IFSCC 2025 full paper (162)

"Complementary effects of saccharide isomerate and dipotassium glycyrrhizate on tight junction barrier dysfunction and nerve activation"

Ryosuke Mogi [1,*], Simayijiang Aimaiti [1], Nanako, Maekubo [1], and Setsuji, Koyama [1]

[1] Chifure Beauty Science Center, Chifure Holdings Corporation, Saitama, Japan

1. Introduction

Itching is a sensation felt on the skin that causes a desire to scratch. Although numerous substances are known to cause pruritus, such as, substance P, cytokines, proteases, histamine is best known to evoke experimental itch when applied to the skin [1]. Recent studies have suggested that the tight junction (TJ) barrier plays a critical role in itch reduction by maintaining homeostatic pruning of epidermal nerves [2].

TJs form intercellular junctions in the second layer of stratum granulosum and control the suppression of an epidermal moisture transpiration and other protective functions, which are essential for maintaining healthy skin [3]. TJs contain several components, including transmembrane proteins (claudin [CLDN]) and TJ plaque proteins (zonula occludens [ZO]).

Histamine is released from mast cells when tissues are inflamed or stimulated by allergens, inducing itching triggered by excitation of a subset of unmyelinated C-fibers [1]. Histamine has also been reported to cause skin barrier dysfunction by downregulating the expression of TJ component proteins [4]. Moreover, histamine accelerates nerve activation by increasing nerve growth factor (NGF) [5] and decreasing semaphorin-3A (Sema3A) levels [6]. Therefore, suppression of both nerve activation and TJ barrier dysfunction induced by histamine is necessary for itch reduction.

Saccharide isomerate (SI) and dipotassium glycyrrhizate (DPG) have recently been used in cosmetics to treat dry and sensitive skin [7,8]. SI is a humectant with a stratum corneum water-retention function. In human studies, SI has been reported to inhibit water evaporation, retain stratum corneum water content, and reduce itching during long-term continuous use [9]. DPG is an anti-inflammatory agent. DPG has been reported to promote the expression of TJ components and suppress the increase in NGF under substance P stimulation in normal human epidermal keratinocytes (NHEKs). In human studies, DPG has been shown to reduce itching after both long- and short-term use [8].

However, the effects of SI and DPG, both individually and in combination, on TJ enhancement and itch reduction under histamine stimulation with SI and DPG, remain unclear. In the present study, we investigated the individual and combined effects of SI and DPG on histamine-induced TJ barrier dysfunction and nerve activation.

2. Materials and Methods

2.1. Cell culture and the reagents

NHEKs were purchased and cultured with a serum-free keratinocyte growth medium (KGM, keratinocyte basal medium (KBM) containing human epidermal growth factor, insulin, hydrocortisone, gentamycin/amphotericin B, and bovine pituitary extract) (Kurabo Industries, Japan). The Ca2+ concentration was 0.15 mM for growth medium and 1.5 mM or 1.8 mM for differentiation medium. When treated with SI (DSM, Netherlands) and DPG (Maruzen Pharmaceuticals, Japan), NHEKs were incubated with 1.5 mM or 1.8 mM Ca2+ in differentiation medium.

2.2. Quantitative real-time polymerase chain reaction (qPCR) analysis

NHEKs were seeded in 24-well plates in the KGM medium. After three days, the medium was changed to a differentiation medium (KGM with 1.8 mM CaCl2) containing 1000 μmol/L histamine and 500 μg/mL DPG or 0.25% SI for 24 h of cultivation.

Total RNA was isolated from NHEKs using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. cDNA was prepared using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA). qPCR was performed using TB Green Premix Ex Taq II (Takara, Japan) and specific primers on a QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific, USA). The following primers were purchased from FASMAC (Japan).

