Oral Intake of Glucosylceramide Improves Relatively Higher Level of Transepidermal Water Loss in Mice and Healthy Human Subjects

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We examined the effect of oral intake of pure glucosylceramide derived from konjac extract on skin barrier function evaluated by transepidermal water loss (TEWL) in hairless mice with sodium dodecyl sulfate (SDS)-induced skin roughness. The difference of TEWL between SDS-treated site and untreated sites in the pure glucosylceramide-fed group was significantly lower than that in control group on day 14 of ingestion. We investigated interleukin-1α (IL-1α) production in the hairless mouse skin, and it was significantly lower in the glucosylceramide-fed group than that of control animals. This reduced IL-1α production should contribute to improvement of skin barrier function. To investigate the effect of oral intake of glucosylceramide in human, we conducted a randomized double-blind placebo-controlled study including 100 healthy subjects whose TEWL in cheek was relatively high. As a result, cheek TEWL was significantly lower in the test product group as compared with the control group in weeks 8 and 12 of ingestion ($p = 0.023$ and $p = 0.002$ respectively).

Key words —— konjac extract, glucosylceramide, transepidermal water loss (TEWL), itch, interleukin-1α (IL-1α), randomized double-blind placebo-controlled study

INTRODUCTION

The stratum corneum of skin has a barrier function to protect the organism from exogenous factors and to prevent water loss. Decrease of barrier function is associated with diseases such as atopic dermatitis (AD),[1–4] which is characterized by skin dryness and intense itching. Impairment of skin barrier function may also be caused by sudden change of the weather[5,6] or contact with chemical agents such as cleansers.[7,8] Severe itching may reduce the quality of life (QOL) through induction of sleep disorders.

Stratum corneum lipids are known to influence skin barrier function and mainly composed of ceramide (40–60%). The other components of stratum corneum lipids include cholesterol, cholesterol ester, free fatty acids, etc. The ceramide content of stratum corneum lipids decreases with aging and is also decreased in patients with atopic dermatitis,[9] and it is known that a decrease of ceramide causes impairment of the barrier function.[10]

Glucosylceramide, which is a glycoside of ceramide, is a major sphingo (glycol) lipid in plants such as soybean, corn, wheat, rice,[11] and *Amorphophallus konjac* (*A. konjac*).[12] Recently, it has been reported that skin barrier function in hairless mice is improved by intake of extracts of food materials containing glucosylceramide; the purity was > 6%.[13] It has also been reported that oral intake of glucosylceramide reduced transepidermal water loss (TEWL)[14] in normal adult[12] or in AD patients.[15,16]

A. konjac, a rich source of glucocylceramide, has been eaten as food for a long time in Japan. Additionally, *A. konjac* is extremely safe with no allergenic effects. Then, we focused our attention

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on konjac extract, which was fractionated from *A. konjac*, and studied its efficacy.

In this study, we investigated the efficacy of glucosylceramide on improving skin barrier function using pure glucosylceramide derived from konjac extract and examined the action mechanism in animal models. Moreover, we confirmed the efficacy of konjac extract including glucosylceramide in human healthy volunteers whose TEWL was relatively high, in a randomised, double-blind placebo-controlled study.

MATERIALS AND METHODS

Animals —— Five-week-old male HR-1 mice were purchased from Hoshino Laboratory Animals. They were used for the experiment at the age of 6–7 weeks. The animals were individually accommodated in plastic cages placed within a barrier system where the temperature was kept at 21–25°C, and lighting was on for 12 hr/day (7:00–19:00). The relative humidity was < 10% in the dry environment study to investigate interleukin-1α (IL-1α) release from skin and was 40–70% in the other studies. The animals were allowed free access to a solid diet (AIN-93G, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water (after ultraviolet and microfilter treatment). During the acclimation period, the relative humidity was kept at 40–70%. The present study was approved by the ethics committee of Shiseido Research Center (Yokohama, Japan) in accordance with the guideline of the National Institute of Health (Bethesda, MD, U.S.A.).

Test Substance —— Konjac extracts (containing 12% glucosylceramide) and konjac-derived glucosylceramide (100% pure product) were supplied from Unitika Ltd. (Kyoto, Japan). Each test substance was dispersed in 1% suspension of tragacanth gum (Wako Pure Chemical Industries, Ltd., Osaka, Japan) to make 30 µg glucosylcelamide/0.5 ml suspension for the mice *in vivo* study determining changes of TEWL. In the studies of IL-1 level, 250 µg glucosylceramide/0.5 ml tragacanth gum was used. One percent of tragacanth gum suspension served as control substance.

