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Oral Intake of Beet Extract Provides Protection Against Skin Barrier Impairment in Hairless Mice

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The epidermis acts as a functional barrier against the external environment. Disturbances in the function of this barrier cause water loss and increase the chances of penetration by various irritable stimuli, leading to skin diseases such as dry skin, atopic dermatitis, and psoriasis. Ceramides are a critical natural element of the protective epidermal barrier. The aim of this study was to evaluate whether the oral intake of beet (*Beta vulgaris*) extract, a natural product rich in glucosylceramide (GlcCer), may prevent disturbance in skin barrier function. When HR-1 hairless mice were fed a special diet (HR-AD), transepidermal water loss (TEWL) from the dorsal skin increased, with a compensatory increase in water intake after 5 weeks. Mice fed with HR-AD had dry skin with erythema and showed increased scratching behaviour. Histological examinations revealed a remarkable increase in the thickness of the skin at 8 weeks. Supplemental addition of beet extract, which contained GlcCer at a final concentration of 0.1%, significantly prevented an increase TEWL, water intake, cumulative scratching time, and epidermal thickness at 8 weeks. These results indicate that oral intake of beet extract shows potential for preventing skin diseases associated with impaired skin barrier function.

Keywords: glycosylceramide; transepidermal water loss; Beta vulgaris; hairless mice; skin barrier impairment; scratching.

INTRODUCTION

A substantial body of data demonstrates that disturbances in skin barrier function cause various skin diseases, as reflected by increases in transepidermal water loss (TEWL). The alteration of genetic and/or environmental factors within the skin barrier, such as lipid lamellae, natural moisturizing factors, and corneodesmosomes, can cause skin barrier impairment (Cork *et al*., 2009). Impaired skin barrier function allows the entry of various environmental allergens and microorganisms, thereby provoking inflammatory reactions and causing pruritus (Baker, 2006). Reactive scratching deteriorates skin barrier function, eventually leading to inflammatory skin diseases (Thaipisuttikul, 1998).

Sphingolipids are essential components of the mammalian permeability barrier (Wertz and Downing, 1982), and their presence in the epidermis is critical for barrier maintenance (Holleran *et al*., 1991). Ceramides, one of the most common sphingolipids, are localized in the stratum corneum (SC) where they make up 50% of the total lipid volume constituting the lipid lamellae of keratinocytes (Elias and Menon, 1991; Feingold, 2007). In epidermal keratinocytes, ceramides and/or their precursors are either delivered by the circulation or they are synthesized, and then incorporated into the lamellar bodies and stored as glucosylceramide (GlcCer) (Vielhaber *et al*., 2001). Once keratinocytes arrive at the outermost layer of the epidermis, the lamellar bodies fuse with the apical membrane of the keratinocytes and the stored GlcCers are secreted into the extracellular space. GlcCers are then enzymatically hydrolysed into ceramides and transported to the lamellar membrane of the SC (Holleran *et al*., 2006). Amounts of epidermal ceramides are regulated by the balance between ceramide-generating enzymes (e.g. serine-palmitoyl transferase, sphingomyelinases, and β-glucocerebrosidase) and ceramide-degradative enzymes (e.g. ceramidases and sphingomyelin acylase) (Vielhaber *et al*., 2001; Hong *et al*., 2007). Reductions in the epidermal ceramides amount due to changes in epidermal enzyme activities can cause various skin diseases, including dry skin, atopic dermatitis (AD), and psoriasis (Imokawa *et al*., 1991; Motta *et al*., 1993; Motta *et al*., 1994; Jensen *et al*., 2004). Therefore, compensating for shortages in ceramide or GlcCers would be expected to attenuate skin barrier impairment in these diseases.

Due to their strong anti-inflammatory actions, topical glucocorticoids have been used for treatment of diseases associated with reduced barrier function, such as AD and psoriasis. They are also known to suppress barrier function due to decreased lipid production, including fatty acid and ceramide (Kao *et al*., 2003). Combinatorial therapy use with moisturizers would be expected to compensate for the adverse effects of glucocorticoids in by facilitating skin hydration, reducing susceptibility to irritation, and restoring the integrity of the SC (Elias and Menon, 1991). Although moisturizers serve as a prominent first-line therapeutic option for patients with AD and other chronic skin diseases, they do not necessarily increase the amount of skin ceramides (Kikuchi and Tagami, 2008). Furthermore, some topical moisturizing products deteriorate skin barrier function since they contain detergents in order to enhance the permeability of the moisturizing molecule (Danby *et al*., 2011; Torma *et al*., 2008; Buraczewska *et al*., 2009). Although ceramides predominant moisturizers are efficacious and safe in treatment of patients with mild to moderate

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AD (Kircik and Del Rosso, 2007; Anderson and Dinulos, 2009; Na *et al*., 2010; Kircik *et al*., 2011), there is no clear evidence that topical ceramides become incorporated into the SC and behave as endogenous ceramides (Caussin *et al*., 2007).