Claudin-1 (CLDN1)
Forward (F): GCATGAAGTGTATGAAGTGCTTGGA
Reverse (R): CGATTCTATTGCCATACCATGCTG

Claudin-4 (CLDN4)
F: CCTGCTAGCAAGAACAGAGTCCAC
R: GCAGGCAGATCCCAAAGTCA

Zonula occludens-1 (ZO-1)
F: CAGCAGAAATGCCTTACCCTCA
R: CACCAAATGCACAACGTACTCAGTC

Nerve growth factor (NGF)
F: AGCGTCCGGACCCAATAACA
R: CCTGCAGGGACATTGCTCTC

Semaphorin-3A (Sema3A)
F: GTCGCAGAGAGCGCTGGTCTATTGG
R: GTAATTGCCTGAATCCTTCTGTTGT

Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ)
F: GGCCCATCATGACATTGGGTA
R: CCACCAAGTAAGAATGGTTGGTGAC

All qPCR analyses were performed in triplicate, and relative expression levels were determined using the 2-ΔΔCt method after normalization to the expression of YWHAZ.

2.3. Immunofluorescence microscopy

NHEKs were seeded in a cover-glass chamber (Greiner, Germany) in KGM. After three days, the growth medium was changed to a differentiation medium (KGM with 1.8 mM CaCl2) containing 1000 µmol/L histamine and 500 µg/mL DPG or 0.25% SI for 48 h of cultivation. The cells were fixed with acetone: methanol = 1:1 (FUJIFILM Wako Pure Chemical, Japan).

After blocking with 0.1% BSA in PBS, the fixed cells were incubated with primary antibodies (rabbit anti-claudin-1 polyclonal antibody (Invitrogen, USA), mouse anti-claudin-4 monoclonal antibody (Invitrogen, USA), and mouse anti-ZO-1 monoclonal antibody (Invitrogen, USA)) overnight at 4 °C and then were incubated with secondary antibodies (goat anti-rabbit IgG Alexa Fluor 488 (Invitrogen, USA) or goat anti-mouse IgG Alexa Fluor 594 (Invitrogen, USA) for 30 min, followed by staining the nuclei with Hoechst 33342 (DOJINDO, Japan), and observed under a fluorescence microscope (KEYENCE, Japan). Images were processed using an image analysis software (KEYENCE, Japan).

2.4. Western Blotting

NHEKs were seeded in 24-well plates in the KGM medium. After 24 h of incubation, the growth medium was changed to a differentiation medium (KGM with 1.8 mM CaCl2) containing 1000 µmol/L histamine and 500 µg/mL DPG or 0.25% SI for 24-48 h of cultivation.

NHEK protein lysates were prepared using the RIPA buffer containing protease/phosphatase inhibitors (Thermo Fisher Scientific, USA). Equal amounts of protein lysates were subjected to sodium dodecyl sulfate-polyacrylamide electrophoresis with 4–20% gradient stain-free gels (Bio-Rad Laboratories, USA) and transferred to low-fluorescence polyvinylidene difluoride membranes using the Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, USA).

The membranes were incubated with the following primary antibodies: rabbit anti-claudin-1 polyclonal antibody (Invitrogen, USA), mouse anti-claudin-4 monoclonal antibody (Invitrogen), mouse anti-ZO-1 monoclonal antibody (Invitrogen, USA), and the secondary antibodies: goat anti-rabbit IgG StarBright Blue 700 (Bio-Rad Laboratories, USA) or goat anti-mouse IgG StarBright Blue 520 (Bio-Rad Laboratories). After washing in water, the blots were analyzed using a ChemiDoc Touch MP (Bio-Rad Laboratories).

Total protein content was measured using Stain-Free Technology (Bio-Rad Laboratories, USA), following the manufacturer's instructions. The TJ protein levels were normalized to the total protein level, and each level was expressed as a percentage relative to the level in control cells.