Determination of Effect of Test Substance on Sodium Dodecyl Sulfate (SDS)-induced Rough Skin of Mice

Administration of Sample —— After 1 week of acclimation, the test substance or the control substance (0.5 ml) was orally administered via a stomach tube to each animal once daily (9:00–10:00) for 14 consecutive days.

SDS Treatment —— Under inhalational anesthesia with sevoflurane (Ishimaru Pharmaceutical Co., Ltd., Osaka, Japan), an unwoven cloth (*ca.* 1.5 × 5 cm) containing 300 µl of 10% aqueous solution of SDS (Wako Pure Chemical Industries, Ltd.) was placed in contact with the left lateral area of the mouse back for 5 min. After the cloth was removed, the left lateral area of the back was gently wiped with absorbent cotton pre-immersed in warm water.

Measurement of Mice TEWL —— Mice (test product group and control group) were orally treated with the test substance or placebo for 14 days. From the 4th day of treatment, SDS was applied daily about 3 hr after the dose (13:00) as described above. One hour after the SDS treatment on the 4th day of SDS treatment, TEWL of each mouse was measured at the right and left lateral areas of the back under identical conditions for all animals, using a Tewameter® TM-300 (Courage and Khazaka Electronics, Cologe, Germany). ΔTEWL was calculated using the equation shown below. In this equation, SDS-treated site is the left lateral area of the back, and SDS-untreated site is the right lateral area of the back.

ΔTEWL (g/m^2·hr) = TEWL of SDS-treated site (left) (g/m^2·hr) − TEWL of SDS-untreated site (right) (g/m^2·hr)

All procedures for measuring skin barrier function and disrupting the barrier were carried out under anesthesia.

Measurement of Skin Cytokine Release —— After measurement of TEWL, each mouse was euthanized without pain. The lateral back skin was collected, and subcutaneous fat removed with a knife. Test samples were prepared according to the method of Wood *et al.*[17] Specifically, skin pieces were placed in wells of a flat-bottomed multi-well (24-well) plate for cell culture (Becton Dickinson Labware, Franklin Lakes, NJ, U.S.A.), cooled in an ice bath. Ice-cooled Dulbecco's modified Eagle's medium (DMEM), 2 ml, was added to each well, and the plate was incubated on ice for 15 min. The culture was harvested, and stored at −80°C until measurement. IL-1α as measured by Quantikine® mouse IL-1α/IL-1F1 enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, U.S.A.). Tumor necrosis factor-α (TNF-α) was measured by mouse TNF-α instant ELISA (Bender MedSystems, Burlingame, CA, U.S.A.). Interferon-γ (IFN-γ) was measured by

mouse IFN-γ instant ELISA (Bender MedSystems).

In addition, the intake period of glucosylceramide was investigated over 29 days.

Determination of Effect of Test Substance on Cytokine Release by Skin from Mice Kept in a Dry Environment —— After 1 week of acclimation, the test substance or the control substance (0.5 ml) was orally administered via a stomach tube to each animal in the glucosylceramide group and the control group ($n = 7$ or 8 in each group) once daily (9:00–10:00) for 29 consecutive days. The relative humidity was kept < 10% in this study to make dry environment.

After the 29-day treatment, each mouse was euthanized without pain. Cytokines were measured as described in "Measurement of skin cytokine release."

Human Study Design —— This study was carried out by KSO Corporation Co., Ltd. (Tokyo, Japan). The study began in November 2006 and ended in March 2007. To minimize the influence of changes in air temperature, weather, etc. on assessment of skin condition, each subject was acclimated to the test laboratory for 40 min, where the temperature and relative humidity were controlled at $21 \pm 2°C$ and 45%, respectively. The study was designed as a placebo-controlled randomized double-blind parallel group comparison.

Candidates were healthy individuals who self-assessed their skin roughness and itching in skin. From 256 candidates, individuals having skin abnormalities were excluded; thus, 120 individuals having high TEWL in cheek were selected. These 120 individuals were randomly divided into two groups (60 individuals/group) in such a manner as to minimize the inter-group differences in TEWL, male-to-female ratio, and age. After this grouping, they were treated in double blind manner. At the start of the study (week 0), individuals found to have skin abnormalities and those who refused to participate in the study or had low TEWL in cheek were excluded; thus the remaining 100 individuals (49 men and 51 women, age 21–59 years) were finally included in the study.

