Photoprotective effects of sphingomyelin-containing milk phospholipids in ultraviolet B-irradiated hairless mice by suppressing nuclear factor- κ B expression

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ABSTRACT

Ceramide-containing phospholipids improve skin hydration and barrier function and are ideal for use in skin care products. In this study, we evaluated the photoprotective effect of milk phospholipids on the skin condition of UVB-irradiated hairless mice. Skin parameters were assessed following oral administration of milk phospholipids. The UVB irradiation induced photoaging in mice. The animals were divided into 5 groups: a control group (oral administration of saline with no UVB irradiation), UVB group (oral administration of saline with UVB irradiation), and 3 UVB irradiation groups receiving the milk phospholipids at 3 different concentrations of oral administration, 50 mg/kg (ML group), 100 mg/kg (MM group), and 150 mg/kg (MH group), for 8 wk. An increase in skin hydration and transepidermal water loss were improved in the 150 mg/kg of milk phospholipid-administered group. Hematoxylin and eosin staining revealed a decrease in epidermal thickness in the milk phospholipid-administered groups (50, 100, and 150 mg/kg of body weight). In particular, the 100 and 150 mg/kg groups showed significant changes in the area, length, and depth of the wrinkles compared with the UVB group. Moreover, the gene expression of matrix metalloproteins was attenuated, and that of proinflammatory cytokines, especially tumor necrosis factor- α , was significantly reduced in the milk phospholipid-administered groups than in the UVB group. The reduced ceramide and increased sphingosine-1-phosphate levels in the skin tissue due to UVB exposure were restored to levels similar to those of the control group following milk phospholipid administration. These results were confirmed to be due to the downregulation of protein expression of nuclear factor

κ -B (NF- κ B) and phosphorylated I κ B- α (inhibitor of κ B α). Collectively, oral administration of milk phospholipids improves skin health through a synergistic effect on photoprotective activity.

Key words: milk phospholipid, photoprotective activity, nuclear factor- κ B (NF- κ B), ultraviolet B irradiation

INTRODUCTION

The skin ages similar to other organs in the body. Aging is divided into intrinsic aging, which occurs when human body functions deteriorate and physiological functions such as hormones change, and extrinsic aging, which occurs upon exposure to various environmental factors including UVB rays. Among them, photoaging induced by UVB rays is the most direct cause of extrinsic aging, and typical phenomena include dry skin, wrinkles, decreased skin elasticity, and pigmentation (Woodby et al., 2020; Krutmann et al., 2021). Skin moisture is the most important factor in maintaining skin health and controlling aging. Lack of adequate skin moisture prevents activation of enzymes that are involved in the generation of lipids and natural moisturizing factors, leading to dry and thick stratum corneum of the skin (Bouwstra et al., 2021). The skin comprises the epidermis, dermis, and subcutaneous tissue. The stratum corneum of the epidermis, which is the outermost layer of the skin, is composed of the following 3 main lipids: ceramide (50%), free fatty acids (25%), and cholesterol (25%; Wartewig and Neubert, 2007). Lipids, which form a lamellar structure in the spaces between keratinocytes, act as a barrier to water permeation, preventing water transpiration and the invasion of external factors. The ceramide content in this lipid layer is extremely high. Sphingomyelin in the skin granular layer is hydrolyzed by sphingomyelinase to produce ceramide (Mizutani et al., 2009; Vávrová et al., 2017).

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Milk contains approximately 4% lipids, and most of them are dispersed in milk in the form of fat globules. These fat globules are covered with a thin membrane called the milk fat globule membrane, approximately half of which consists of lipids. Of these lipids, approximately 70% are triglycerides, approximately 30% are phospholipids, and trace amounts are cholesterol. Phospholipids in milk consist of lipids with a glycerol or sphingosine backbone (Fong et al., 2007). Sphingomyelin, which accounts for a large amount of milk phospholipids, increases ceramide levels in the body (Ohlsson et al., 2010), which is not only involved in epidermal hydration but also in the maintenance of skin barrier function (Brod, 1991). Dietary sphingomyelin is hydrolyzed to ceramide and sphingosine in the small intestine after ingestion, and a portion of it is reused for sphingolipid synthesis (Schmelz et al., 1994). Sphingolipids upregulate the expression of enzymes involved in ceramide synthesis (Duan et al., 2012) and are involved in the enhancement of ceramide levels in the skin of hairless mice (Haruta-Ono et al., 2012b).