Since sphingolipids are found in many natural foodstuffs, dietary ceramides have become prominent among various therapeutic interventions (Vesper *et al*., 1999). Daily intake of GlcCer from natural crop extracts has been suggested to improve skin barrier impairments in animal models of AD. For example, oral administration of extracts from maize, rice bran, and konjac have been reported to be effective in a murine model of AD (Tsuji *et al*., 2006; Tsuji *et al*., 2006; Uchiyama *et al*., 2008). These extracts improved impairments in skin barrier function, as evidenced by some dermal index in a similar dose (0.03–0.1%). In contrast to animal studies, the efficacy of ceramide is still controversial in the clinical setting. In a human double-blind study using 100 healthy subjects with mildly dry skin, oral intake of konjac-derived ceramide for 12 weeks significantly reduced TEWL values and relieved skin itching (Uchiyama *et al*., 2008). Similarly, a double-blind study using 51 women with dry skin, oral wheat extract for 12 weeks significantly prevented barrier impairment and had a tendency to reduce skin dryness and redness (Guillou *et al*., 2011). Furthermore, oral konjac-derived ceramide significantly reduced TEWL from the necks and inner elbows of patients with mild to moderate AD (Miyanishi *et al*., 2005). On the other hand, beet derived-ceramide did not improve TEWL in 35 female subjects with mildly dry skin, but it did improve skin elasticity (Hori *et al*., 2010). The above reports indicate that differences in moisturizing actions between different crops could be caused by variations in ceramide composition (Takakuwa *et al*., 2005; Hori *et al*., 2010). The basic structure of ceramide consists of long-chain sphingoid bases with amide-linked fatty acid chains of C14–C26 in length (Pruett *et al*., 2008; Mizutani *et al*., 2009). With respect to sphingoid bases, rice bran, wheat germ, and konjac-derived ceramide consist principally of 8-*cis*-unsaturated bonds, such as d18:$2^{4t,8c}$ and d18:1^{8c}, while beet-derived ceramide consists of 8-trans-unsaturated bonds, such as d18:$2^{4t,8t}$, d18:1^{8t}. Fatty acid chains of beet ceramide are mostly palmitic acids, while other crops have chains consisting of typically 16–24 carbon atoms (Tahara *et al*., 1986; Hori *et al*., 2010). This may confer an advantage on beet extract as a source of GlcCer since the major fatty acids found in mammalian epidermal ceramides are palmitic and linoleic acids (Rogers *et al*., 1996).

To compare the effects of beet extract against extracts from other ceramide-containing crops on skin barrier function, we employed the simple and reproducible HR-AD diet model in hairless mice, and recorded symptoms that closely resemble those occurring in human AD (Fujii *et al*., 2005).

MATERIALS AND METHODS

Animals. The 4-week-old male HR-1 hairless mice used in this study were obtained from Nihon SLC (Hamamatsu, Japan). The mice were housed under conventional conditions with four mice/plastic cage with woodchips. Animals were acclimatized under standard conditions, with a 12-h light:dark cycle, a constant temperature of 24°C, and 40% relative humidity for 7 days in the animal research facility before experimentation. They were given an AIN-93G control diet (Research Diets Inc., NJ) and drinking water ad libitum. The experimental protocol for this study was approved by the Institutional Animal Care and Use Committee of the Hamamatsu University School of Medicine.

Diets. AIN-93G and HR-AD diets were purchased from Research Diet Inc. and Nihon Nosan Co. (Yokohama), respectively. Extract of beet (*Beta vulgaris*) was kindly provided by Meiji Food Material (Tokyo) and Nippon Beet Sugar Mfg. (Tokyo). Beet extract was obtained from residual pulp, and industrial waste of sugar beets from which beet sugar was first extracted. The ethanol extract of the residual pulp was beet extract, and it was analysed by TLC according to previously published report (Takakuwa *et al*., 2005). Beet extract powder contains approximately 50% β-cyclodextrin, with the reminder being composed mainly of various lipids of which GlcCers constitute 4.0%. GlcCers of beet extract mainly consist of a sphingoid base of *trans*-4, *trans*-8-sphigadienine with the fatty acid chain being palmitic acid (d18:$2^{4t,8t}$). A 2.5% volume of beet extract powder was mixed into HR-AD powder and shaped into pellets (beet extract-supplemented HR-AD). High-performance liquid chromatography analysis indicated that the beet extract contained GlcCer at a final concentration of 0.1%. The ingredients of each diet are summarized in Table 1.

Experimental design. Seven days after their arrival at the animal research facility, the mice were divided into three weight- and TEWL-matched groups with eight mice in each group: the normal diet group (AIN-93G), HR-AD diet group, and beet extract-supplemented HR-AD group. Body weight, and consumption of drinking water, and food in each cage were measured every week.

Table 1. Ingredients in the normal and special diets fed to HR-1 hairless mice

Ingredient	Normal %	HR-AD	Beet Extract
Water	ND	1.60	1.60
Crude fibre	5.00	4.40	4.29
Crude lipid	7.0	0.40	1.2
Glucosylceramide	<0.00003	<0.00003	0.1
Crude protein	20.7	21.6	21.8
Crude carbohydrate	63.90	66.75	66.6
		mg/kg	
Mn	10.05	80.85	78.90
Zn	42.89	132.33	129.22
Fe	54.52	289.70	282.69
Cu	4.54	20.59	20.14
Se	0.18	0.30	0.30
Ca	5198	9641	9400
P	3133	8275	8217
Na	992	2057	2010
Mg	557	186	191

Measurement of TEWL. TEWL was measured under constant humidity and temperature using a H4300-S apparatus (NIKKISO-YSI, Tokyo), which consisted of a semi-closed chamber of 8-mm diameter. TEWL was recorded with the probe placed on the dorsal skin around the scapula. TEWL was measured repeatedly for appropriate durations of time, and two values were adopted when the difference between them was less than 20%.

