Skin-improving function of disaccharide polysulfate, with increased effects on epidermal moisturizing and dermal elastic factors, and decreased effects on pigmentation factors

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Abstract

We tested the human skin barrier function, moisturizing function, brightening function, elasticity function and efficacy of disaccharide polysulfate with a molecular weight of less than 1,000, which can penetrate healthy stratum corneum. We measured the transepidermal water loss (TEWL) of a three-dimensional human epidermis model cultured for 3 days after topical application of disaccharide polysulfate, then observed the effects on TEWL suppression. The mRNA levels of proteins involved in intercellular lipid transport, storage in the stratum corneum, in moisture retention, and involved in the degradation of FLG were observed increased by measuring with RT-qPCR. Furthermore, disaccharide polysulfate inhibited the elongation of dendritic processes of cultured human melanocytes and increased type 1 collagen and fibrillin 1 in cultured human fibroblasts. Based on our cell culture experiments, disaccharide polysulfate has excellent barrier functions, moisturizing, brightening and skin elasticity promoting effects. Disaccharide polysulfate, at lower concentrations than heparinoid, increased the stratum corneum water content. In a randomized, placebo-controlled, double-blind study, participants with low stratum corneum water content applied a lotion and emulsion containing disaccharide polysulfate to their faces for 4 weeks. Disaccharide polysulfate increased the stratum corneum water content and skin elasticity, and decreased both the TEWL and melanin index. In addition, improvements in skin translucence, pore size and blackheads in pores were observed. These results suggest that, beyond their moisturizing effects, cosmetics containing disaccharide polysulfate act on the epidermis by increasing barrier factors, decreasing pigmentation factors, and increasing collagen fibers and microfibrils, thereby ameliorating dry, dull and inelastic skin.

Keywords: disaccharide polysulfate, moisturizing, brightening, elasticity

1. Introduction

The skin has an important barrier function that prevents stimulation from the external environment and moisture evaporation from inside the body. The main factors maintaining this barrier function are the cornified cell envelope (CE) of stratum corneum cells, natural moisturizing factors (NMF) produced by the breakdown of proteins such as filaggrin (FLG) and intercellular lipids of the stratum corneum. NMF are primarily composed of amino acids and their derivatives, and are essential for skin moisturization. NMF are derived from FLG and are closely associated with skin hydration. FLG is a protein involved in differentiation that aggregates keratin fibers and the structural proteins of the stratum corneum, and strengthens the internal structure of the stratum corneum [1]. FLG is degraded by proteolytic enzymes, such as calpain-1 (CAPN1), caspase-14 (CASP14) and bleomycin hydrolase (BLMH), thus forming NMF [2]. The degradation of FLG into NMF is shown in **Figure 1**. Recently, abnormalities in the FLG gene have been found to be a major factor in the pathogenesis of ichthyosis vulgaris and atopic dermatitis [3, 4].

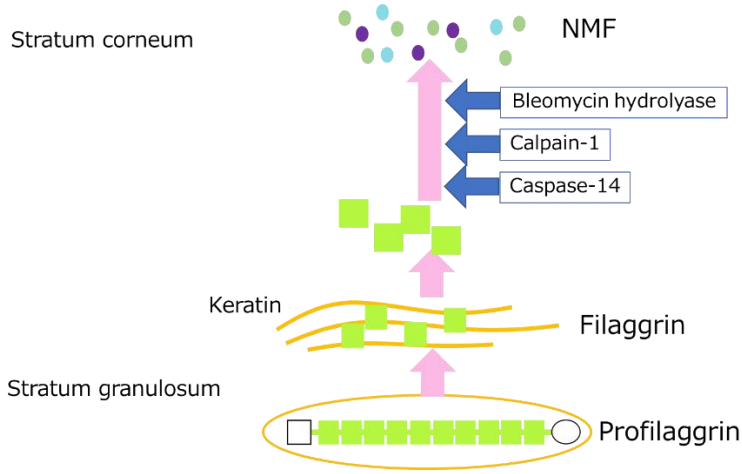


Figure 1. Degradation pathway of FLG to NMF in the stratum granulosum and stratum corneum.

CASP14, CAPN1 and BLMH are FLG-degrading enzymes. Low levels of CASP14 are often identified in patients with atopic dermatitis and psoriasis [5]. CAPN1, an μ-calpain, is activated by calcium ions. BLMH is universally present in all tissue, and its highest

concentrations are found in the skin. Patients with atopic dermatitis have been reported to have diminished levels of skin BLMH [6].

ATP binding cassette subfamily A member 12 (ABCA12) is a critical transmembrane lipid transfer protein in keratinocytes that plays an important role in lamellar granule lipid transport [7]. Spontaneous ABCA12 mutation has been shown to interfere with lipid transport into lamellar granules, thus causing ichthyosis [7]. ABCA12 is expressed between the stratum spinosum and stratum granulosum, and this transporter helps localize and store synthesized intracellular lipids within lamellar granules, and eventually secrete stored lipids from cells into the intercellular space of the stratum corneum [8]. Spontaneous mutations in, or other damage to, the ABCA12 gene interfere with intercellular lipid transport and proper lipid storage within the lamellar granules [7]. Eventually, intercellular lipid stores are diminished, thereby impairing barrier function.

Heparinoid, a mucopolysaccharide polysulfate, has a molecular weight of more than 5,000 ~~m.w.~~ and cannot penetrate healthy stratum corneum [9]. Therefore, in medicine, it is used for the treatment of inflammatory diseases, progressive palmar keratoderma, sebum deficiency (senile xeroderma), chilblains, hypertrophic scars and keloids, to moisturize and promote blood circulation [10]. Disaccharide polysulfate, with a molecular weight of less than 1,000 ~~m.w.~~, can penetrate healthy stratum corneum [11].

Solar lentigo (SL), also known as a sun-induced freckle or senile lentigo, is a hyperpigmentation that occurs increasingly with age in sun-exposed areas, such as the face, forearms, back, and the dorsal sides of the hands. Many studies have been conducted on SL from both clinical and histological viewpoints [12, 13]. We have also observed an increase in the cell size and elongation of dendrites of melanocytes in the SL lesions [14]. These morphological changes in melanocytes are consistent with increased melanization due to the differentiation of melanocytes in the SL lesions [14]

Dermal elasticity is conferred by elastic and collagen fibers, which are part of the extracellular matrix [15,16]. Elastic fiber primarily comprises elastin, which is fibrillar and cross-linked in many places. Elastic fiber turnover is very slow, similarly to that of collagen fibers [17,18]. Collagen is also known to decrease in aging skin [19]. It is also known to be denatured by UV rays [20]. Consequently, the fibers degenerate and deteriorate with age, thereby causing loss of dermal elasticity and consequently skin sagging and elasticity loss. Degeneration and deterioration of elastic and collagen fibers with aging render the skin unable to expand and contract; this inability is considered the direct cause of sagging and loss of elasticity.