2.5. Transepithelial Electrical Resistance (TEER) measurement

NHEKs were seeded onto 12-well polyester membrane Transwell-Clear inserts (Corning, USA) and cultured in DMEM. After three days, the growth medium was changed to differentiation medium (KGM with 1.5 mM CaCl2) containing 1000 µmol/L histamine and 500 µg/mL DPG or 0.25% SI. Changes in TEER were monitored using the Millicell Electrical Resistance System 2 (Millipore, USA) to evaluate the barrier function every 24 h for three days.

2.6. Statistical analysis

Data are presented as the mean ± standard error (SE) and were analyzed using Dunnett's multiple comparison test. Statistical analysis was performed using Statcel 4 software (OMS Publishing Inc., Japan). Statistical significance was set at $*p < 0.05$ or $**p < 0.01$.

3. Results

3.1. SI and DPG regulated gene expressions in TJ and nerve-related factors in histamine-induced NHEKs

We investigated the effect of SI and DPG on the mRNA expressions of CLDN1, CLDN4, ZO-1, NGF, and Sema3A in histamine-induced NHEKs using qPCR. Compared to the untreated group under histamine conditions, SI increased CLDN4 and decreased NGF mRNA levels, whereas DPG increased CLDN1, ZO-1, and Sema3A, while also decreasing NGF mRNA levels. Treatment with both SI and DPG increased CLDN1, CLDN4, ZO-1, and Sema3A and decreased NGF expression (Figure 1). These results, summarized in Table 1, suggest that the combination of SI and DPG regulates TJ and nerve-related factors in a complementary manner (Table 1).