Analysis of scratching behaviour. Scratching behaviour was assessed 8 weeks after starting the HR-AD diet. The HR-1 mice were placed in an observation chamber (cubic cage, 15 cm on the side) for at least 20 min as an acclimatization period. Scratching behaviour was then recorded with a digital video recorder for 60 min and analysed later in a blind manner. One scratching event was defined as a sequence of several to more than ten scratching movements, which consisted of more than five repetitions of quick scratching movements using the hind paws. Since individual scratching movements are difficult to distinguish, the frequency and duration of each scratching event was analysed. When the mice scratched their head and dorsal skin with their hind paws, this scratching behaviour was counted and analysed separately. Cumulative scratching duration was defined as the total time spent scratching in 60 min. The proportion of head scratching against total scratching was defined as the head-scratching ratio.

Skin inspection. On the first day and 2, 4, and 6 weeks after HR-AD loading, the severity of dermatitis on the head and the dorsal part of the body was evaluated based on erythema and scaling/dryness (none = 0, mild = 1, moderate = 2, and severe = 3). At 8 weeks after HR-AD loading, the whole body was photo-recorded, and scoring was reconfirmed in a blind manner.

Isolation of organs, histochemical evaluation, and serum analysis. At 8 weeks after initiating the HR-AD diet, mice were anaesthetized with ether, and approximately 1 mL of blood was withdrawn from the vena cava. The whole liver and spleen were isolated, and skin sections were removed from the head and dorsal area around the scapula. The liver and spleen specimens were weighed, and the skin was fixed with 10% formalin phosphate buffer. Collected blood samples were incubated at room temperature and then centrifuged at $2300 \times g$ for 10 min. The supernatant was used for mineral and IgE assays. Serum Mg^{2+} and Ca^{2+} concentrations were analysed with an auto-analyser system (Hitachi 7070, Tokyo) after appropriate dilution. Serum IgE concentrations were analysed with an enzyme immune assay kit (Morinaga, Tokyo). Fixed skin specimens were embedded in paraffin, cut into 4-μm sections, and stained with haematoxylin-eosin or toluidine blue. After inspection of the whole specimen, a typical section was randomly chosen (sections with broken histological structure were excluded from consideration), and its image was photo-recorded with a microscope (BX51, Olympus, Tokyo). Each section was analysed using WinROOF v5.0 (Mitani Co., Tokyo). Sections stained with haematoxylin-eosin were used to measure thickness of the SC, epidermis, and dermis. The density of mast cells in the dermis was measured in 0.04-mm^2 cross-sections with toluidine blue staining.

Statistical analysis. Values were expressed as mean ± standard deviation (SD) for eight mice. Multiple comparisons were performed by analysis of variance (ANOVA) with post hoc tests. Differences of $p < 0.05$ were considered statistically significant.

RESULTS

Body weight and food/water consumption.

In the normal diet group, mean body weight reached a plateau after 6 weeks (Fig. 1A). In the HR-AD diet group, mean body weight began to decrease at 8 weeks (Bonferroni post hoc test, $p < 0.01$). The beet extract-supplemented HR-AD diet prevented decreases in body weight compared to the HR-AD diet at 6, 7 ($p < 0.05$), and 8($p < 0.001$) weeks.

Mice in the normal diet group consumed around 0.2 mL/g body weight/d (corresponding to about 6 mL/body/d) of drinking water throughout the 8 weeks (Fig. 1B). In contrast, the HR-AD diet increased water intake more than 2–3-fold after 6 weeks. The HR-AD diet significantly increased water consumption compared to the normal diet (two-way ANOVA, $p < 0.001$) during the experimental period, and supplementation of beet extract to the HR-AD diet significantly prevented this increased consumption ($p < 0.01$). During the experimental period, mice consumed approximately 0.12 g/g body weight/d of food, and this value did not differ among experimental groups (data not shown).

Measurement of TEWL.

In the normal diet group, TEWL was constant throughout the 8 weeks (i.e. 10–20 g/m^2/h). The HR-AD diet significantly increased TEWL after 5 weeks ($p < 0.01$; Fig. 1C), and after 8 weeks TEWL was almost 3-fold higher in HR-AD fed mice than in mice fed a normal diet. The beet extract-supplemented diet completely prevented increases in TEWL ($p < 0.001$), to values comparable to those of the normal diet group.

Inspection of the skin.

Fig. 2 shows photographs of typical HR-1 mice from each group. In the HR-AD group, scale formation on the skin was apparent in several mice at 4 weeks and was marked after 6 weeks. Mean dryness values were 0.25, 1.25, and 1.75 at 4, 6, and 8 weeks of treatment, respectively. The HR-AD diet also caused mild erythema on the head but not on the body. Mean erythema values were 0.75 at 6 weeks and 1.0 at 8 weeks. The HR-AD fed mice showed a tendency to develop alopecia compared to those of the other groups. Neither the normal diet nor the beet extract-supplemented HR-AD diet caused changes in skin conditions during the experimental period. The severity scores of skin conditions were increased in HR-AD fed mice, and this was significantly prevented by the addition of beet extract supplement (Table 2).

Scratching behaviour.