In this study, milk phospholipids used were polar lipids obtained by ethanol treatment of milk whey powder and contained high levels of phospholipids, mainly phosphatidylcholine and phosphatidylethanolamine, and sphingolipids, mainly sphingomyelin (Lee et al., 2020). Milk phospholipids are a food material rich in ceramide precursors that can protect the skin from UVB rays. Therefore, milk phospholipids were orally administered to UVB-irradiated hairless mice to evaluate its photoprotective effects.

MATERIALS AND METHODS

Materials and Animals

Milk phospholipids were obtained from Solus Advanced Materials Co., Ltd. and consisted of $25.0 \pm 5.0\%$ (mean $\pm$ SD) of phospholipids ($7.5 \pm 1.5\%$ of phosphatidylcholine, $6.5 \pm 1.5\%$ of phosphatidylethanolamine, $1.3 \pm 0.7\%$ of phosphatidylserine, and $6.5 \pm 1.0\%$ of sphingomyelin), $1.5 \pm 0.5\%$ of lactosylceramide, $0.9 \pm 0.6\%$ of glucosylceramide, and $0.3 \pm 0.1\%$ of GD3 ganglioside (Supplemental Table S1; <https://doi.org/10.6084/m9.figshare.17157776.v1>; Jo, 2021).

Male hairless mice (Skh: HR-a, 8 wk old) were purchased from the Central Lab Animal Inc. and subjected to a 1-wk acclimatization period. The breeding environment of the experimental animals was maintained at $23 \pm 2^\circ\text{C}$, $55 \pm 10\%$ humidity, and a 12-h light-dark cycle. Mice were fed with a mouse-only feed (Purina), and drinking water was provided ad libitum. The breeding and management of mice was based on the Guide for the Care and Use of Laboratory Animals, and

this study was approved by the Institutional Animal Care and Use Committee at Korea University (KUIA-CUC-2020-0054).

Experimental Design

Hairless mice were randomly divided into 5 groups (6 mice/group) based on their average BW. Mice were divided into oral administration of saline without UVB irradiation (control), oral administration of saline with UVB irradiation (UVB), oral administration of low-dose milk phospholipids with UVB irradiation (ML), oral administration of medium-dose milk phospholipids with UVB irradiation (MM), and oral administration of high-dose milk phospholipids with UVB irradiation (MH) groups. Once daily, mice in groups ML, MM, and MH were orally administered 50, 100, and 150 mg/kg of BW of milk phospholipids, respectively.

To induce photoaging, UVB and milk phospholipid-administered mice (ML, MM, and MH) were irradiated with UVB at the same time, 3 times per week, using a UVB lamp (UB800, Waldman Licht Technik GmbH). Mice were exposed to 1 minimal erythral dose (MED; $75 \text{ mJ}/\text{cm}^2$) in the first and second weeks, 2 MED in the third week, 3 MED in the fourth week, and 4 MED from the fifth week onwards. Milk phospholipids were orally administered with UVB exposure for a total of 8 wk, and mice were killed using CO_2 gas after the end of the experiment.

Measurement of Skin Hydration Level, Transepidermal Water Loss, and Wrinkle Formation

Skin hydration level and transepidermal water loss (TEWL) were measured using a Corneometer CM825 (Courage and Khazaka Electronic GmbH) and Tewameter TM300 (Courage and Khazaka electronic GmbH), respectively. The dorsal side of the animal was photographed with a digital camera (EOS 600D, Canon), and a replica was generated using a silicone polymer (SILFLO impression material, Flexico) for wrinkle analysis and evaluation. The skin template was fixed at an incident angle of light of 20° , and the wrinkle shadow contrast image was photographed using the Visioline program, imaged, and analyzed using the Skin Viosimeter SV600 software (Courage and Khazaka Electronic GmbH). The area, number, length, and depth of the wrinkles were determined.

Histopathological Analysis

After killing the animals, the dorsal skin was incised, attached to filter paper, and fixed in 10% neutral

formalin for hematoxylin and eosin and immunohistochemical staining. Tissues were paraffin-embedded by the general tissue processing method, and 6- μ m sections were cut and stained with hematoxylin and eosin. Immunohistochemical analysis for collagen was performed as described by Yamamoto et al. (2008) using rabbit anti-type I collagen antibodies (1:400; Abcam). All histopathological analyses were performed using a stereomicroscope (Axio Zoom v.16; Carl Zeiss) and the ImageJ software (version 1.8.0; National Institutes of Health).

