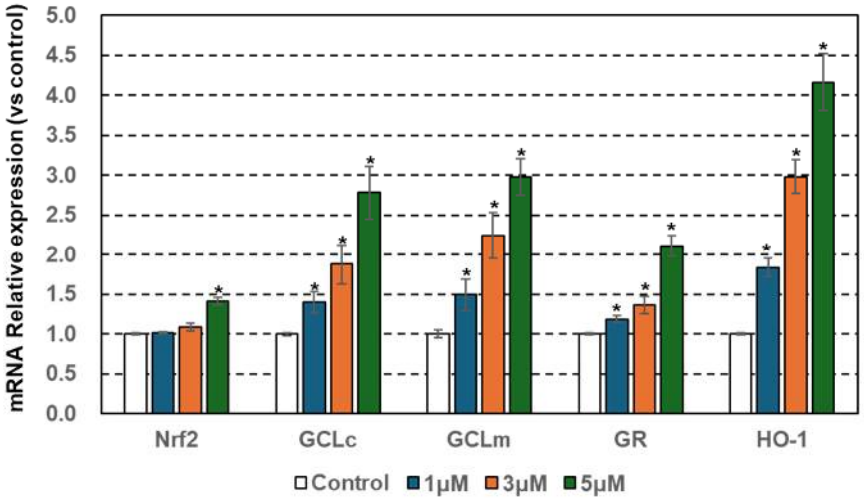

Figure 4. Up-regulation of PA on the mRNA expression of F mRNA expression of antioxidative function-related genes (Nrf2, GCLc, GCLm, GR, HO-1) on NHEKs.

Each value reported represent means ± SD.

Values were significantly different from the control group, at $p < 0.05$ (*).

3-4. Stimulation of **PA** of filaggrrin and involucrin, loricrin, and glutathione synthesis on NHEKs

The amount of filaggrrin and involucrin, loricrin, and glutathione in the NHEKs lysate were increased by treated with **PA** in a concentration dependent manner (**Figure 5**). The maximal increase of 128%, 141%, 128% and 147% on filaggrrin and involucrin, loricrin, and glutathione production was seen in **PA** with a concentration of 5 μM.