All mice except 1 in the normal diet group exhibited scratching behaviour during the60-min observation

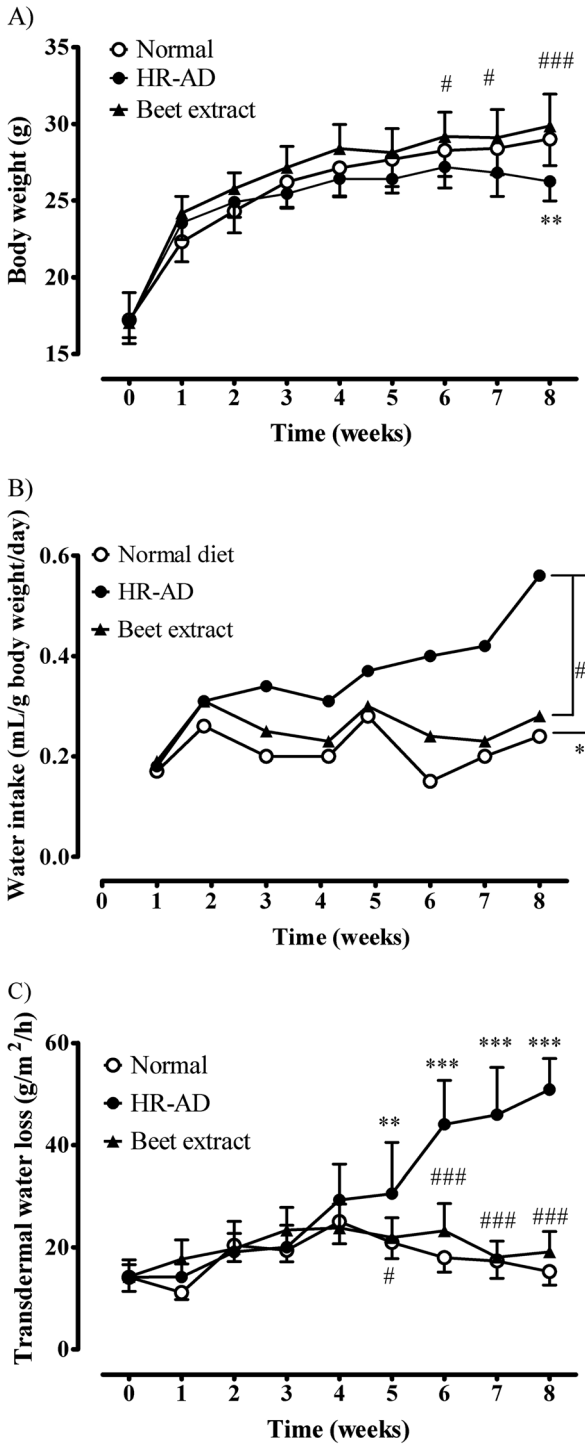

Figure 1. A) Change in body weight among mice fed a normal (○), HR-AD (●), and beet extractsupplemented HR-AD (▲) diet during the study period (mean ± SD, $n = 8$). **: Significantly different from the normal diet at $p < 0.01$; #, ###: Significantly different from the HR-AD diet at $p < 0.05$ and $p < 0.001$, respectively (Bonferroni's post hoc test). B) Change in water intake in HR-1 mice during the study period. Values represent the mean of eight animals. ***: Significantly different from the normal diet ($p < 0.001$, ANOVA). ##: Significantly different from the HR-AD diet ($p < 0.01$, ANOVA). C) Transepidermal water loss in HR-1 mice fed a normal (○), HR-AD (●), and beet extract-supplemented HR-AD (▲) diet during the study period. Each symbol and vertical bar represents the mean ± SD of eight animals. **, ***: Significantly different from the control group at $p < 0.01$ and $p < 0.001$, respectively. #, ###: Significantly different from the HRAD diet at $p < 0.05$ and $p < 0.001$, respectively (Bonferroni's post hoc test).

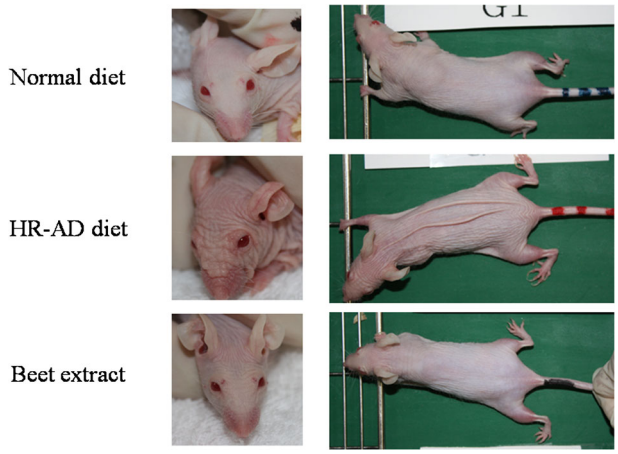

Figure 2. Typical dry skin lesions in HR-1 mice fed a normal, HR-AD, and beet extract-supplemented HR-AD diet at 8 weeks. HR-AD mice exhibited scales and erosion on the neck, face, and ears.

Table 2. Skin scores in HR-1 hairless mice receiving different diets

	Dryness				Erythema			
Grade	0	1	2	3	0	1	2	3
Normal	8	0	0	0	8	0	0	0
HR-AD	0	6	2	0 ***	0	8	0	0 ***
Beet extract	8	0	0	0 #	8	0	0	0#

Values are frequencies.
***,
#: Significantly different (Dunn test, $p < 0.001$) from normal and HR-AD, respectively.

period at 8 weeks after HR-AD loading. In the normal diet group, mean cumulative scratching time was approximately 40 s and consisted of 7.8 scratching bouts of 4.8-s duration (Fig. 3). The HR-AD diet significantly ($p < 0.05$) increased cumulative scratching time and its duration by 2–3-fold. In the HR-AD fed mice, scratching behaviour was preferentially increased at the head position ($p < 0.001$) but was decreased at the dorsal position. Beet extract normalized these changes in scratching behaviour. Mice fed the normal diet scratched both the head and dorsal skin to the same extent, with a head scratching ratio of 45.8 ± 22.0%. The head scratching ratio was increased significantly by the HR-AD diet (94.8 ± 4.0%, ANOVA, $p < 0.001$) and was prevented (41.1 ± 26.2%, $p < 0.001$) by the beet extract supplement.