RNA Extraction and Real-Time Quantitative PCR

Total RNA was extracted from the skin tissues using TRIzol (Life Technologies Inc.) as per the manufacturer's instructions. Complementary DNA was synthesized from 1 μ L of RNA of each sample using the iScript cDNA Synthesis kit (Bio-Rad Laboratories Inc.). To determine mRNA expression of genes encoding MMP, TIMP, and proinflammatory cytokines, real-time quantitative PCR was performed using primers used in a previous study (Suh et al., 2020) and SYBR Green (iQ SYBR Green Supermix, Bio-Rad Laboratories, Inc.). The target gene information was as follows: *Mmp2* (NM_008610.3), *Mmp3* (NM_010809.1), *Mmp9* (NM_013599.4), *Mmp13* (NM_008607.2), *Timp1* (NM_011593.2), *Timp2* (NM_011594.3), *Il4* (NM_021283.2), *Il6* (NM_031168.1), *Il10* (NM_010548.2), and *Tnfa* (NM_013693.3). Data were analyzed by the comparative cycle threshold ($2^{-\Delta\Delta C_T}$) method (Livak and Schmittgen, 2001), and results were normalized to *Gapdh* mRNA levels.

Analysis of Ceramide by High Performance Thin-Layer Chromatography

Skin tissues (100 mg) were homogenized by adding 1 mL of chloroform and methanol (2:1, vol/vol). After centrifugation ($2,800 \times g$, 10 min at 25°C), a chloroform layer was obtained, and the solution was volatilized and used as the sample (Shimoda et al., 2012). High performance thin-layer chromatography silica gel plates (10 $\times$ 20 cm, Merck) were used to separate the ceramide mixture (Matreya LLC) and samples in the following solvent conditions: chloroform:methanol:water (40:10:1) to 2.0 cm, chloroform:methanol:acetic acid (94:4:1.5), from 2.0 to 7.0 cm, and *N*-hexane:diethyl ether:acetic acid (65:35:1), from 7.0 cm to the top. Then, 3% (wt/vol) aqueous cupric acetate containing 8% (vol/vol) phosphoric acid was dispensed, and

the ceramide content was analyzed after drying in an oven at 160°C for 15 min (Uchida et al., 2000).

Analysis of Sphingosine-1-Phosphate by HPLC

Extraction and derivatization of sphingosine-1-phosphate (S1P) from skin tissues was performed as described by Min et al. (2002) and Kim et al. (2011), with some modifications. Skin tissues (100 mg) were homogenized by adding 500 μ L of PBS and centrifugation at $4,800 \times g$ for 5 min at 25°C. The pH was adjusted to 10 to 11 by adding 700 μ L of methanol, 300 μ L of 1 M NaCl, 600 μ L of CHCl_3 , and then 35 μ L of 6 N NaOH. After centrifugation of the sample ($4,800 \times g$, 5 min at 25°C), the lower layer was obtained, and the solution was volatilized. Then, the samples were added to 500 μ L of 0.15 M methanolic KOH and vortexed at 37°C for 20 min. After, we added the solution to 500 μ L of CHCl_3 , 100 μ L of 2 N NH_4OH , and 400 μ L of alkaline water (100 μ L 2 N NH_4OH /100 mL water), and vortexed at 37°C for 1 h. After centrifugation at $4,800 \times g$ for 3 min at 25°C, washing twice with 800 μ L of alkaline water, and volatilizing the solution, the obtained lipid extraction samples were dissolved in 120 μ L of methanol. The *o*-phthalaldehyde derivatization reagent [Sigma; 50 mg of ortho-phthalaldehyde, 1 mL of ethanol, 100 μ L of 2-mercaptoethanol, and 50 mL of 3% (wt/vol) boric acid solution] was added to 20 μ L of the sample and incubated at 25°C for 30 min for derivatization.

The S1P analysis was performed using the e2695 Waters HPLC system (Waters) equipped with a YMC-Pack Pro C18 column (4.6 mm $\times$ 150 mm) and fluorescence detector (Waters, e2475). The flow rate was 0.5 mL/min, and the injection volume was 20 μ L. The mobile phase was methanol:distilled water (92:8, vol/vol) with 0.1% triethylamine, and the chromatogram of S1P was analyzed at excitation and emission wavelengths of 340 and 455 nm, respectively. The concentrations of S1P standards (Sigma) were calculated using individual calibration curves (Moon et al., 2013).