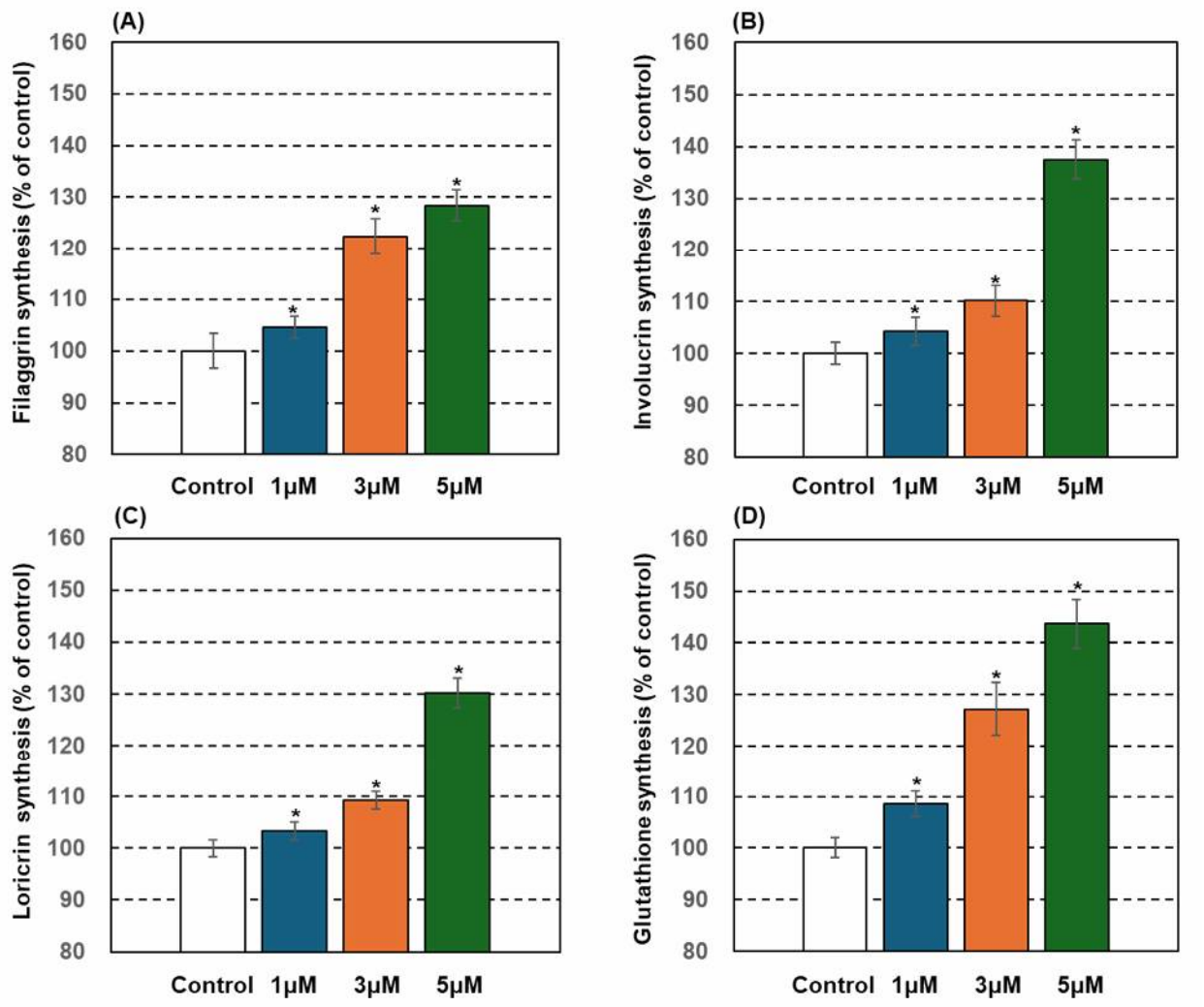

***Figure 5**. Effects of PA on the filaggrin (**A**), involucrin (**B**), loricrin (**C**), and glutathione (**D**) synthesis in NHEKs. Each value reported represent means ± SD.*

Values were significantly different from the control group, at $p < 0.05$ (*).

3-5. Stimulation of **PA** of the mRNA expression of dermal function-related genes on NHDFs

Treatment of NHEKs with **PA** showed the significant upregulation of mRNA expression of epidermal function-related genes, COL1A1, COL1A2, Fibrillin-1, MFAP-4, EMILIN-1 in a dose dependent manner (**Figure 6**). **PA** stimulated upregulation of mRNA expression of COL1A1, COL1A2, Fibrillin-1, MFAP-4, EMILIN-1 at a concentration of 5 μM by 1.40, 1.50, 2.31, 1.36, 2.05, and 1.31 times. Especially, **PA** showed the higher up-regulation of mRNA expression of Fibrillin-1 and EMILIN-1, which icontributes to the formation of elastin fibers.

3-6. Stimulation of **PA** of type I collagen and elastin synthesis on NHDFs

The amount of type I collagen and elastin on the NHDFs were increased by treated with **PA** in a concentration dependent manner (**Figure 7**). The maximal increase of 166% and 181% on type I collagen and elastin production was seen in **PA** with a concentration of 5 μM.

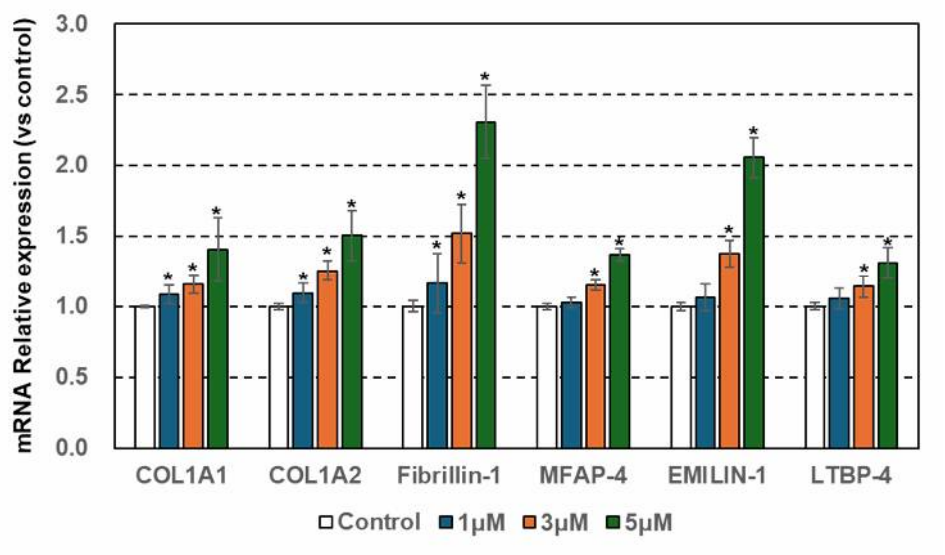

Figure 6. Up-regulation of PA on the mRNA expression of F mRNA expression of dermal function-related genes (COL1A1, COL1A2, Fibrillin-1, MFAP-4, EMILIN-1) on NHDFs.