Test beverage (containing konjac extract) or placebo beverage was ingested for a period of 12 weeks. The 12-week ingestion period was followed by a 4-week non-ingestion period. Each subject was instructed to visit the hospital immediately before the start of ingestion (week 0), and at 4, 8, and 12 weeks after the start of ingestion and at 4 weeks after the end of ingestion (completion of the study) for evaluation of the skin condition. This study was carried out in accordance with the Declaration of Helsinki and the Ethical Guidelines on Epidemiological Studies.

Test Beverage —— Emulsified konjac extract (containing 0.3% glucosylceramide) was purchased from Unitika Ltd. The test beverage was prepared with emulsified konjac extract to contained 1.8 mg glucosylceramide, sweetener, sour seasoning, and flavor in 340 ml water. The placebo beverage was prepared with the same amounts of sweetener, sour seasoning, and flavor as the test beverage, so that they would not differ from each other in taste.

Evaluation of Human TEWL —— TEWL was evaluated at the cheek, forearm and back using an evaporimeter (Model EP-2, Servomed, Stockholm, Sweden). Measurement was continued for 120 seconds, and the most stable value was adopted as TEWL at a given occasion of measurement for a given subject. The variation in this parameter from baseline (Δ) was compared between the two groups.

Evaluation of Itching Sensation —— Evaluation was made over the entire face, entire arm, and entire leg. The itching scale used in this study measures the severity of itching at a specific point in time, using a horizontal visual analogue scale (VAS). Possible scores range from 0 (no itching) to 10 (severe itching). The variation in the rating from the baseline (Δ) was compared between the two groups.

Statistics —— Data were expressed as mean ± S.D. Student's t-test was used for inter-group comparison of TEWL, itch data, and IL-1α level. Dunnett's test was used for inter-group comparison of mouse TEWL. Analysis of covariance (ANCOVA) was performed on absolute TEWL. $p < 0.05$ was regarded as statistically significant.

RESULTS

Identification of Active Component of Konjac Extract

To identify the active component of konjac extract, we determined the effect on TEWL of pure glucosylceramide, which is the candidate active component of konjac extract in mice.

As shown in Fig. 1, ΔTEWL in the 100% pure product (100% glucosylceramide) ingestion group was significantly ($p = 0.002$) lower than the control group whereas that, in the konjac extract ingestion group was lower than control group ($p = 0.084$). Each ΔTEWL was 15.9 ± 4.5 g/m^2·hr in the con-
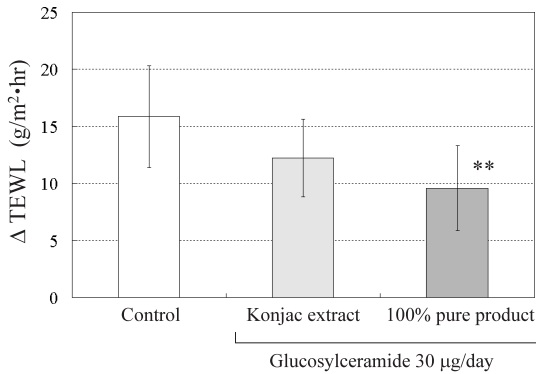
Fig. 1. Effect of Administration of Glvcosylceramide Solution to Mice on TEWL of SDS-treated Skin

Mice (test product group and control group: $n = 10$) were orally treated with test substance or placebo for 14 days. From day 4 of treatment, SDS was applied using the method described in Materials and Methods. One hour after SDS treatment on day 4 of SDS treatment, TEWL was measured under identical conditions, using a Tewameter. Columns indicate values of ΔTEWL in the control group, the groups given konjac extract containing 12% glucosylceramide (glucosylceramide: 30 μg/day), and the groups given 100% pure product (=glucosylceramide) (glucosylceramide: 30 μg/day), as indicated. Dunnett's test was used for comparison between three groups. Data are presented as mean ± S.D. Asterisks indicate a statistically significant difference, $^{**}p < 0.01$.

trol group, 12.2 ± 3.4 g/m^2·hr in the konjac extract ingestion group, and 9.6 ± 3.7 g/m^2·hr in the pure glucosylceramide ingestion group. Hence konjac-derived pure glucosylceramide was stronger than konjac extract in inhibitory effect of TEWL, suggesting that the elevation of TEWL associated with SDS-induced skin roughness was not suppressed by any other component except glucosylceramide contained in the ingested konjac extract.