Serum Mg^{2+}, Ca^{2+}, and IgE levels.

No serum samples showed evidence of haemolysis, as assessed by visually examining its colour. Serum Mg^{2+} concentrations in mice fed the HR-AD diet were significantly lower ($p < 0.001$) than those of the normal diet group (Table 3). The beet extract diet did not inhibit decreases in serum magnesium levels, which remained significantly lower ($p < 0.001$) than those in mice fed the normal diet. In contrast, serum Ca^{2+} concentrations were slightly increased ($p < 0.05$) in mice fed the HR-AD diet but were still within the normal range. The supplemental beet extract significantly prevented this increase in serum Ca^{2+} levels ($p < 0.05$). HR-AD treatment increased ($p < 0.05$) serum IgE levels, but supplemental addition of

beet extract to the diet did not reverse this effect. A more than 100-fold increase in serum IgE levels was previously reported in a similar model that was associated with marked increases in eosinophils and mast cells (Fujii *et al*., 2005). Therefore, our model likely represents the transition from dry skin into AD. The difference in pathophysiological status between our model and that of the previous study is likely due to differences in experimental conditions (i.e. temperature, humidity, wood chips, and cage capacity).

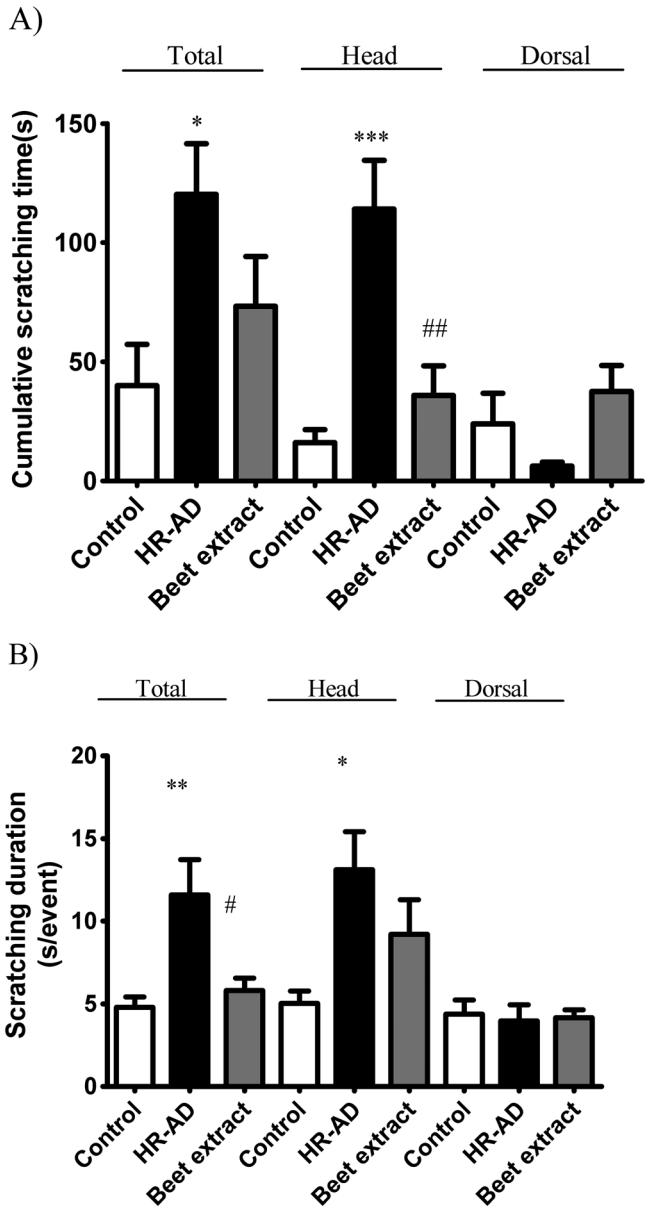

Figure 3. A) Cumulative scratching time and B) scratching duration in HR-1 mice fed a normal, HR-AD, and beet extract-supplemented HR-AD diet. Scratching behaviour was evaluated for 60 min. Each value represents the mean ± SD of eight animals. *, **, ***: Significantly different from the control group at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. #, ##: Significantly different from the HR-AD diet at $p < 0.05$ and $p < 0.01$, respectively (Tukey's multiple comparison test).

Histochemical examination.

Tissue weights in the normal, HR-AD, and beet extract-supplemented HR-AD groups were 110 ± 38, 97 ± 21, and 107 ± 32 mg for the spleen and 1.46 ± 0.18, 1.61 ± 0.17, and 1.77 ± 0.17 g for the liver, respectively. There were no differences between any groups. Tissue weight adjusted for body weight also did not differ among the three groups.

Fig. 4 shows photographic images of typical head skin sections stained with haematoxylin-eosin. The skin sections of HR-AD fed mice revealed marked thickening of the epidermal and SC layers, which was prevented by supplementation with the beet extract (Fig. 5A, B). Dermis thickness did not differ among the groups. Both the head and dorsal skin showed similar levels of thickening. Mast cells were identified in the dermis by toluidine blue staining, and the number of mast cells (expressed as mean ± SD) did not differ significantly among the groups (ANOVA; Table 3). Degranulation of mast cells also did not differ among the groups. No remarkable inflammatory changes, such as eosinophil migration or skin, were observed in any group.