Western Blotting

Protein expression in skin tissues was investigated as previously described (Suh et al., 2020). Antibodies against I κ B α (1:1,000, #9242, Cell Signaling Technology), phospho-I κ B α (1:1,000, #2859, Cell Signaling Technology), α -tubulin (1:1,000, #2125, Cell Signaling Technology), NF- κ B (1:1,000, #6956, Cell Signaling Technology), GAPDH (1:1,000, #5174, Cell Signaling

Figure 1. Effects of milk phospholipids on skin hydration (A) and transepidermal water loss (TEWL, B). CON = oral administration of saline with no UVB irradiation; UVB = oral administration of saline with UVB irradiation; ML, MM, and MH = oral administration of low-dose (50 mg/kg of BW), medium dose (100 mg/kg of BW), and high-dose (150 mg/kg of BW) milk phospholipids, respectively, with UVB irradiation. AU = arbitrary units. Data are expressed as means $\pm$ SE ($n = 6$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ for control vs. UVB group without milk phospholipids group using Tukey's multiple comparison test.

Technology), and anti-rabbit IgG (1:1,000, #7074, Cell Signaling Technology) were used.

Statistical Analysis

The results are expressed as mean and standard error. The significance of data between the UVB and experimental groups was verified by the Tukey's multiple comparison test using SPSS software version 12.0 for Windows (SPSS Inc.).

RESULTS

Effect of Milk Phospholipids on Skin Hydration and TEWL

Skin barrier function is an important indicator of skin homeostasis. Thus, skin hydration and TEWL were analyzed. Skin hydration was significantly lower, and TEWL was significantly higher in the UVB group than in the control group (CON, $P = 0.0001$ and $P = 0.0005$, respectively; Figure 1). Milk phospholipid administration suppressed the UVB irradiation-induced decrease in skin hydration and suppressed the increase in TEWL. In particular, skin hydration and TEWL were significantly different between the MH and UVB groups ($P = 0.0001$ and $P = 0.0314$, respectively).

Effect of Milk Phospholipids on Epidermal Thickness and Collagen Distribution

Hematoxylin and eosin and immunohistochemical staining were performed to evaluate the effect of milk phospholipid intake on epidermal thickness and collagen distribution following UVB irradiation (Figure

2). After 8 wk of UVB irradiation and administration of milk phospholipids, hematoxylin and eosin-stained sections of skin samples obtained from mice were analyzed for epidermal thickening. The thickness of the epidermis was significantly greater in the UVB group than in the nonirradiated control group ($P = 0.0001$, Figure 2B). Consistent with visual observations of the hematoxylin and eosin-stained samples, oral administration of milk phospholipids significantly decreased UVB irradiation-induced increase in epidermal thickness ($P = 0.0004$, $P = 0.0002$, and $P = 0.0001$, respectively) in a dose-dependent manner. In addition, skin of control group had collagen fibers homogeneously distributed, and collagen distribution was significantly increased by UVB irradiation, as indicated by collagen distribution ratios of the UVB and control groups ($P = 0.0002$, Figure 2D). Collagen distribution tended to decrease in the milk phospholipid-administered groups (ML, MM, and MH), but the difference was not significant.

Effect of Milk Phospholipids on Skin Wrinkle Formation

To evaluate the inhibitory effect of milk phospholipids on UVB irradiation-induced wrinkle formation, the wrinkle area, mean wrinkle length, and mean wrinkle depth, which are wrinkle-forming factors, were measured (Figure 3). The UVB irradiation caused significant differences in the wrinkle area, mean wrinkle length, and mean wrinkle depth, as indicated by data of the UVB and control groups ($P = 0.0001$, $P = 0.0002$, and $P = 0.0003$). Oral administration of medium-dose and high-dose milk phospholipids suppressed UVB irradiation-induced wrinkle formation (area, $P = 0.0002$

Figure 2. Effects of milk phospholipids on epidermal thickness (A, B) and relative collagen distribution (C, D). CON = oral administration of saline with no UVB irradiation; UVB = oral administration of saline with UVB irradiation; ML, MM, and MH = oral administration of low-dose (50 mg/kg of BW), medium dose (100 mg/kg of BW), and high-dose (150 mg/kg of BW) milk phospholipids, respectively, with UVB irradiation. Data are expressed as means $\pm$ standard error ($n = 6$). *** $P < 0.001$ for control vs. UVB group without milk phospholipids group using Tukey's multiple comparison test.

and $P = 0.0001$; length, $P = 0.0356$ and $P = 0.0002$; depth, $P = 0.0002$ and $P = 0.0002$).