Effect of Konjac Extract on Cytokine Production in Skin Roughness Mice

It has been reported that epidermal IL-1α in mice bred under dry conditions [low relative humidity ($< 10\%$) environment] is higher than in those kept under normal conditions of humidity.[18] Therefore the hypothesis that improvement of skin barrier function by konjac extract involves modulation of the IL-1α level in skin was tested by examining the effect of glucosylceramide on release of IL-1α in the skin of mice bred under dry conditions. Furthermore, effects of glucosylceramide on IL-1α production in SDS skin roughness mouse were investigated. As shown in Fig. 2(A), the amount of IL-1α released in the skin was 148 ± 27 pg/ml·cm^2 in the control group and 98 ± 25 pg/ml·cm^2 in the glucosylceramide treatment group ($p = 0.003$), in-
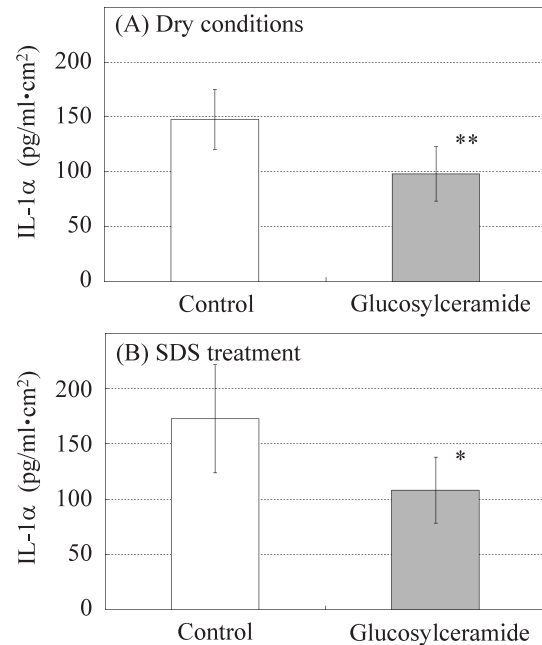

Fig. 2. Effects of Glucosylceramide on IL-1α Production in Mice Skin Roughness

(A) Mice (test product group and control group: $n = 7$–8) were fed with glucosylceramide: 250 μg/day (konjac-derived glucosylceramide) under dry conditions (relative humidity $< 10\%$) for 29 days. The skin was then collected from each mouse and the level of IL-1α measured. (B) Mice (test product group and control group: $n = 4$–5) were orally treated with glucosylceramide: 250 μg/day (konjac extracts) or the placebo for 14 days. Mice were treated with SDS for 4 days as described in Materials and Methods. One hour after SDS treatment on day 4 of SDS treatment, the skin was collected from each mouse and the level of IL-1α measured. □ indicates the control group and ▓ indicates the glucosylceramide ingestion group. Student's t-test was used for comparison between two groups. Data are presented as mean ± S.D. Asterisks indicate a statistically significant difference, $^{*}p < 0.05$, $^{**}p < 0.01$.

dicating that release of IL-1α in skin under dry conditions was significantly suppressed by ingestion of konjac-derived glucosylceramide. As shown in Fig. 2(B), the amount of IL-1α released in the skin was 173 ± 49 pg/ml·cm^2 in the control group and 108 ± 30 pg/ml·cm^2 in the glucosylceramide treatment group ($p = 0.043$), indicating that release of IL-1α in skin treated with SDS was significantly suppressed by ingestion of konjac-derived glucosylceramide. These results suggest that glucosylceramide improves the skin barrier function through suppressing the formation of IL-1α.