DISCUSSION

The three main lipid components, i.e. cholesterol, free fatty acids, and ceramides, are essential for maintaining skin barrier homeostasis. Abnormal skin lipid profiles cause impaired skin barrier function, which can lead to common skin diseases, such as AD and psoriasis (Imokawa *et al*., 1991; Motta *et al*., 1993; Motta *et al*., 1994; Jensen *et al*., 2004). In the present study, an HR-AD diet, in which total lipid and magnesium content were minimal, led to impaired skin barrier function characterized by increases in TEWL and scratching behaviour. Increased TEWL led to compensatory keratinocyte proliferation resulting in SC deposition and epidermal thickening. These histological and functional skin impairments were prevented by supplemental addition of beet extract into the HR-AD diet.

Table 3. Serum concentrations of Mg^{2+}, Ca^{2+}, and IgE and dermal density of mast cells in HR-1 hairless mice

	Normal	HR-AD	Beet extract
Serum Mg^{2+} level (mg/dL)	2.95 ± 0.37	1.33 ± 0.26 ***	1.43 ± 0.17 ***
Serum Ca^{2+} level (mg/dL)	9.14 ± 0.74	10.28 ± 0.77*	9.38 ± 1.00#
Serum IgE level (ng/mL)	1.46 ± 0.18	1.61 ± 0.17 *	1.77 ± 0.17
Dermal mast cells (cells/mm^2)	246.9 ± 96.8	259.4 ± 66.7	234.4 ± 120.2

Values represent mean ± SD of 8 mice.

*,

***: Significantly different from the normal diet at $p < 0.05$, $p < 0.001$, respectively.

#: Significantly different from the HR-AD diet at $p < 0.05$ (Tukey's multiple comparison test).

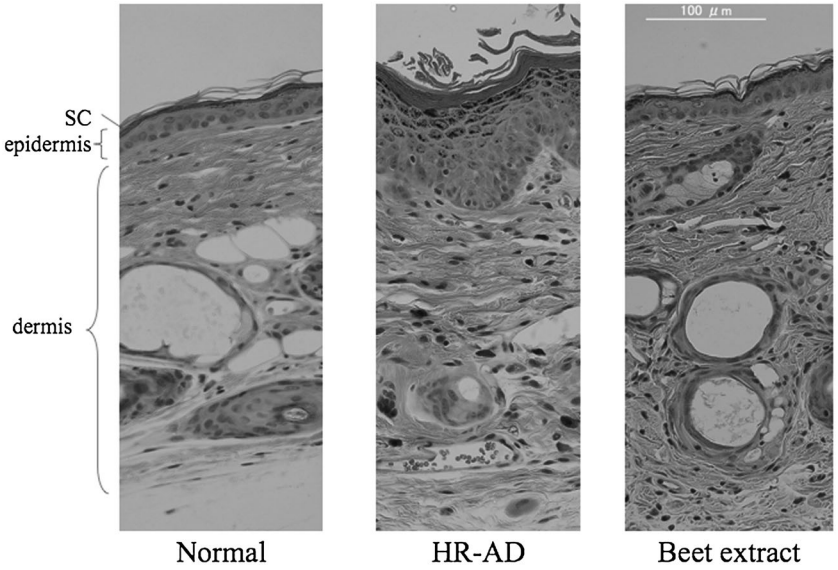

Figure 4. Image of haematoxylin-eosin staining of the head skin of HR-1 mice fed a normal, HR-AD, and beet extract-supplemented HR-AD diet. There is minimal infiltration of leukocytes and no change in mast cells in the dermis. Deposition of the stratum corneum layer and marked epidermal hyperplasia are seen in HR-AD fed mice.

Regardless of their origin, ceramide-containing crops, including beet, have been shown to improve skin barrier impairments at least in mice. The lack of efficacy of beet extract previously observed in humans could be partly explained by study conditions such as differences in the severity of illness, room temperature/humidity, or daily dietary habits. Otherwise, lack of efficacy could be caused by utilization of sphingoid base, since it has been reported that some cultured human cell lines differentially respond to ceramides and sphingoid bases. For example, konjac-derived ceramide facilitates collagen I production, while beet extract promotes the production of fibronectin but not collagen (Hori *et al.*, 2010). In human keratinocytes, sphingoid bases have different effects on gene expression of transglutaminase-1 and 3 which are enzymes involved in cross-linking several insoluble proteins (e.g. involucrin and loricrin) in the SC (Hasegawa *et al.*, 2011). In Caco-2 cells, sphingosine is preferentially accumulated into the cells, compared to sphingadienine, which is found in plants. In another report, plant-derived ceramide, i.e. phytoceramide, but not animal-derived ceramide, activated peroxisome proliferator-activated receptors, transcription factors that regulate lipid and glucose metabolism (Murakami *et al.*, 2011). Together, these reports imply that, due to intermolecular interactions, mixed intake of various GlcCer molecules may be beneficial by enhancing potency or reducing side effects, phenomenon similar to that which occurs with licorice and soybean (Uto *et al.*, 2012; Seber *et al.*, 2012). However, further studies are required to determine the precise benefits of beet extract over other ceramide-containing crops or purified GlcCer.