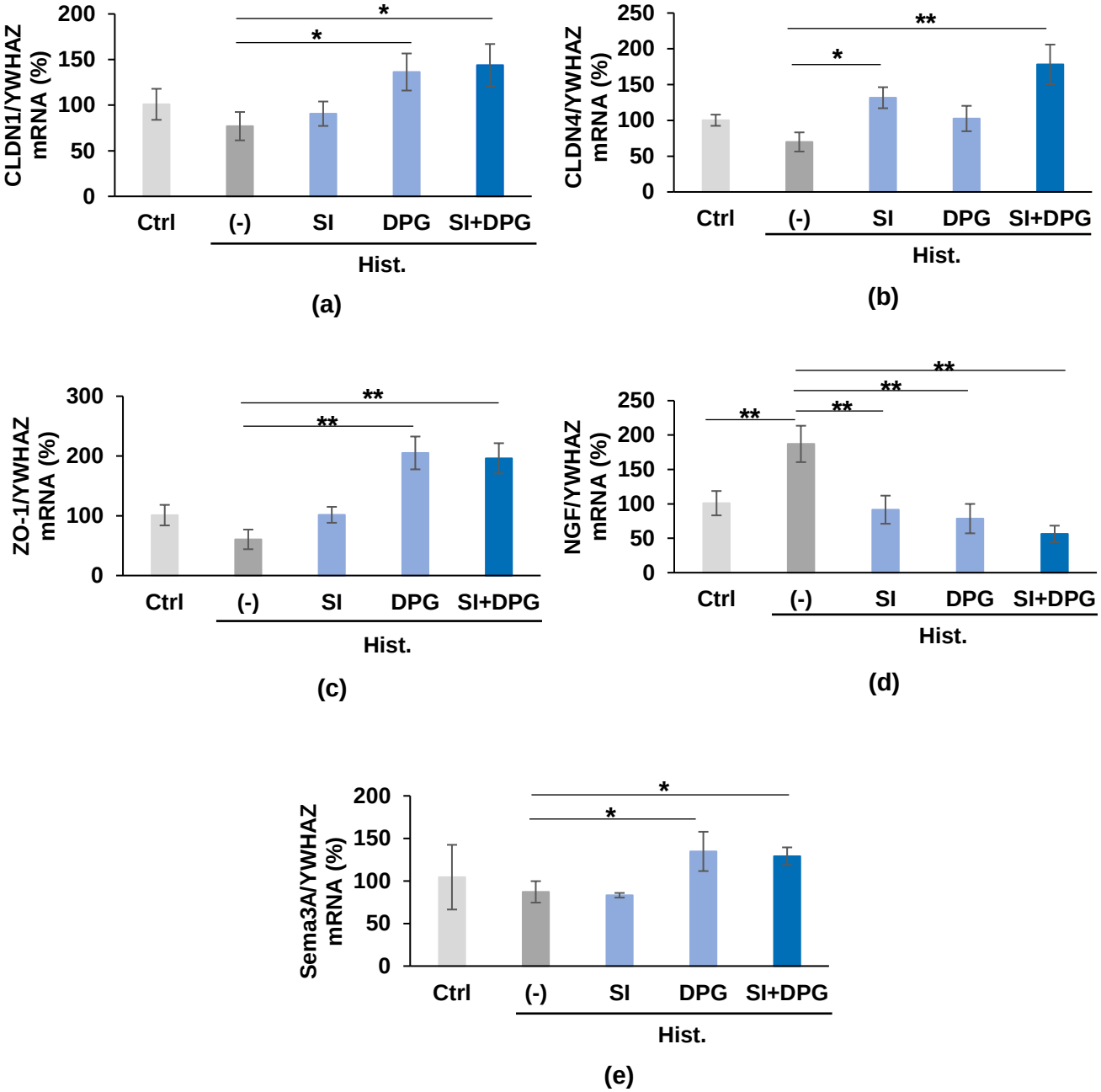

Figure 1. Combination of SI and DPG regulated the mRNA expressions of TJ-related factors ((a) CLDN1; (b) CLDN4; (c) ZO-1) and nerve-related factors ((d) NGF; (e) Sema3A) in histamine induced NHEKs. NHEKs were treated with or without 1000 μmol/L histamine, 500 μg/mL DPG, or 0.25% SI for 24 h. The mRNA expressions of CLDN1, CLDN4, ZO-1, NGF, and Sema3A were measured by qPCR. Each bar represents the mean ± SE, n=3, *p<0.05, **p<0.01, vs. Hist using Dunnett's test.

Table 1. Comparison of SI and DPG mRNA levels in the histamine-induced and deduced NHEKs, ⇑: Significantly increased, ↗: Tendency to increase, ⇒: Unchanged, ⇓: Significantly decreased.

vs Hist.	SI	DPG	SI + DPG
CLDN1	↗	⇑	⇑
CLDN4	⇑	↗	⇑
ZO-1	↗	⇑	⇑
NGF	⇓	⇓	⇓
Sema3A	⇒	⇑	⇑

3.2. SI and DPG enhanced protein levels and localization of TJ proteins in histamine-induced NHEKs

Next, we investigated the effects of SI and DPG on the protein levels of CLDN1, CLDN4, and ZO-1 in histamine-induced NHEKs using western blotting. Compared with the results obtained in histamine-induced NHEKs, SI increased the protein levels of CLDN4. Treatment with both SI and DPG increased CLDN4 protein levels and tended to increase CLDN1 and ZO-1 protein levels (Figure 2 (a)~(d)), and promoted their localization to the membrane (Figure 2 (e)).