Fibrillin 1 is a component of dermal elastin fibers (elastic fibers). Although fibrillin 1 has a similar distribution to that of elastin, it is particularly expressed and distributed in

oxytalan fibers and fine fibers (microfibrils approximately 10 nm in diameter) that are oriented from epidermal basal cells to the dermis downwards.

The objective of this study was to investigate whether topical application of disaccharide polysulfate with an average molecular weight of less than 1,000, to three-dimensional reconstructions of the human epidermis (3D epidermis models) might promote the synthesis of FLG. In addition, the contribution of FLG-degrading enzymes under increasing NMF doses was assessed by measurement of the mRNA levels of these enzymes and associated factors. We also examined the effectiveness of the lightening and elasticity function of disaccharide polysulfate on the skin.

2. Materials and Methods

2.1. Quantification of mRNA in a 3D epidermis model with topically applied disaccharide polysulfate

2.1.1. 3D epidermis model

The 3D epidermis model (LabCyte EPI-MODEL) was purchased from Japan Tissue Engineering Co. Ltd.

2.1.2 Application of disaccharide polysulfate to the 3D epidermis model

Twenty microliters of the prepared aqueous solution was topically applied to the 3D epidermis models (n = 3), then incubated at 37°C for 3 days.

2.1.3. RNA extraction and mRNA quantification by real-time PCR with the 3D epidermis model

RNeasy Protect Mini Kit (Qiagen N.V., Venlo, Netherlands) was used for RNA extraction and One-Step SYBR PrimeScript RT-PCR kit II (Takara Bio Inc. Ltd.) and Quant Studio 5 (Thermo Fisher Scientific Inc.) were used for analysis with the ΔΔCt method. Experiments were performed three times.

2.2. Immunohistochemical staining of the 3D epidermis models

2.2.1. Culture of the 3D epidermis models

Twenty microliters each of the 5 mg/mL aqueous solution of filter sterilized disaccharide polysulfate (S-sence Inc.., Yokohama, Japan) and ultrapure water (control) was topically applied to the 3D epidermis models (n = 3) and incubated at 37°C for 3 days.

2.2.2. Tissue section preparation

Embedding agent for frozen tissue section production (Tissue-Tek O.C.T. Compound; Sakura Finetek Japan Co., Ltd) was added to an embedding dish. After being completely frozen, the samples were thinly sliced to 10 μm with a cryostat (Leica Biosystems Nussloch GmbH) and collected on glass slides.

2.2.3. FLG immunofluorescence

The primary antibody (human anti-FLG rabbit polyclonal antibody (Atlas Antibodies AB) in PBS solution) was added to the tissues placed on glass slides and incubated for 1 h. After samples were washed with PBS, the secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG (H+L); Thermo Fisher Scientific Inc.) in PBS solution was added and allowed to stand for 1 h. After a washing step, nuclear staining was performed with DAPI (1000-fold dilution; Dojindo Laboratories) for 5 min. Each sample was washed with pure water and drained. The samples were observed and photographed under an inverted fluorescence microscope (IX70, Olympus Corporation). The experiment was performed on three tissue sections.

2.3. Effects of topical application of disaccharide polysulfate solution on the amounts of free amino acids in 3D epidermal models

2.3.1. Topical application of disaccharide polysulfate solution on 3D epidermal models

The 3D epidermal models (24-well, Japan Tissue Engineering Co., Ltd.) were cultured in dedicated medium for 1 day in an incubator. The medium was changed, 50 μL of aqueous solutions of disaccharide polysulfate (0.05%, 0.1% and 0.2%) and heparinoid (0.1% and 0.3%) were applied, and the cells were cultured for 3 days

2.3.2. *Measurement of* free amino acids and total amino acids

The 3D epidermal model was washed twice with PBS, and then 500 μL of 10 mM HCl (550-fold dilution of 20% HCl) was added to the epidermal model for 24 h at room temperature to extract free amino acids. Subsequently, 20 μL of the extract or amino acid standard diluted with 10 mM HCl was placed in a 96 well plate, 80 μL of Fluoraldehyde Reagent Solution (Thermo Scientific) was added, and the amount of free amino acids was measured at 360 nm excitation and 460 nm fluorescence. From the calibration curve, the amount of amino acids in the sample was determined. To correct for variations due to differences in the number of cells analyzed, the amount of free amino acids was normalized to the total amino acid amount.

The amount of total amino acids was determined by the addition of 1 mL of 6 M KOH and hydrolysis at 95°C for 24 hours to obtain the amount of amino acids with the method described above.

2.4. Effects of topical application of disaccharide polysulfate solution on human stratum corneum water content and transepidermal water loss (TEWL) in a 3D epidermal model

2.4.1. Effects of topical application disaccharide polysulfate solution on human stratum corneum water content

Stratum corneum water content was measured with a SKICON-200EX (Yayoi Co. Ltd.,

Tokyo, Japan) instrument, at high-frequency conduction at 24°C and 40%. After measurement of the stratum corneum water content, 10 μL of test solution (water, disaccharide polysulfate 0.05%, 0.1% or 0.2%; heparinoid 0.1% or 0.3%) was topically applied to six locations (1.5 cm × 1.5 cm) on the flexed sides of the forearms of five healthy adults, and spread with the side of a pipette. The change in the moisture content of the stratum corneum was measured with a SKICON-200EX instrument after the product was allowed 15 min to naturally penetrate the skin, and again after 30 min.

2.4.2. Effects of topical application disaccharide polysulfate solution on TEWL in a 3D epidermal model

A 3D epidermal model (24-well, Japan Tissue Engineering Co., Ltd.) was cultured in dedicated medium for 1 day in an incubator. Aqueous solutions of disaccharide polysulfate (0.05%, 0.1% or 0.2%) or heparinoid (0.1% or 0.3%) were topically applied in 40 μL volumes and incubated for 3 days. TEWL was measured with a Tewameter (Courage + Khazaka electronic GmbH instrument with a 24-well culture insert probe as previously described [19, 20] at 24°C and 40%.

2.5. Effects of disaccharide polysulfate on dendrites of human melanocytes

2.5.1. Culture of human melanocytes

In the center of a 35-mmφ dish (14-mmφ polylysine-coated; Matsunami Glass), 200 μL of melanocyte growth medium (DermaLife Ma Comp kit, Kurabo) was added. The cells were cultured in an incubator, and after cell adhesion, 2 mL of melanocyte growth medium was added to the culture.

2.5.2 Culture of human melanocytes in test medium

Melanocytes were cultured in 2 mL of melanocyte growth medium or 2 mL of melanocyte growth medium containing leukotriene C4 (LTC4; 500 nM) with each test substance (250 μg/mL) for 4 days, then photographed under a microscope (20× magnification).