Each value reported represent means ± SD.

Values were significantly different from the control group, at $p < 0.05$ (*).

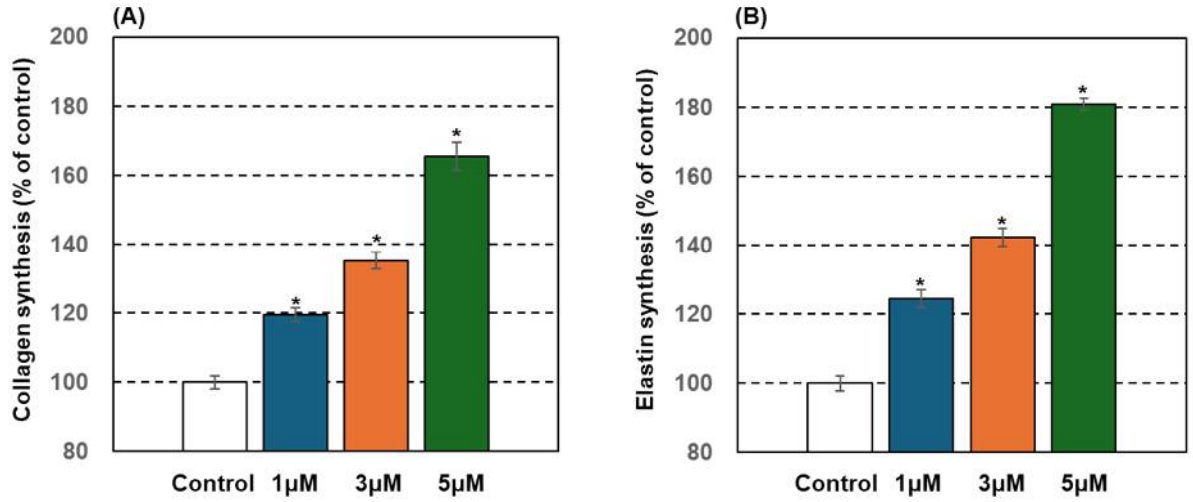

Figure 7. Effects of PA on the collagen (**A**) and elastin (**B**) synthesis in NHDFs.

Each value reported represent means ± SD.

Values were significantly different from the control group, at $p < 0.05$ (*).

3-7. Stimulation of the mRNA expression and protein synthesis of filaggrrin and involucrin, loricrin with treatment of **CRPA** on epidermal model

Treatment of epidermal human skin model with **CRPA** showed the significant upregulation of mRNA expression (**Figure 8**) and protein synthesis (**Figure 9**) of Filaggrin, Involcrin, and Loricrin in a dose dependent manner. Treatment of **CRPA** containing 0.20% **PA** stimulated upregulation of mRNA expression of Filaggrin, Involcrin, and Loricrin by 2.41, 1.53, and 2.26 times. The maximal increase of 125%, 116%, and 112% on filaggrrin and involucrin, loricrin, and glutathione production was seen in treatment of **CRPA** containing 0.20% **PA**. Especially, treatment of **CRPA** showed the higher up-regulation of mRNA expression and protein synthesis of Filaggrin.