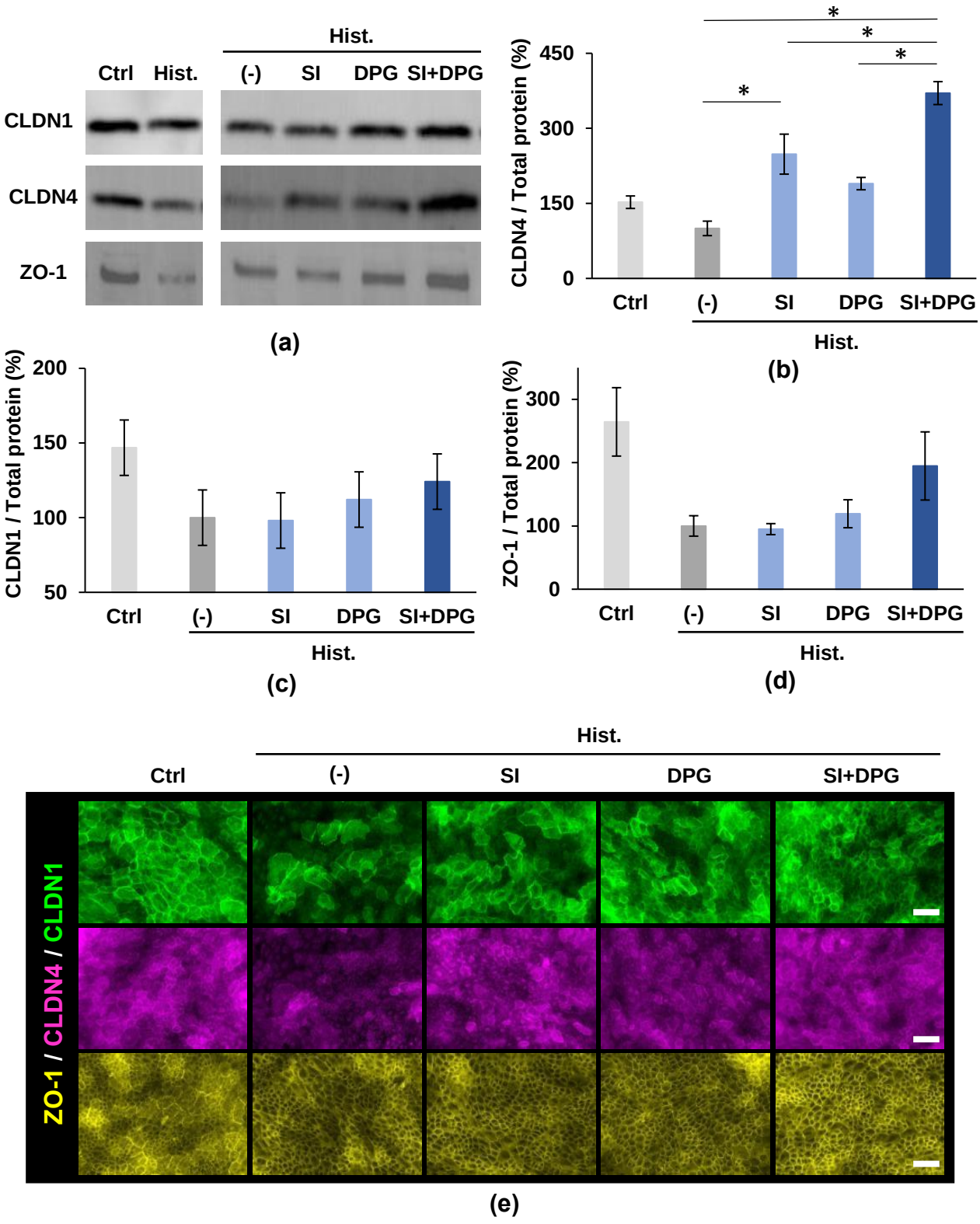

Figure 2. Combination of SI and DPG enhanced protein levels and membrane localization of TJ-related factors in histamine-induced NHEKs. NHEKs were treated with or without 1000 μmol/L histamine, 500 μg/mL DPG, or 0.25% SI for 24–48 h: (a)~(d) Protein levels of CLDN1 (24 h), CLDN4, ZO-1 (48 h) in total cell extracts were analyzed by western blotting. Each bar represents the mean ± SE, n=3, *p<0.05, vs. Hist using Dunnett's test; (e) localization of CLDN1, CLDN4, and ZO-1 at 48 h was detected using laser microscopy. Scale bars represent 100 μm.