2.6. Effects of disaccharide polysulfate on type 1 collagen and fibrillin 1 in cultured human fibroblasts

2.6.1. mRNA quantification by real-time PCR

Human dermal fibroblasts (TIG-111) were seeded at 100,000 cells/well in 12-well plates and cultured in 2 mL DMEM (Thermo Fisher Scientific Inc.) containing 2% FBS for 1 day. Subsequently, the medium was replaced with 2 mL DMEM containing 0.2% FBS, 125 μg/mL disaccharide polysulfate solution, 125 μg/mL disaccharide solution or solvent (PBS; control), and the cells were incubated for 3 days. RNA extraction and quantification of

mRNA by real-time PCR were performed as in Section 2.1.3.

2.6.2. Type I collagen assays

Human dermal fibroblasts were cultured with 2 mL DMEM containing 0.2% FBS, 125 μg/mL disaccharide polysulfate solution, 125 μg/mL disaccharide solution or solvent (PBS; control) for 3 days. Cells were counted with a Cell Counting Kit-8 (Dojindo Laboratories). One hundred microliters of diluted collagen for calibration or sample was placed in a high-adsorption plate (Immulon 96 well plate) and incubated at 4°C for 1 day to coat the plates. After blocking with 1% bovine serum albumin solution, the amount of type I collagen was measured using biotin-conjugated anti-collagen type I antibody (Rockland; 20 μg/mL) and streptavidin-horseradish peroxidase (HRP, Prozyme Inc.; 10,000 fold dilution). The 2, 2′-azinobis (3-ethylbenzothiazoline-6-sdfonic acid) ABTS solution (0.3 mg/mL) was allowed to react for 15 min. Stop solution (50 μL/well) was added. The absorbance was measured at 405 nm in a microplate reader.

2.7. Effects of topical application of lotion and emulsion containing disaccharide or polysulfate on dry skin in humans

After approval from the ethics committee, we sufficiently explained the aim, details and methods of the study to 26 female participants 33–59 years of age, then obtained their written informed consent to participate in the study. The study was conducted on healthy participants with low stratum corneum water content, high water evaporation and low lipid content. Informed consent was obtained from all participants in the study. The TEWL, water content in the stratum corneum and the lipid content of the skin surface were measured in a room kept at constant temperature and humidity, and the participants completed questionnaires regarding the condition of their skin. This was a randomized, placebo-controlled, double-blind, parallel comparative study on the topical application of lotions and emulsions, conducted over 8 weeks between November 2021 and December 2021. Participants were randomly allocated into one of two groups based on their age, TEWL, water content in the stratum corneum and lipid content of the skin surface measured before topical application, which were used as stratification factors for stratified block randomization, with 13 participants per group. Both test products (active group: lotion and emulsion containing 0.05% disaccharide polysulfate; placebo group: lotion and emulsion with no active components) were indistinguishable in their external appearance. Participants applied the lotion and emulsion to their faces in the amounts normally used,

twice daily, in the morning and night, for 4 weeks. No other lotion or emulsion was allowed to be used during the study period. The participants were asked to use their usual cleanser, make-up remover, ~~lotion,~~ and foundation, and were not allowed to switch to other products during the study. After the participants washed their faces in the face-washing room, they acclimated for 30 min in the waiting room. The temperature in the face-washing room, waiting room and measurement room was set to 21.8°C ± 0.2°C, and the humidity was set to 45.8% ± 1.3%.

TEWL was measured on the left cheek with a Tewameter TM300 device (Courage + Khazaka electronic GmbH). The water content in the stratum corneum was measured on the cheek with a SKICON-200EX instrument. The blood oxygen saturation index (Hb SO_2 index (%)) was measured with a CM-700d spectrophotometer (Konica Minolta) and the skin analysis software CM-SA. The melanin index was measured with a Mexameter (Courage + Khazaka electronic GmbH) instrument. Skin elasticity (R7) was measured with a Cutometer CT580 (Courage + Khazaka electronic GmbH) instrument. Skin translucence, pore size and blackheads in pores were evaluated visually on three levels by an expert.

2.8. Statistical analysis

For numerical data, t-tests were performed, and $P < 0.05$ was considered statistically significant.

For efficacy evaluation in human trials, the difference between each subject before (W0) and after (W4) topical application of the lotion and emulsion was evaluated with a paired t-test, or the difference between each measurement in the group with 0.05% disaccharide polysulfate added to the lotion and emulsion (active group) and the group without the addition (placebo group) was evaluated with an unpaired t-test. Non-numerical data were subjected to Wilcoxon signed rank test (W0 vs. W4) or Mann-Whitney's U test (active vs. placebo), with $P < 0.05$ being considered statistically significant and $P < 0.1$ being considered a trend.

3. Results

3.1. Moisture effects of disaccharide polysulfate

3.1.1. mRNA quantification of IVL, TGM1, FLG, CASP14, CAPN1, BLMH and ABCA12 in the 3D epidermis models

In the 3D epidermis models, 0.25 mg/mL disaccharide solution or disaccharide polysulfate solution was topically applied, and the cells were cultured for 3 days. mRNA

was extracted, and real-time PCR was performed. The primers were specific to the differentiation markers IVL and TGM1; FLG; the FLG-degradation enzymes CASP14, CAPN1 and BLMH; and the lipid transporter ABCA12.

The results of the quantitative mRNA analysis are shown in **Figure 2**. All values were normalized to those of ACTB. FLG is responsible for aggregating keratin proteins, the skeletal proteins of the stratum corneum. FLG is degraded into NMF, which is essential for moisturization. The mRNA levels of IVL, TGM1, FLG, CASP14, CAPN1, BLMH and ABCA12 were significantly higher in the disaccharide polysulfate-treated 3D epidermis model than in the solvent (control)-treated 3D epidermis model. In contrast, the mRNA levels of these enzymes were not significantly higher in the disaccharide-treated epidermis than in the solvent (control)-treated 3D epidermis model.