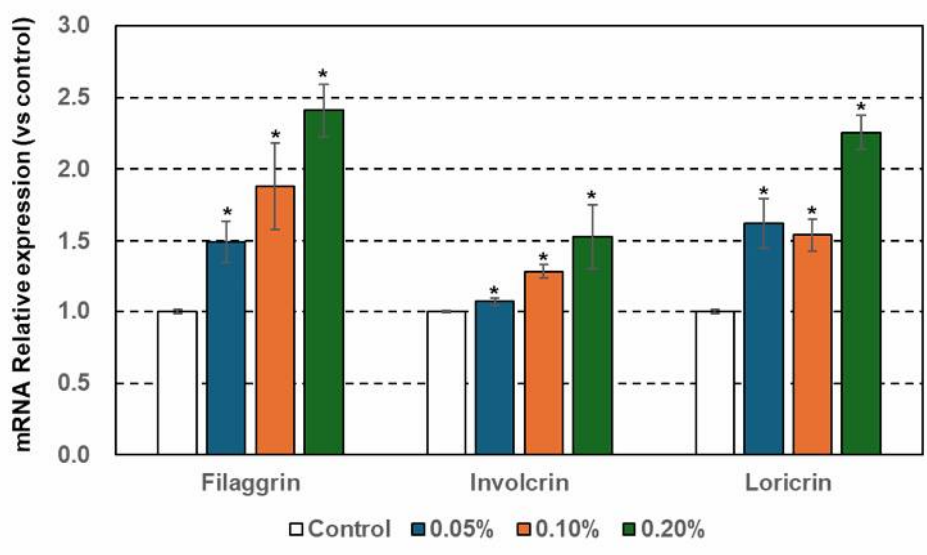

Figure 8. Up-regulation of treatment of CRPA on the mRNA expression of F mRNA expression of epidermal function-related genes (Filaggrin, Involucrin, Loricrin) on epidermal skin model.
Each value reported represent means ± SD.
Values were significantly different from the control group, at $p < 0.05$ (*).

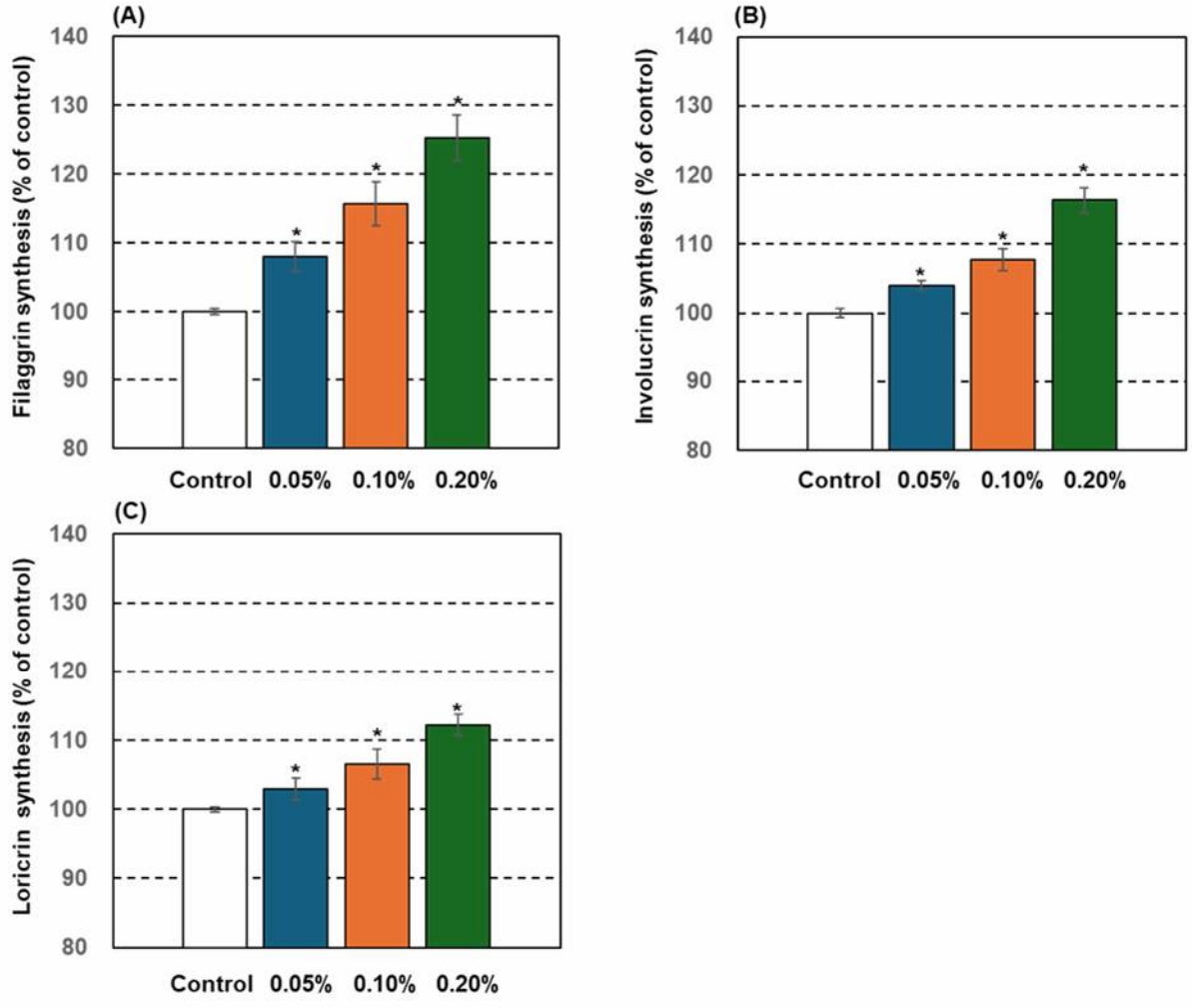

Figure 9. Effects of treatment of CRPA on the filaggrin (**A**), involucrin (**B**), and loricrin (**C**) synthesis on epidermal skin model.