A deficiency of epidermal ceramides may be the most likely mechanism of impaired skin function in HR-AD fed mice. Chemical analysis of the three different diets used in the present study revealed that the lipid component in the HR-AD diet was less than 10% of that in the normal diet, and that the beet extract-supplemented diet still had less lipids than the normal diet (Table 1). The major lipid element of the normal diet was soybean oil, which is composed of linoleic and oleic acids (unpublished data). The GlcCer concentration of the beet extract-supplemented diet was 0.1%, whereas it was less than 0.1 ppm in the HR-AD and normal diets (Table 1). Although the amount of lipids in the beet extract-supplemented diet remained low compared to the normal diet, it completely prevented the symptoms associated with impaired skin barrier function in HR-AD fed mice. When dietary ceramide is reduced, endogenous synthesis, via a multi-step *de novo* pathway from palmitoyl-CoA and serine, is expected to increase in order to maintain its levels (Spiegel and Merrill, 1996). The HR-AD diet contained the minimum amount of lipids necessary for HR-1 mice to maintain vital activity but was insufficient for *de novo* ceramide synthesis to occur. Thus, beet extract supplementation of the diet prevents impaired skin function, possibly by compensating for the lack of ceramide production. An earlier study by Tsuji and colleagues showed a beneficial effect of rice germ, which also contained 0.1% GlcCer, on impaired skin barrier function (Tsuji *et al.*, 2006). In their study, hairless mice were fed an HR-AD diet for 4 weeks in order to impair skin barrier function, after which the mice were changed to a normal diet, with or without rice germ. When rice germ was added to the normal diet, TEWL returned to basal levels 7 days earlier than in mice fed the normal diet alone. In contrast to the current study, the normal diet in this study contained sufficient lipids. Tsuji and colleagues also demonstrated the effectiveness of maize, a crop that also contains GlcCer, on another model of skin barrier impairment of mice. The use of maize supplement for 5 weeks prevented skin barrier impairment induced by repeated tape-stripping (Tsuji *et al.*, 2006). Most food containing GlcCer, including rice germ, maize, and beet extract, consist of a wide range of fatty acids and sphingoid bases, each of which prevent skin barrier impairments at similar doses. Thus, GlcCer is the most likely common element in these crops to prevent impaired skin barrier function.

Dietary ceramides are believed to be actively transported across the gastrointestinal wall and provided to the peripheral. Sphingolipids in beet extract are probably absorbed from the gastrointestinal tract in a similar manner to other lipids. An early report by Nilsson showed that orally administered labelled sphingomyelin was

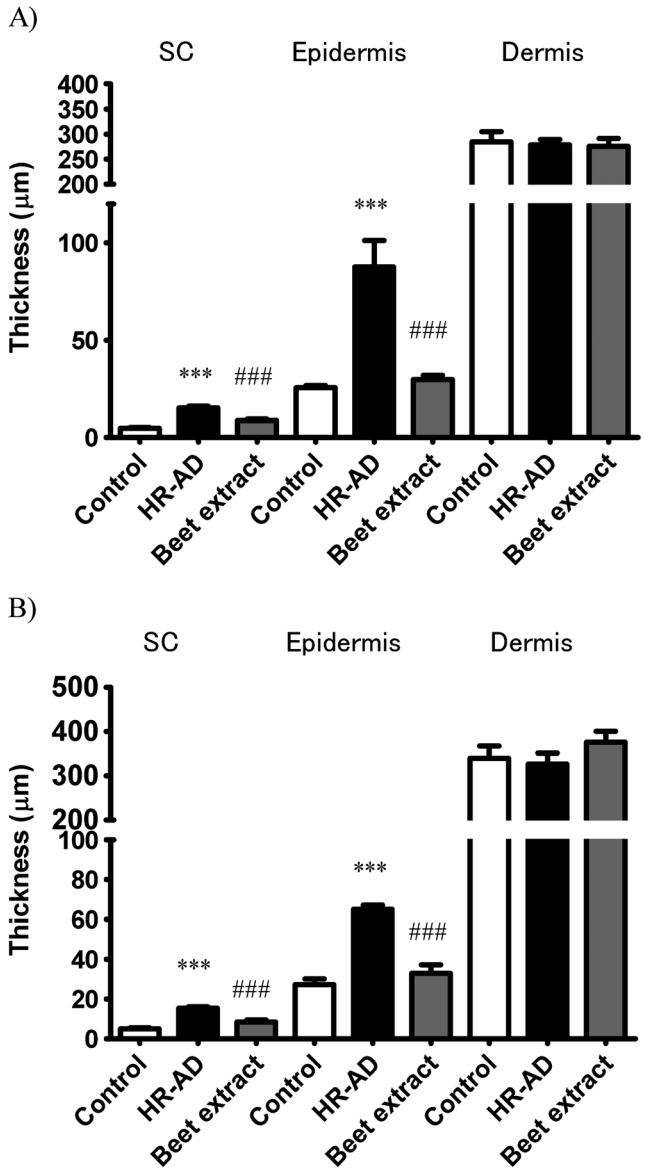

Figure 5. Epidermal thickening in HR-1 mice fed a normal, HR-AD, and beet extract-supplemented HR-AD diet. Thickness was measured from images of haematoxylin-eosin staining of A) the head and B) dorsal area. Each value represents the mean ± SD of eight animals. ***: Significantly different from the normal diet group at $p < 0.001$. ###: Significantly different from the HR-AD diet at $p < 0.001$ (Tukey's multiple comparison test). SC, stratum corneum.

absorbed into the body via the gastrointestinal tract, with only 25% being found in the faeces. Of the absorbed sphingomyeline, 80–90% was metabolized to ceramide, possibly by intraluminal sphingomyelinase (Nilsson, 1968). In studies using lymphatic duct-cannulated rats, Sugawara and colleagues reported that free and complex forms of GlcCer can be detected in the lymph after administration of dietary maize (Sugawara *et al*., 2010). Recently, Duan and colleagues showed that dietary GlcCer reversed the damage to skin barrier function by up-regulating ceramide synthases in the epidermis (Duan *et al*., 2012). Incorporated GlcCer is also known to enhance production of involucrin, one of the major precursor proteins in cornified envelopes of keratinocytes in the SC (Hasegawa *et al*., 2011).