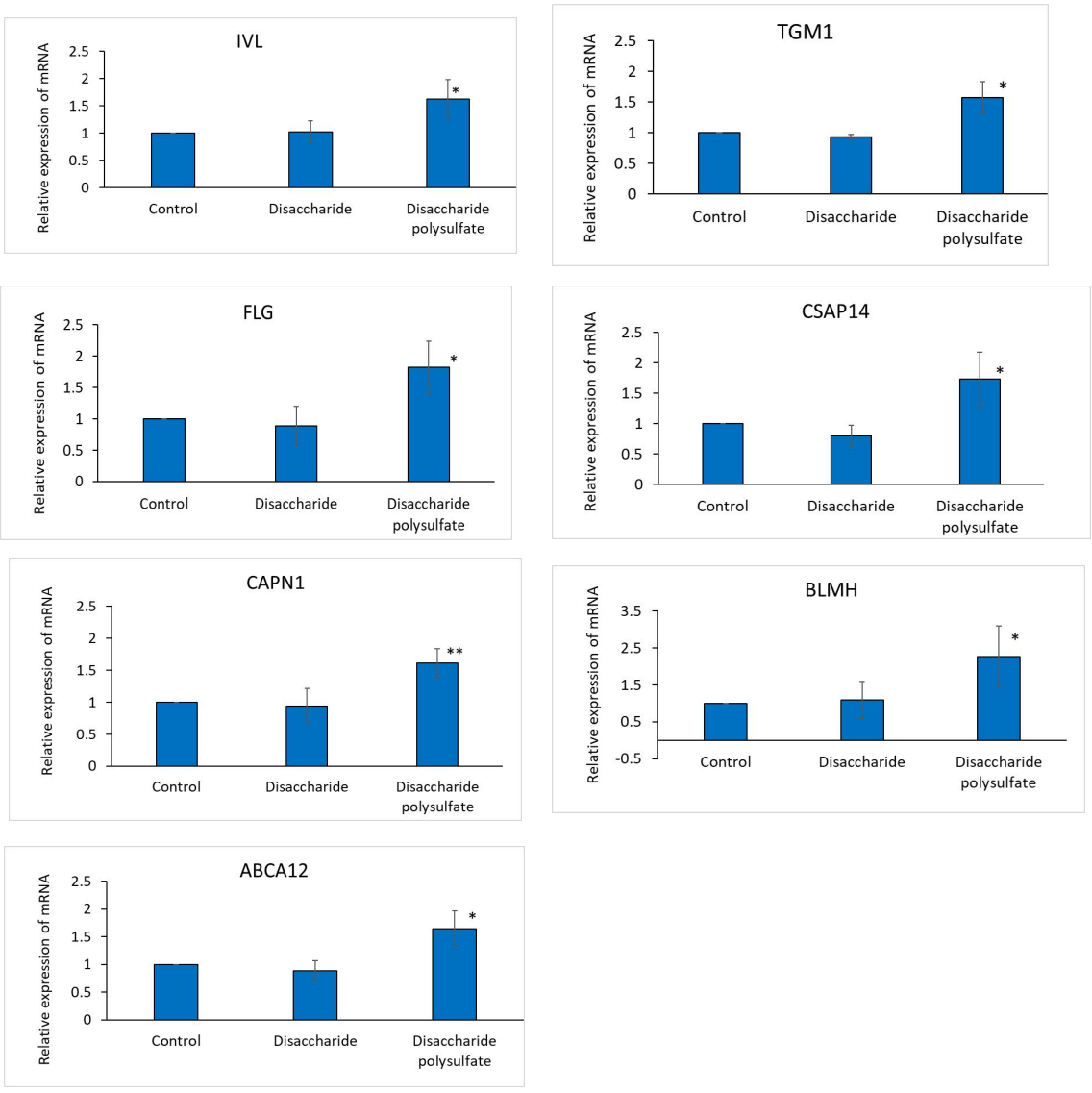


Figure 2. Effects of topical application of disaccharide polysulfate on the mRNA levels of IVL, TGM1, FLG, CASP14, CAPN1, BLMH and ABCA12 in the 3D epidermis models. n = 3, mean ± standard deviation, * $P < 0.05$ vs. control. ** $P < 0.01$ vs. control.

3.1.2. Immunohistochemical staining of FLG in the 3D epidermis models

To examine whether the immunoreactivity of FLG was greater in the presence of disaccharide polysulfate, we used a fluorescent antibody against FLG. The results of nuclear staining and FLG staining are shown below **(Figure 3**). The fluorescence intensity of immunoreactive FLG was greater near the stratum corneum in the 3D epidermis model with topically applied disaccharide polysulfate than the model with topically applied solvent (control).

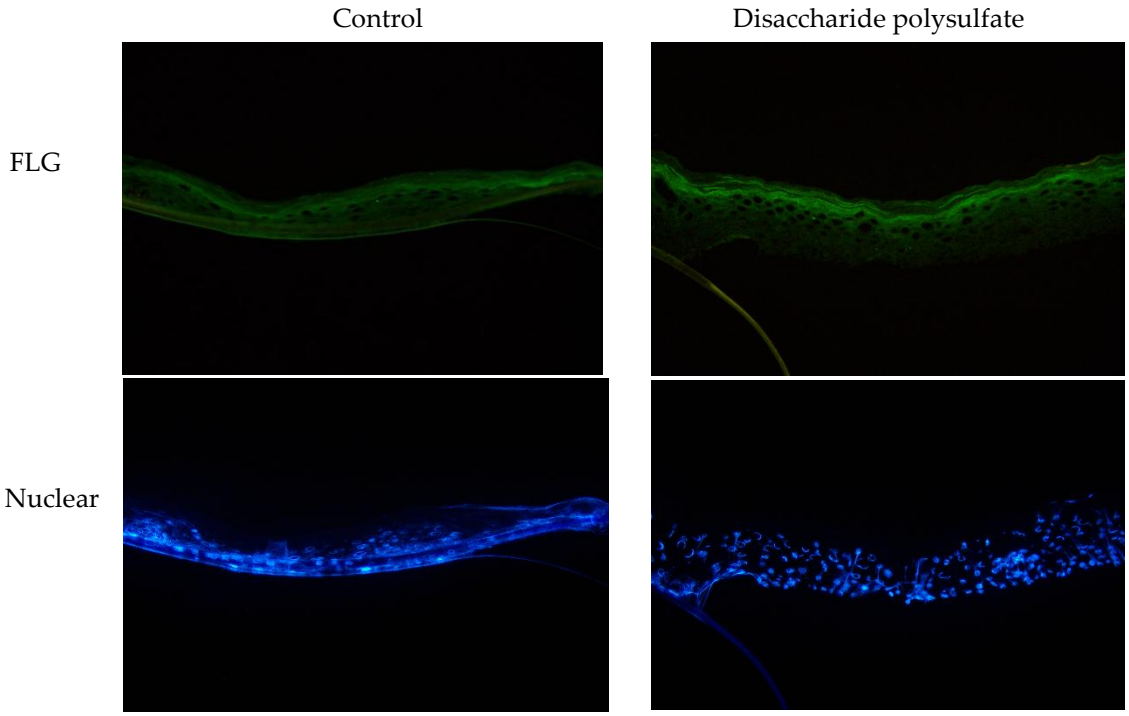


Figure 3. Immunoreactive FLG in 3D epidermis models with topically applied disaccharide polysulfate or solvent (control).

3.1.3. Free amino acids in a 3D epidermal model with topical application of disaccharide polysulfate or heparinoid

After 3 days of incubation of the epidermal model with disaccharide polysulfate solution or heparinoid solution, the amount of free amino acids relative to total amino acids was calculated. The groups treated with disaccharide polysulfate solution at 0.05%, 0.1% or 0.2% showed significantly higher amounts of free amino acids than the control solution

groups (**Figure 4**). Application of 0.3% heparinoid solution, compared with control solution, resulted in significantly higher free amino acid levels, whereas no significant difference was observed at the 0.1% concentration **(Figure 4**).