3.3. SI and DPG enhanced barrier function in histamine-induced NHEKs

To investigate whether SI and DPG contribute to skin barrier function, we measured the TEER values in histamine-induced NHEKs treated with SI and DPG. Compared to the results obtained in histamine-induced NHEKs, SI increased the TEER values at 72 h (Figure 3 (a)), and DPG increased the TEER values at 24–48 h (Figure 3 (b)). Their combination increased the TEER by 24–72 h (Figure 3 (a), (b)). These results suggest that combination of SI and DPG regulates the TEER values in a complementary manner (Table 2).

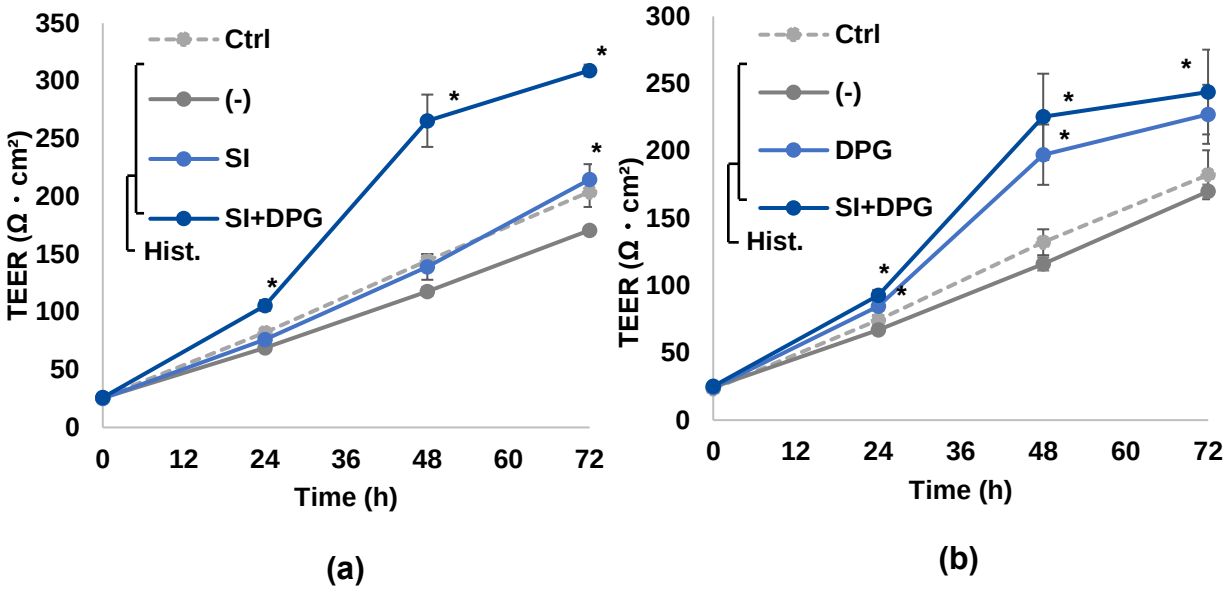

Figure 3. The combined effects of SI and DPG on skin barrier function in histamine-induced NHEKs. The TEER values (24-72 h) were measured using a Milli cell ERS-2: (a) SI alone and SI with DPG groups; (b) DPG alone and SI with DPG groups. Each bar represents the mean ± SE, n=3, *p<0.05, vs. Hist using Dunnett's test.

Table 2. Comparison of the TEER values during treatment with SI and DPG and the values obtained in histamine-induced NHEKs, ⇑: Significantly increased, ⇗: Tendency to increase, ⇒: Unchanged.

vs Hist.	SI	DPG	SI + DPG
24h	⇒	⇑	⇑
48h	⇒	⇑	⇑
72h	⇑	⇗	⇑

4. Discussion

In previous studies, SI was reported to inhibit water evaporation, retain stratum corneum water content, and reduce itching during a long-term continuous use [7,9]. DPG has been reported to promote the expression of TJ-related factors and suppress the increase in NGF under substance P stimulation in NHEKs. DPG has also been shown to reduce itching with long- and short-term use in human studies [8].