In addition, TNF-α and IFN-γ levels of both skin roughness conditions were below the determination limit.
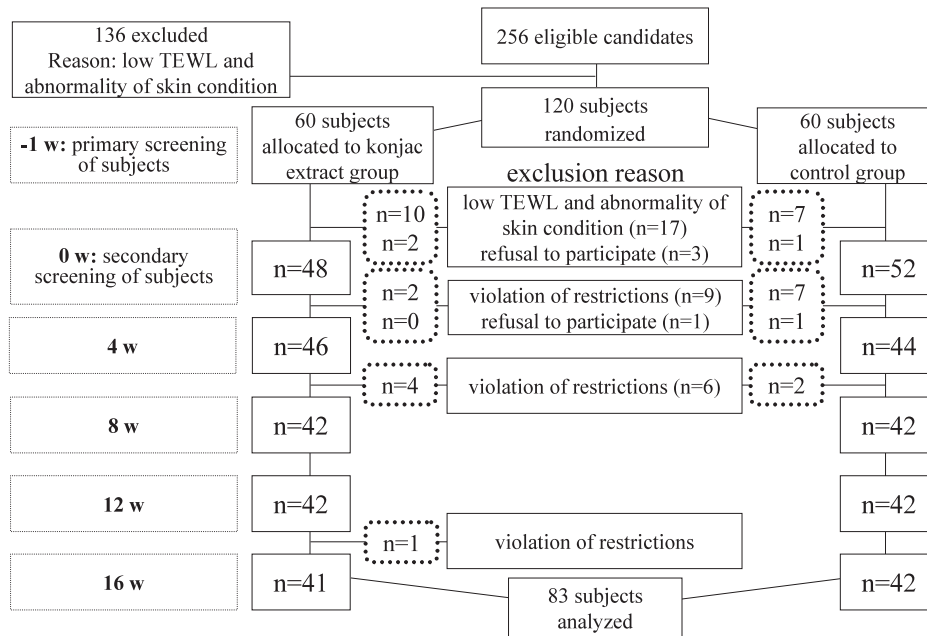
Fig. 3. Flow Chart for Selection of Subjects

Table 1. Inclusion Criteria

1. Men and women aged ≧ 20 years
2. Individuals conscious of skin roughness by drying, and whose symptoms of itching are found in winter
3. Individuals who experience rough symptoms on the skin after bathing and washing face and arms
4. Individuals whose cheek TEWL at the time of measurement was > 27 (the targeted value) at 0 weeks
5. Individuals whose skin conductance (40–55 as the targeted value) was low at 0 weeks
6. Individuals who abstain from drinking alcohol from the day before measurement to the measurement end
7. Individuals who agree to participate in the stydy
8. Individuals judged appropriate for the study by attendant physician

Table 2. Exclusion Criteria

1. Individuals with abnormalities of skin areas planned to be tested
2. Individuals considered likely to develop allergy to the test substance
3. Individuals with chronic cutis symptoms such as atopic dermatitis
4. Patients receiving drug therapy as outpatients
5. Individuals participating in other studies within the past 3 months
6. Habitual smokers
7. Patients with asthma
8. Pregnant women, women desiring to become pregnant, or lactating women
9. Individuals having used cosmetics or the like possessing moisture-retaining effects on the skin areas planned to be tested in this study within 1 month before the sart of the study
10. Individuals taking or intending to take drugs against pollinosis
12. Individuals who used a ceramide-containing supplement within the past 1 month
13. Individuals who used ceramide- or retinol-containing cosmetics within the past 1 month
14. Individuals judged inappropriate for the study by attending physician

Subject Selection and Baseline Data in Human Study

The effect of glucosylceramide in improving skin barrier function was evaluated in healthy volunteers, using konjac extract. The subjects were 100 healthy individuals who were recruited under the inclusion criteria listed in Table 1, and who were not excluded under the exclusion criteria listed in Table 2. Upon completion of the study, subjects found to fall under any of the exclusion criteria, those who had violated the instructions (Table 3), and these whose compliance with the ingestion protocol was poor were excluded from evaluation if deemed necessary at a case evaluation meeting held before the

key was opened. As shown in Fig. 3, excluding these subjects who dropped out of the study or violated the protocol, 83 subjects were included in the final analysis.