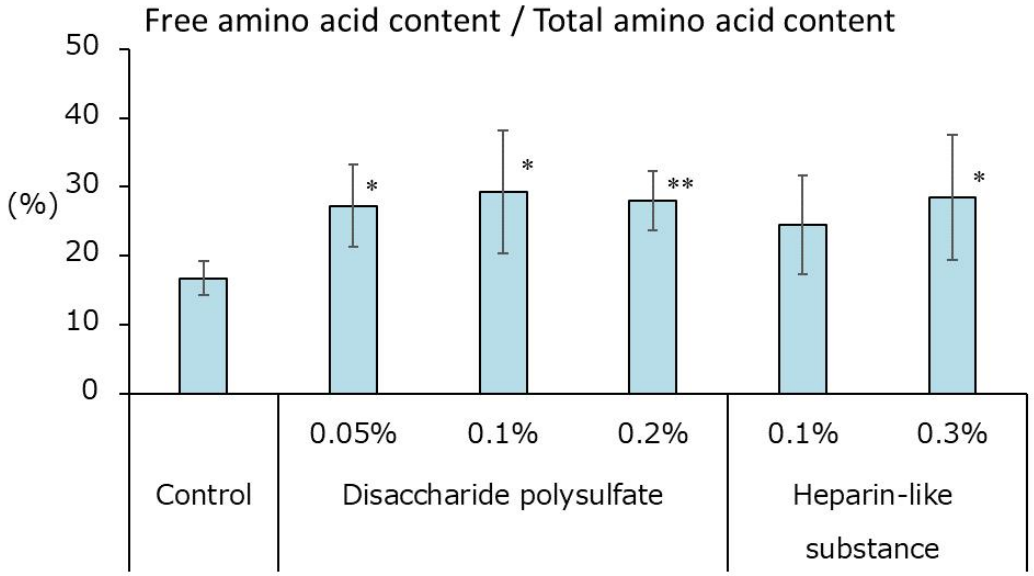


Figure 4. Free amino acids relative to total amino acids in a 3D epidermal model with topical application of disaccharide polysulfate solution, heparinoid solution or solvent solution (control). n = 4, mean ± standard deviation, * $P < 0.05$ vs. control. ** $P < 0.01$ vs. control.

3.1.4. Stratum corneum water content and TEWL after topical application of disaccharide polysulfate solution

At 15 and 30 min after topical application of disaccharide polysulfate solution at 0.05%, 0.1% or 0.2% or solvent solution (control) to human skin, the stratum corneum water content was significantly greater at the disaccharide polysulfate sites than the control sites. In contrast, the stratum corneum water content at 0.1% and 0.3% heparinoid solution-treated sites was significantly higher than that at control-treated sites after 15 min, but no significant difference was observed between the heparinoid solution-treated and control-treated sites after 30 min (**Figure 5**).

TEWL was measured 3 days after topical application of 0.05%, 0.1% or 0.2% disaccharide polysulfate solution, 0.1% or 0.3% heparinoid solution, or solvent solution (control) to a human 3D epidermal model. Lower TEWL was observed in the treatment groups than the control group. In contrast, no significant difference in TEWL was observed between the 0.1% and 0.3% heparinoid solution-treated sites and the control-treated sites

after 3 days (**Figure 5**).

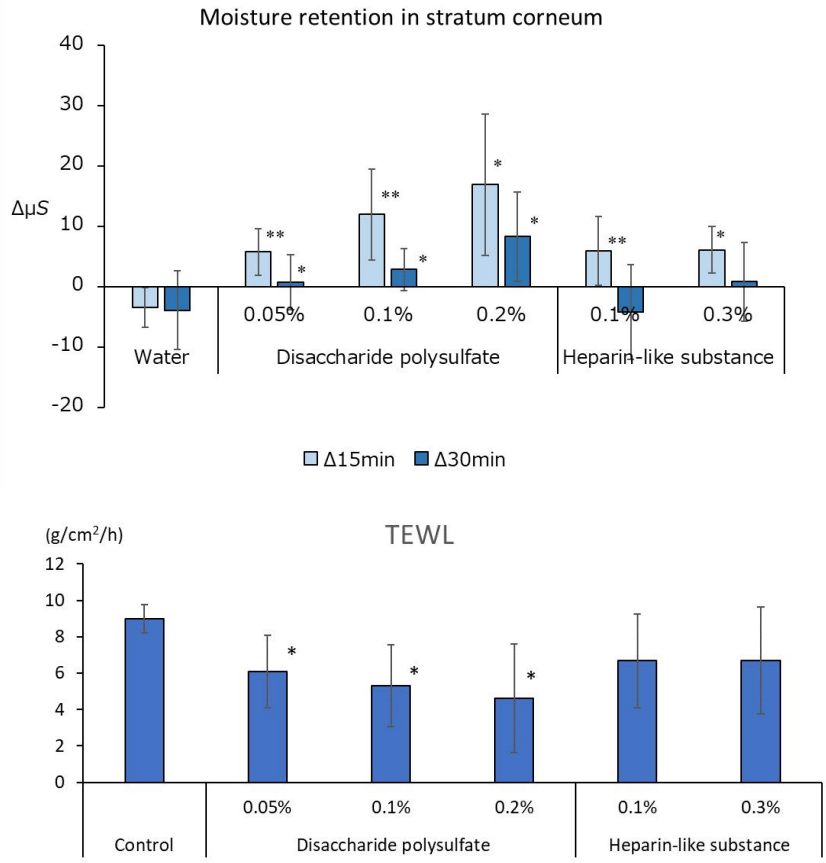


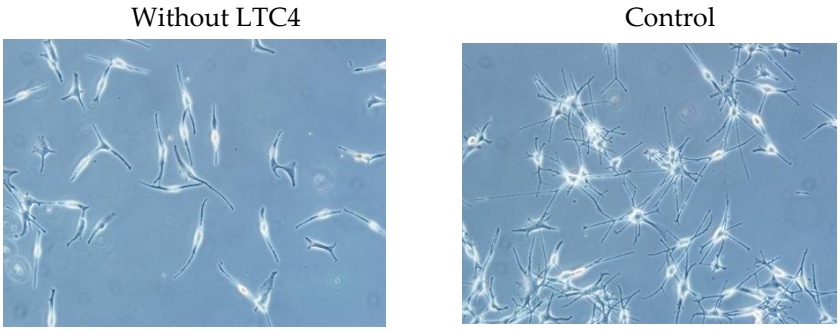
Figure 5. Increase in stratum corneum water content and decrease in TEWL after topical application of disaccharide polysulfate solution. n = 5, mean ± standard deviation, * $P < 0.05$ vs. control. ** $P < 0.01$ vs. control.