In this study, we investigated the individual and combined effects of SI and DPG on both the TJ barrier dysfunction and nerve activation induced by histamine in NHEKs.

Regarding the mRNA expression of TJ-related factors, SI increased CLDN4, and DPG elevated CLDN1 and ZO-1 expression. Moreover, their combination increased CLDN1, CLDN4, and ZO-1 expression. Similar trends were confirmed for the protein and membrane localization of CLDN1, CLDN4, and ZO-1. These results suggest that SI and DPG individually increase the expression of different TJ-related factors, and their combination complement each other to enhance the expression levels and membrane localization, consequently increasing the TEER. In a previous study, the TJ barrier was shown to play a critical role in itch reduction by maintaining the homeostatic pruning of epidermal nerves [2]. Thus, the combination of SI and DPG may improve the TJ barrier and maintain homeostatic pruning of epidermal nerves. Regarding the mRNA expression of nerve-related factors, SI and DPG decreased NGF expression, whereas DPG increased Sema3A expression. The same effect was observed when both components were combined.

These results suggest that the combination of SI and DPG inhibits nerve overactivity by effectively regulating the expression of nerve-related factors. In a previous study, NGF was shown to be involved in the release of histamine from mast cells [5]. Thus, the combination of SI and DPG inhibited histamine-induced NGF activation, suggesting that it may also inhibit histamine release from mast cells. Our results suggest that the combination of SI and DPG is a potential agent for itch reduction through TJ barrier improvement and nerve regulation.

The barrier function-enhancing and neuroactive inhibitory effects of SI and DPG have been confirmed in vitro, but their combined effects in humans are not yet known. Therefore, clinical studies on the effects of SI- and DPG-containing formulations are currently underway.

5. Conclusion

The combination of SI and DPG ameliorated histamine-induced TJ barrier dysfunction and nerve overactivation by regulating the mRNA and protein expression of CLDN1, CLDN4, ZO-1, NGF, and Sema3A, and improving barrier function. We hope that our results will help produce quality skincare products for people with sensitive skin and lead to their quality of life (QOL) improvement.

6. References

[1] Shim, W.S., Oh, U. Histamine-induced itch and its relationship with pain. Molecular Pain. 4 (2008) 29.
[2] Sonoko, T. *et al.* Homeostatic pruning and activity of epidermal nerves are dysregulated in barrier-impaired skin during chronic itch development. Scientific Reports. 9 (2019) 8625.
[3] Gschwandtner, M. *et al.* Histamine suppresses epidermal keratinocyte differentiation and impairs skin barrier function in a human skin model. Allergy 63 (2013) 37-47.
[4] Kubo, A., Nagao, K., Amagai, M. Epidermal barrier dysfunction and cutaneous sensitization in atopic diseases. J Clin Invest. 122 (2012) 440–447.
[5] Naoko, K., Shinichi, W. Histamine enhances the production of nerve growth factor in human keratinocytes. Journal of Investigative Dermatology. 121 (2003) 570-577.
[6] Shoko, Fukamachi. *et al.* Modulation of semaphorin 3A expression by calcium concentration and histamine in human keratinocytes and fibroblasts. Journal of Dermatological Science. 61 (2011) 118-123.
[7] Padberg, G. Effect of the carbohydrates scleroprotein interaction on water‐binding by the horny layer. J Soc Cosmet Chem. 23 (1972) 271-279.
[8] Yashiki, K., Ohto, N., Kawashima, Y., Nakahara, T. The Effect of Dipotassium Glycyrrhiza on Sensitive Skin Care. Journal of Socirty of Cosmetic Chemists of Japan. 50 (2017) 334-339.
[9] Smith, W. Reduction of AHA irritation potential by inclusion of a saccharide isomerate. SOFW-Journal. 121 (1995) 1013-1017.