Among these 83 subjects, there was no significant difference of mean age between the test product group (40.6 ± 9.1 years) and the control group (39.5 ± 8.9 years). Cheek TEWL at week 0 was 26.3 ± 6.8 g/m^2·hr in the test product group and 24.9 ± 8.6 g/m^2·hr in the control group ($p = 0.408$), and forearm TEWL at week 0 was 7.6 ± 2.0 and

Table 3. Instructions to Subjects during the Study Period

1. Only cosmetics which were being used before the study began may be used during the study.
2. Cutting of hair or shaving of the test areas is prohibited for 2 weeks before each occasion of measurement.
3. The use of bathing agent or the like is prohibited.
4. The use of medicines, quasi-medicines of new category, and herbal mixtures is prohibited as a rule.
5. Ingestion of food supplements or foods with health-promoting function (specific health-promoting foods), othe than those that were being used routinely before the study, is prohibited.
6. Excessive alcohol consumption (consumption beyond the customary level) is prohibited.
7. No alcohol may be consumed on the day before each occasion of measurement.
8. Outdoor sports, exposure to artificial ultraviolet ray lamps, and other acts that might cause sunburn are prohibited. Care needs to be taken during daily living to avoid direct exposure of the test skin areas to ultraviolet rays indoors and outdoors. Concretely, it si advisable to protect the test skin areas with a cap or with clothes, or by routinely used sun-screening agents or the like.
9. Hard exercise is prohibited on the day of measurement until measurement for that day is completed.
10. Stimulating foods (hot foods, etc.) such as curry, red pepper, Tabasco, etc. may not be ingested on the day of measurement.
11. Use of thermal undershirts or the like, which exert special heat insulating effects, is prohibited.
12. A bath should be taken before going to bed on the day before measurement and bathing on the day of measurement (prior to measurement) is prohibited.

8.0 ± 2.0 g/m^2·hr, respectively ($p = 0.477$). Therefore there was no significant difference of baseline data between the test and control groups. Sex and age composition of the analysis subjects is shown in Table 4.

Effect of Konjac Extract on TEWL and Itching Sensation

As shown in Fig. 4(A), cheek ΔTEWL at week 8 was −1.2 ± 5.2 g/m^2·hr in the test product group and 1.5 ± 5.5 g/m^2·hr in the control group ($p = 0.023$), whereas that at week 12 was −2.9 ± 5.5 and 0.9 ± 5.6 g/m^2·hr, respectively ($p = 0.002$). In ANCOVA, involving correction for baseline data, ΔTEWL differed significantly between the two groups at weeks 8 and 12 of ingestion, irrespective of the difference in baseline data ($p = 0.035$ and $p = 0.004$, respectively). TEWL in the forearm was also measured, but no significant difference was noted between the two groups. In addition, the back ΔTEWL was significantly lower in the test product group (0.3 ± 2.2 g/m^2·hr) as compared with the control group (1.4 ± 2.4 g/m^2·hr) at weeks 12 of ingestion ($p = 0.032$).

The degree of improvement of itching sensation following ingestion of konjac extract was evaluated in the face and arm of subjects by horizontal VAS. As shown in Fig. 4(B), Δitchiness of the arm was ameliorated in the konjac extract group after 4, 8, and 12 weeks of ingestion and at 4 weeks after the end of ingestion (week 16) ($p = 0.003, 0.059, 0.041$, and 0.058). No marked effect on itching sensation of the face was found. Whereas, Δitching sensation of the leg was lower in the test product group as compared with the control group at weeks 4 and 8 of ingestion ($p = 0.075$ and 0.084).

Table 4. Sex and Average Age of Analysis Subjects

Sex	Control group		Konjac extract group		Total	
	n	Age years	n	Age years	n	Age years
Male	21	38.2 ± 9.7 (23 − 59)	19	41.5 ± 10.6 (23 − 59)	40	39.8 ± 10.1 (23 − 59)
Female	21	40.8 ± 8.0 (30 − 57)	22	39.9 ± 7.7 (28 − 56)	43	40.3 ± 7.8 (28 − 57)
All subjects	42	39.5 ± 8.9 (23 − 59)	41	40.6 ± 9.1 (23 − 59)	83	40.1 ± 8.9 (23 − 59)