3.2. Inhibitory effects of disaccharide polysulfate or disaccharide on dendrite and melanosome formation in melanocytes

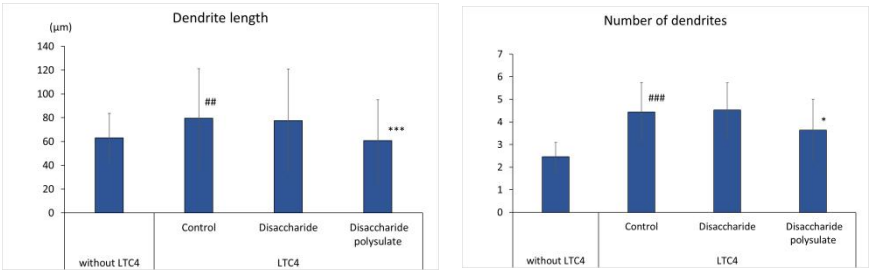
Figure 6a shows the effects of 250 μg/mL disaccharide polysulfate or 250 μg/mL disaccharide on melanocytes activated by LTC4. Disaccharide sulfate inhibited dendrite formation on LTC4-activated melanocytes, but disaccharide did not inhibit dendrite formation on LTC4-activated melanocytes. We measured the effect of 250 μg/mL disaccharide polysulfate on the length and number of dendritic processes of melanocytes, which increased with LTC4, and found that disaccharide polysulfate significantly inhibited the length and number of dendritic processes (**Figure 6b**). In contrast, disaccharide at 250 μg/mL had no inhibitory effect (**Figure 6b**). In addition, disaccharide polysulfate inhibited

tyrosinase-related protein 1, a membrane protein in melanosomes, whereas disaccharide did not (**Figure 6c**).

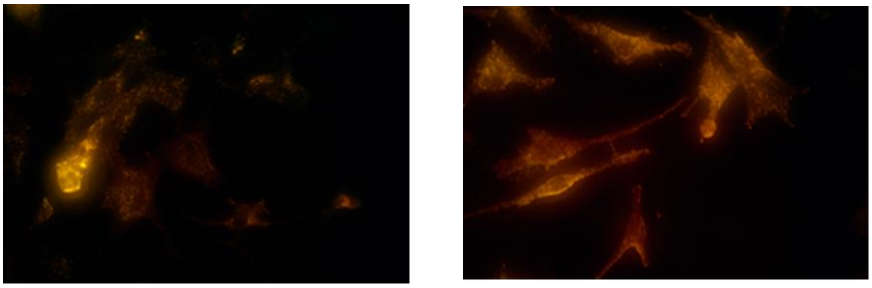
(a) Microscopic image



(b) Dendrite formation (length and number)



(c) Melanosome formation (TRP-1)



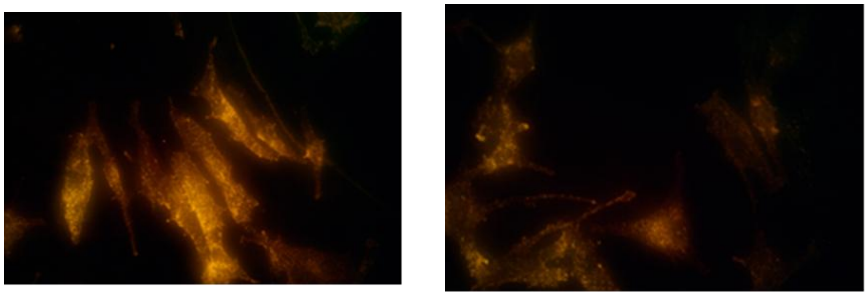
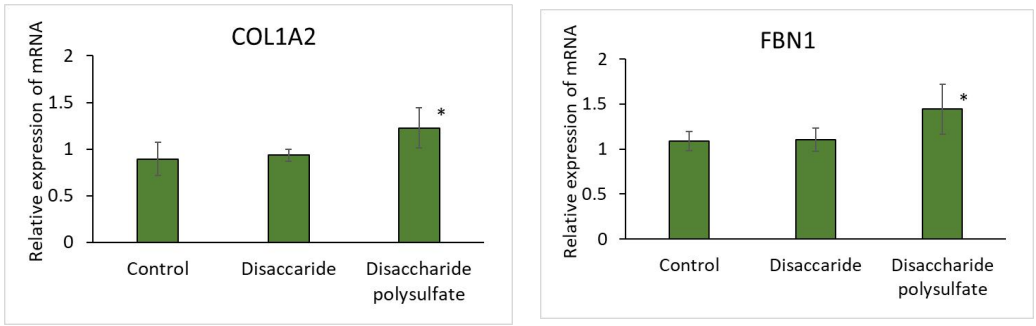


Figure 6. Inhibitory effects of disaccharide polysulfate on dendrite formation (length and number) and melanosome formation (TRP-1) in melanocytes. (a) Microscopic image. (b) Dendrite formation (length and number). (c) Melanosome formation (TRP-1).

3.3. Effects of disaccharide polysulfate on the levels of type 1 collagen and fibrillin 1 in cultured human fibroblasts

Figure 7a shows the effects of 125 μg/mL disaccharide polysulfate or 125 μg/mL disaccharide on the mRNA levels of type 1 collagen and fibrillin 1 in fibroblasts. Type 1 collagen and fibrillin 1 significantly increased with 125 μg/mL disaccharide polysulfate compared with control treatment, and a significant difference was observed for type 1 collagen and fibrillin 1 between disaccharide polysulfate and control treatment. In contrast, disaccharide at 125 μg/mL had no effect. Type I collagen 1n the culture supernatant significantly increased with 25 μg/mL disaccharide polysulfate treatment but not disaccharide treatment (**Figure 7b**).

(a) type 1 collagen fibrillin 1



(b)

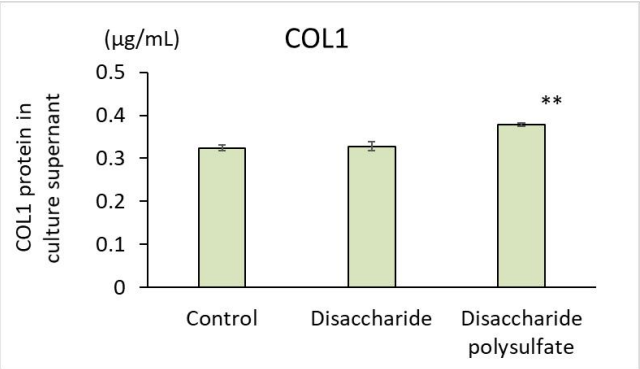


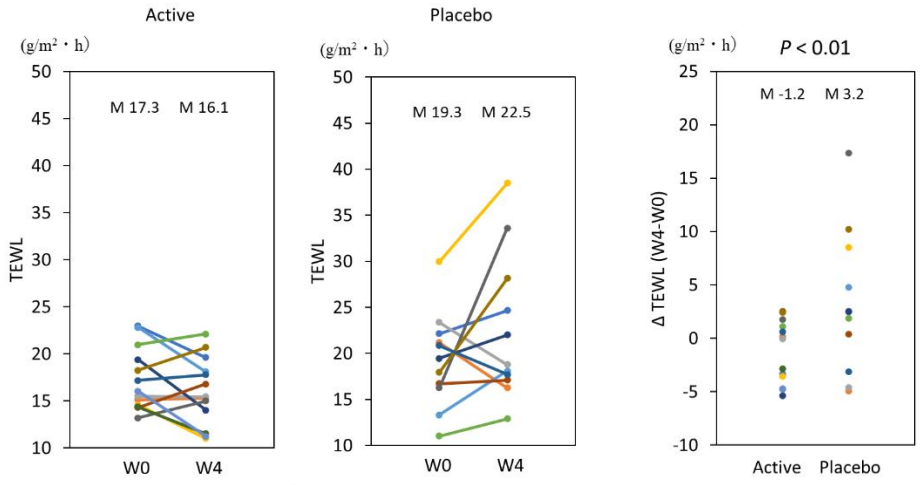
Figure 7. (a) Effects of disaccharide polysulfate on the mRNA levels of type I collagen and fibrillin 1. (b) Effects of disaccharide polysulfate on the levels of type I collagen. n = 3, mean ± standard deviation, * $P < 0.05$ vs. control. ** $P < 0.01$ vs. control.