Each value reported represent means ± SD.

Values were significantly different from the control group, at $p < 0.05$ (*).

4. Discussion

PA significantly promoted proliferation on NHEKs and NHDFs, upregulation of mRNA expression of epidermal function-related genes (AQP3, Claudin1, Filaggrin, Involucrin, Loricrin), of antioxidative function-related genes (Nrf2, GCLc, GCLm, GR, HO-1) in NHEKs, and dermal function-related genes (COL1A1, COL1A2, Fibrillin-1, MFAP-4, EMILIN-1) in NHDFs. Furthermore, **PA** significantly stimulated the production of filaggrin, involucrin, loricrin, glutathione in NHEKs, and type I collagen and elastin in NHDFs. When **CRPA** containing **PA** was applied to a epidermal skin model, similar improvement effects were observed at the cellular level.

Tight junctions are also key contributors to the epidermal barrier, and claudin-1, which is a main component of tight junctions in the epidermis, is crucial for transepidermal water loss (TEWL) and skin barrier function [3]. Fillagrin is a precursor of a natural moisturizing factor (NMF) that shows a skin moisturizing ability, and also plays the role of join together the keratin in NHEKs. Filaggrin monomers are further degraded into natural moisturizing factors that are thought to maintain hydration of the upper stratum corneum and reduce the pH of the skin surface [4, 5]. Cornified envelope plays a role of a physical barrier in the skin, and also has been formed crosslinked proteins such as involcurin and loricrin. The degradation of collagen and elastin is weakening the skin barrier because collagen is an important factor not only of skin barrier but also of process key of wrinkle formation, which is a common phenomenon of aged skin.Therefore, stimulation of these factors is indicated to be enhanced skin barrier.

PA is reported to inhibit growth of the fungi *Sclerotinia sclerotiorum* and *Alternaria solani* as well as the oomycete *Phytophthora sojae*, through its activity on the glucan layer. Chemical genomics using *Saccharomyces cerevisiae* showed that loss of cell wall synthesisand maintenance genes conferred increased sensitivity to **PA**. Morphological analysis revealed that cells treated with **PA** behaved similarly to cells treated with other cell wall-targetingdrugs and mutants with deletions in genes involved in processes related to cell wall biogenesis.

From the above reports, it is expected that **PA** will localize in the skin cell membrane and be highly sensitive to its improving effects on cells. The results of this study also confirmed that **PA** is effective not only for the components that maintain skin homeostasis in various types of skin cells, but also for various phenomena at the cellular level in skin aging and photoaging, suggesting its high sensitivity to those cells. Furthermore, the effectiveness of **PA** was confirmed in the test results of a skin epidermis model, suggesting that it also has a high affinity for the stratum corneum.

These results indicated that **PA** has a high affinity for skin cells, and so is a promising effective agent in cosmetic formulations offering an effective approaches such as the increase of skin hydration, formation of skin barrier and normalization of turnover on skin corneum, improvement of prevention and improvement of acne symptoms, anti-wrinkle formation and so on.

5. Conclusion

We clarified poacic acid (**PA**) significantly promoted proliferation on NHEKs and NHDFs, upregulation of mRNA ex-pression of epidermal function-related genes (AQP3, Claudin1, Filaggrin, Involucrin, Loricrin), of antioxidative function-related genes (Nrf2, GCLc, GCLm, GR, HO-1) in NHEKs, and dermal function-related genes (COL1A1, COL1A2, Fibrillin-1, MFAP-4, EMILIN-1) in NHDFs. Fur-thermore, **PA** significantly stimulated the production of filaggrin,

involucrin, loricrin, glutathione in NHEKs, and type I collagen and elastin in NHDFs. stimulation of these factors is indicated to be not only improved skin homeostasis and enhanced skin barrier, but also improved physicochemical properties such as elasticity by promoting the re-construction of skin tissue, and improved the relationship between the epidermis and dermis. **PA** may be a novel effective agent for cosmetic formulation to immediately and precisely targets skin cells, improving skin homeostasis and exerting various effects on the cellular level against skin aging for the new approach of localized penetration into the skin cell membrane on cosmetics.

6. Acknowledgments

I sincereky thank Haruo Oshima for his valuable information and advice when I began this research.

7. References

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