Magnesium content could be another factor that contributes to skin barrier impairment by an HR-AD diet. An earlier study suggested that magnesium plays a relevant role in skin barrier function and a low-magnesium diet has been shown to produce an AD-like skin rash in hairless rats (Neckermann *et al*., 2000). The application of magnesium salts accelerated recovery 3 h after impairment of the skin barrier (Denda *et al*., 1999). Furthermore, magnesium is an activator of ceramide-associated enzymes, such as neutral sphingomyelinase, which catalyse the conversion of sphingomyelin into ceramides (Goni and Alonso, 2002). In the present study, there was a decrease in blood magnesium levels (to approximately 50% of normal levels) in mice that were fed the HR-AD diet, which contains approximately one-third the magnesium content of the normal diet. Despite its effects in restoring barrier function, serum magnesium concentrations did not recover with the beet extract supplement. Therefore, normalization of plasma magnesium levels is not required for the beneficial effect of beet extract in preventing skin barrier impairment, although magnesium deficiency may be a trigger in causing impaired skin barrier function in the HR-AD diet model.

In the present study, the dose required to prevent impairment of skin barrier function in mice was within a practical range. The amount of food consumed by the mice (0.12 g/g body weight/d) and the GlcCer content in the beet extract-supplemented diet (0.1%), implies that daily GlcCer intake was approximately100 mg/kg body weight, whereas the GlcCer intake in the normal and HR-AD diets was less than 0.03 mg/kg body weight (i.e. the detection limit). Based on the body surface area normalization method, the effective dose in human could be expected to be about 10% of the mouse dose (i.e. 10 mg/kg body weight) (Reagan-Shaw *et al*., 2008). Thus, the GlcCer amount needed to maintain skin barrier homeostasis in humans (body weight: 50–70 kg) could be expected to be 0.5–0.7 g/body/d. It has been estimated that a healthy human normally consumes about 0.4 g of sphingolipids in the daily diet (Vesper *et al*., 1999). Thus, the present study suggests that a normal daily diet almost satisfies the need for sufficient sphingolipids to maintain skin barrier function. Conversely, an unbalanced diet raises the possibility for impairment of skin barrier function to occur. Research on food in the US has revealed that 70% of food-derived sphingolipids are of animal origin and 30% are of plant origin (Vesper *et al*., 1999). The ceramide component of sphingolipids from animal-derived food is greater than that from plant, with the rank order of eggs > cheese > meat > > fish. However, in order to get sufficient ceramide from the diet without ingesting excess cholesterol, humans should use plant-origin foods. Since beet extract contains only a low amount of lipids other than ceramide, it is an ideal supplement for people who suffer from diseases associated with impaired skin function.

HR-1 mice fed an HR-AD diet increased their water intake, possibly as a consequence of an increase in TEWL and the need to maintain the balance between water consumption and loss from the body. Differences in daily water consumption are presumed to come from drinking water when the water, since the dietary water content is constant among the three groups. Similarly, differences in daily water loss are assumed to be due to TEWL, since loss of water by respiration, urinary and faecal excretion, and sweating, is constant among the groups. Thus, TEWL should be equal to the volume of drinking water, and increases in TEWL in mice on the

HR-AD diet should be accompanied by corresponding increases in drinking water volume (i.e. a 3-fold increase). In contrast, during the late phase of the experimental period, increases in body weight were blunted in mice on the HR-AD diet, even though the total energy intake from each diet and food consumption was almost identical in each group. This decrease in body weight may be attributable to an increase in energy expenditure, secondary to increased scratching behaviour (2-fold) in HR-AD fed mice.

Interestingly, mice fed the HR-AD diet had significantly increased head scratching vs. dorsal skin scratching behaviour, resulting in the development of skin erythema. Biased scratching behaviour in this model could be a useful index for evaluating scratching behaviour, although the meaning of this change is unclear. One possible reason for biased scratching is that mice scratched the dorsal skin with the hind paw only, whereas they often groomed the head skin with the forepaw, in addition to scratching with the hind paw. Such repeated irritation to the head skin seems to induce even more scratching in this area. Although skin scaling and erythema were observed on the head skin, neither marked increases in serum IgE levels nor inflammatory histochemical changes were observed in HR-AD fed mice. A more than 100-fold increase in serum IgE levels, associated with marked increases in eosinophiles and mast cells, was reported in a similar model (Fujii *et al*., 2005). Therefore, we believe our model to represent the transition state between dry skin and allergic dermatitis. Although in humans, not all case of AD are accompanied a rise in serum IgE (Tokura, 2010), IgE is still the most reliable index of disease in experimental animal models of AD (Matsuoka *et al*., 2003; Matsumoto *et al*., 2004; Fujii *et al*., 2005). We attribute the differences in pathophysiological status between our model and that previously reported to be due to differences in experimental conditions (i.e. temperature, humidity, woodchips, and cage size).

In conclusion, in this study, we found that a prophylactic beet extract-supplemented oral diet prevents skin barrier impairment. Although ceramides are known to be ideal moisturizing agents, they do not achieve full efficacy upon topical application. As an alternative approach, oral intake of beet extract showed great potential in treating skin diseases, possibly through its active GlcCer component. This effect was commonly found in ceramide-containing crops, at least in mouse model. Therefore, beet extract is valuable as a healthy supplement for maintenance of skin barrier function and prevention of various skin diseases.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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