3.4. Effects of topical application of lotion and emulsion containing disaccharide polysulfate on dry skin in humans

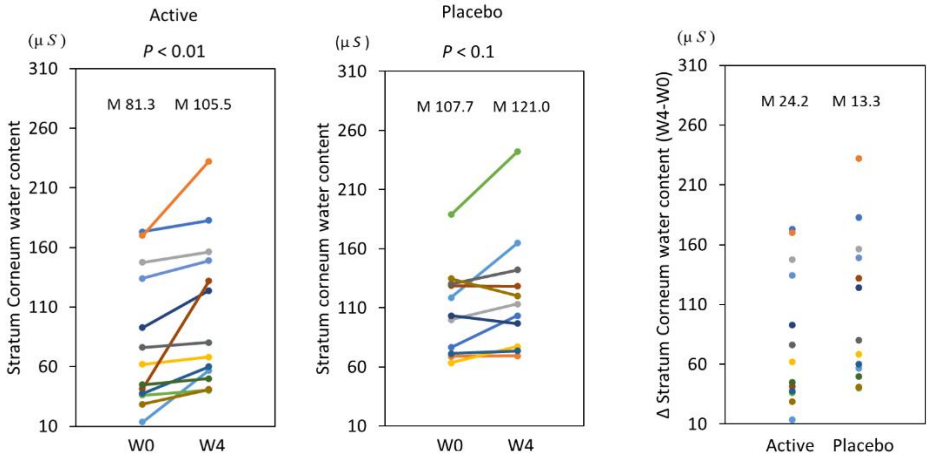
Two placebo group members dropped out during the study period and were excluded from the analysis because of a lack of data from the 4-week measurements. Thus, 13 participants in the active group and 11 participants in the placebo group were included in the statistical analysis. The average age in the active group was 44.7 ± 9.8 years (mean ± S.D.), and that in the placebo group was 45.4 ± 9.2 years (mean ± S.D.). No significant differences were observed between the active and placebo groups in terms of age, TEWL, stratum corneum water content, blood oxygenation index, melanin index and skin elasticity before topical application.

Comparison of the changes in TEWL before and after application indicated a significant improvement in TEWL in the active group compared with the placebo group (**Figure 8a**). The amount of water in the stratum corneum increased significantly in the active and placebo groups after 4 weeks of daily application, but no significant difference was observed between the active and placebo groups (**Figure 8b**). The blood oxygenation index increased significantly in the active group after 4 weeks of daily use, but no significant difference was observed between the active and placebo groups (**Figure 8c**). The melanin index decreased significantly in the active group after 4 weeks of daily use, but no significant difference was observed before and after daily use in the placebo group. No significant difference in the melanin index between the active and placebo groups was observed (**Figure 8d**). Elasticity increased significantly in the active group after 4 weeks of daily use, but no significant difference was observed in the placebo group before and after daily use. A trend toward a difference was observed between the active group and the placebo group (**Figure 8e**).

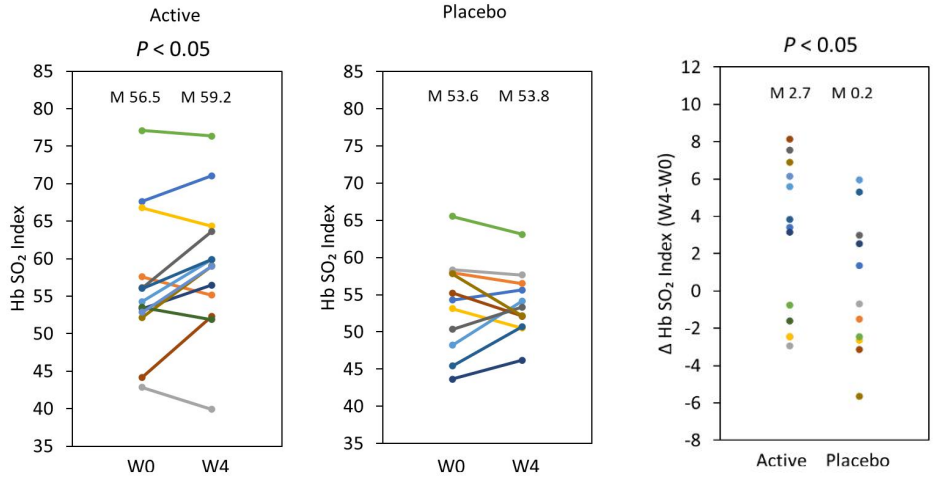
(a)


Active
Placebo
(g/m² · h)
TEWL
M 17.3 M 16.1
M 19.3 M 22.5
W0 W4
Δ TEWL (W4-W0)
P < 0.01
M -1.2 M 3.2
Active Placebo

(b)


Active
Placebo
(μS)
P < 0.01
P < 0.1
Stratum Corneum water content
Stratum corneum water content
M 81.3 M 105.5
M 107.7 M 121.0
W0 W4
Δ Stratum Corneum water content (W4-W0)
M 24.2 M 13.3
Active Placebo

(c)


Active
Placebo
P < 0.05
Hb SO2 Index
M 56.5 M 59.2
M 53.6 M 53.8
W0 W4
Δ Hb SO2 Index (W4-W0)
P < 0.05
M 2.7 M 0.2
Active Placebo