n = No. of subjects. Upper section shows mean ± S.D.; lower section shows age range.
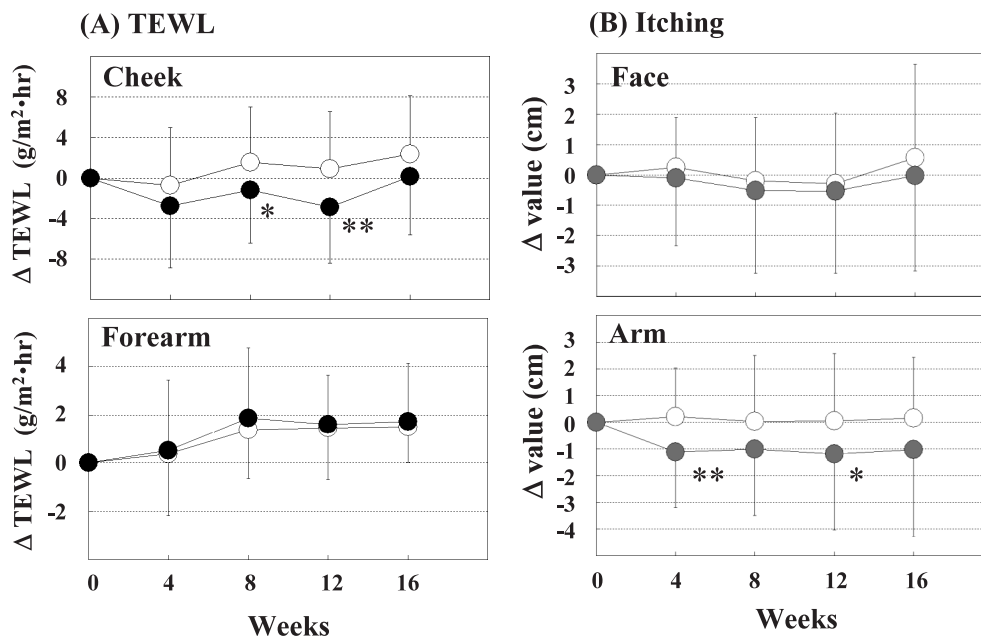
(A) TEWL

(B) Itching

Fig. 4. Effect of Konjac Extract-containing Beverage

(A) TEWL at cheeks and forearms was measured by evaporimeter at weeks 0, 4, 8, and 12 during ingestion period and week 16 in follow-up period. Variation of TEWL relative to baseline level (ΔTEWL) is shown. (B) Severity of itching sensation over faces and arms was evaluated by VAS at weeks 0, 4, 8, and 12 during ingestion period and week 16 in follow-up period. Variation of severity relative to baseline level (Δvalue) is shown. ○ indicates placebo; ● indicates test product. Student's *t*-test was used for comparison between two groups. Data are presented as mean ± S.D. Asterisks indicate a statistically significant difference, $^{*}p < 0.05$, $^{**}p < 0.01$.

DISCUSSION

Oral intake of pure glucosylceramide improved skin roughness caused by SDS treatment in mice. This result is consistent with previous studies showing that corn and rice extracts containing glucosylceramide improve the skin condition in mouse models of chronic skin roughness induced by HR-AD and acute skin roughness induced by tape stripping.[13] Thus although there is considerable indirect evidence that glucosylceramide is effective in ameliorating skin roughness caused by environmental factors, the present study demonstrates clearly for the first time that the effect of konjac extract in decreasing TEWL is attributable to glucosylceramide contained in the extract. We additionally demonstrated that ingestion of glucosylceramide reduces the skin tissue level of IL-1α (a factor that exacerbates skin roughness) in a mouse model of skin roughness induced by a low relative humidity (< 10%) environment. In addition, we have previously observed that ingested glucosylceramide or its metabolite is distributed to the skin (data not shown). These findings suggest that glucosylceramide may reduce TEWL by suppressing induc-tion of IL-1α in response to changes in the external environment. However, the possibility remains that glucosylceramide may play a more direct role in improving barrier function, because an increase of stratum corneum ceramide was observed following ingestion of glucosylceramide for 4 weeks.[19]

Although it has been reported that barrier function is improved by uptake of konjac extract in patients with atopic dermatitis,[15, 16] we found that these effects are also observed in healthy volunteers whose daily life and activities during the study were restricted to only a small degree. Thus konjac extract also appears effective for improving skin condition in healthy subjects. However, the pattern of improvement was not similar between face and arm. Although there is not clear explanation for this effect, the correlation curve of TEWL and itching may be non-linear and the curve may differ from site to site. Further studies on IL-1α levels in skin of subjects who have taken konjac extract, and on the regulatory mechanism of IL-1α roductions are planned.

In conclusion, test beverage containing konjac extract improved skin condition including barrier function, alleviated itching sensation in volunteers who took it for 12 weeks, and inhibited IL-1α pro-

duction in skin. Therefore konjac extract may alleviate sleeplessness caused by itching, which should help people to lead more active daily lives and improve their QOL.

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