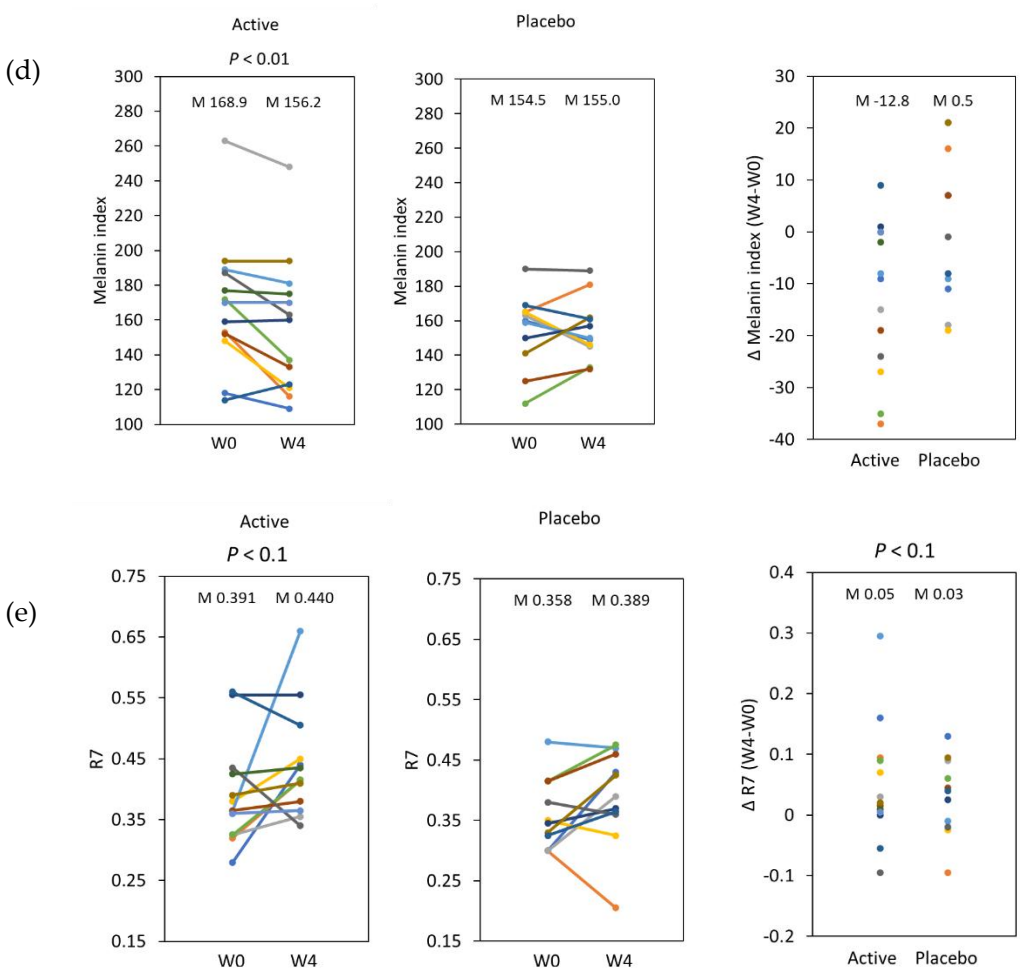


Figure 8. Effects of topical application of lotion and emulsion containing disaccharide polysulfate on dry skin in humans. TEWL (a), water content in the stratum corneum (b), blood oxygenation index (c), melanin index (d) and elasticity of the skin surface (e) were analyzed between the groups treated with lotion and emulsion containing 0.05% disaccharide polysulfate (active group) and placebo lotion and emulsion (placebo group); n=13 (active) and n=11 (placebo). M represents the mean value.

Translucency tended to improve in the active group, but not in the placebo group, after 4 weeks of daily use. Furthermore, no difference was observed between the active and placebo groups after 4 weeks of daily use (**Figure 9a**). Pore size significantly improved in the active group after 4 weeks of daily use, whereas the placebo group showed a trend toward improvement. No significant difference was observed between the active and placebo groups after 4 weeks of daily use (**Figure 9b**). Blackheads in pores were significantly ameliorated in the active group after 4 weeks of daily use, whereas no significant difference was observed in the placebo group. A significant difference in blackheads in pores was observed between the active and placebo group after 4 weeks of

daily use (**Figure 9c**).

(a)

Skin translucence		-2	-1	0	1	2	W0 vs. W4	Active vs. Placebo
Active	W4-W0	0	0	10	3	0	$P < 0.1$	N.S
Placebo	W4-W0	0	0	9	2	0	N.S	

(b)

Pore size		-2	-1	0	1	2	W0 vs. W4	Active vs. Placebo
Active	W4-W0	0	6	7	0	0	$P < 0.05$	N.S
Placebo	W4-W0	0	3	8	0	0	$P < 0.1$	

(c)

Blackheads in pores		-2	-1	0	1	2	W0 vs. W4	Active vs. Placebo
Active	W4-W0	0	8	5	0	0	$P < 0.01$	$P < 0.01$
Placebo	W4-W0	0	1	8	2	0	N.S.	

Figure 9. Effects of topical application of lotion and emulsion containing 0.05% disaccharide polysulfate on dry skin in humans. The changes in skin translucence (a), pore size (b) and blackheads in pores of the skin surface (c) were analyzed between the groups treated with lotion and emulsion containing 0.05% disaccharide polysulfate (active group) and placebo lotion and emulsion (placebo group); n=13 (active) and n=11 (placebo).

4. Discussion

The increase in stratum corneum water content by topical application of disaccharide polysulfate was thought to be due to the promotion of FLG biosynthesis and FLG degradation in the human epidermis, as suggested by increased mRNA levels of FLG and its degrading enzymes in a 3D epidermal model, as well as an increase in FLG protein confirmed by immunofluorescence. The improvement in TEWL after topical application of disaccharide polysulfate might have been due to enhanced lipid transport into the lamellar granules by ABCA12, given the increase in ABCA12 expression. In the experiments in 3D epidermis models, IVL and TGM1 mRNA levels increased after the topical application of disaccharide polysulfate, thereby promoting differentiation.

Furthermore, immunofluorescence staining indicated that topical application of disaccharide polysulfate increased the fluorescence intensity and the area of FLG protein expression in the 3D epidermis model. When disaccharide polysulfate was applied topically in the 3D epidermis model, the mRNA level of CASP14 increased. The results

suggested that disaccharide polysulfate increases CASP14 by promoting final epidermal differentiation. CAPN1 is also involved in the degradation of FLG, because topical administration of disaccharide polysulfate to a 3D epidermal model increased CAPN1 mRNA. In addition, the mRNA level of BLMH [2], which is thought to be involved in the degradation of deiminated FLG to amino acids, was significantly increased by disaccharide polysulfate treatment.

LTC4, an inflammatory and allergenic factor, has been reported to promote dendrite formation and increase TRP-1 in cultured human melanocytes [21]. Disaccharide polysulfate promotes dendrite formation in cultured human melanocytes in the presence of LTC4. The inhibition of the promotion of TRP-1 and the increase in TRP-1, and the absence of these effects after disaccharide treatment, suggest that topical application of disaccharide polysulfate inhibits the activity of human melanocytes. The increase in transparency and the decrease in the melanin index might have been due to the inhibition of melanosome formation by melanocytes and the distribution of melanin to surrounding keratinocytes.

The enhancement of type I collagen production and the increase in fibrillin 1 mRNA in cultured human fibroblasts after disaccharide polysulfate treatment suggested an increase in type I collagen fibers and microfibrils in the dermis. The increase in skin elasticity after topical application of disaccharide polysulfate might have been due to this increase in type I collagen fibers and microfibrils.

5. Conclusions

Beyond their moisturizing effects, cosmetics containing disaccharide polysulfate were found to ameliorate dry, dull and inelastic skin by increasing factors associated with barrier function, decreasing factors associated with pigmentation, increasing collagen fibers and microfibrils.

6. Conflict of Interest Statement.

The authors declare no conflict of interest.

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