Effect of Milk Phospholipids on MMP and TIMP Expression

The MMP are representative enzymes that degrade extracellular matrix proteins, especially collagen, whereas TIMP inhibit the action of collagen-degrading enzymes (Fisher et al., 1996; Vincenti and Brinckerhoff, 2002). The UVB irradiation increased the expression of genes encoding MMP, promoting wrinkle formation (Figure 4). The expression of *Mmp2*, *Mmp3*, *Mmp9*, and *Mmp13* in the UVB group was significantly increased compared with that in the control group because of UVB irradiation (Figure 4, $P = 0.0424$, $P = 0.0005$, $P = 0.0007$, $P = 0.0001$, respectively). In contrast, the expression of *Timp1* and *Timp2* was lower in the UVB

group than in the control group. Expression of *Timp1* in the UVB group was significantly decreased compared with that in the control group ($P = 0.0001$). The UVB irradiation-induced increase in the expression of genes encoding MMP was decreased in the ML, MM, and MH groups compared with the UVB group. In addition, *Timp2* expression in the MM and MH groups were higher than that in the UVB group, but there were no significant differences. Oral administration of milk phospholipids was more effective in regulating the expression of genes encoding MMP than genes expressing TIMP.

Effect of Milk Phospholipids on Proinflammatory Cytokine Production

The mRNA expression of the proinflammatory cytokines *Il4*, *Il6*, *Il10*, and *Tnfa* after UVB irradiation were

Figure 3. Effects of milk phospholipids on skin wrinkle area (A), mean wrinkle length (B), and mean wrinkle depth (C). Photographs and replicas of the mouse dorsal skin (D). CON = oral administration of saline with no UVB irradiation; UVB = oral administration of saline with UVB irradiation; ML, MM, and MH = oral administration of low-dose (50 mg/kg of BW), medium dose (100 mg/kg of BW), and high-dose (150 mg/kg of BW) milk phospholipids, respectively, with UVB irradiation. Data are expressed as means $\pm$ standard error ($n = 6$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ for control vs. UVB group without milk phospholipids group using Tukey's multiple comparison test.

significantly increased in the UVB group compared with the control group (Figure 5). The expression of proinflammatory cytokines in the ML, MM, and MH groups was similar to that of the control group, demonstrating the suppression of UVB irradiation-induced increase in proinflammatory cytokine expression. The expression of *Il10*, which encodes an immunosuppressive cytokine, was significantly decreased in the ML, MM, and MH groups compared with the UVB group in a dose-dependent manner (Figure 5C). The expression and function of cytokines that mediate the inflammatory response induced by UVB rays were restored to normal levels by milk phospholipids.

Effect of Milk Phospholipids on Ceramide and S1P Contents in Skin Tissues

When irradiated with UVB, the ceramide content in the UVB group was lower than that in the control group, but the difference was not significant (Figure 6A). The UVB irradiation-induced decrease in the ce-

ramide content was reversed by milk phospholipids, but the difference was not significant compared with that in the UVB group.

Figure 6B shows that the S1P content in the UVB group was significantly increased compared with that in the control group ($P = 0.0247$). Furthermore, milk phospholipids considerably decreased the S1P content compared with the UVB group.

Effect of Milk Phospholipids on NF- κ B Activation

Figure 7 shows protein expression of NF- κ B and I κ B [I κ B α and phosphorylated (p)-I κ B α]. The protein levels of NF- κ B and p-I κ B α were significantly increased by UVB irradiation, as demonstrated by data of the UVB and control groups ($P = 0.0001$ and $P = 0.0074$, respectively). The increase in protein level of NF- κ B by UVB irradiation was significantly decreased by milk phospholipids (ML, MM, and MH) in a dose-dependent manner ($P = 0.0001$, $P = 0.0001$, and $P = 0.0001$, respectively). The increase in protein level of p-I κ B α by

UVB irradiation was also significantly decreased by milk phospholipids (ML, MM, and MH) in a dose-dependent manner ($P = 0.0410$, $P = 0.0070$, and $P = 0.0001$, respectively). However, the effect of milk phospholipids on the I κ B α protein level showed the opposite trend to the change of the NF- κ B and p-I κ B α protein levels. Milk phospholipids reduced the expression of NF- κ B, a photoaging inducer of reactive oxygen species (ROS) generated by UVB irradiation, at the protein level.

DISCUSSION

The milk phospholipids used in this study were polar lipids extracted from whey powder and were composed of phospholipids, mainly phosphatidylcholine and phosphatidylethanolamine, and sphingolipids, mainly sphingomyelin; thus, they were rich in ceramide precursors, which are necessary for moisturizing the skin (Lee et al., 2020). Milk fat is a dietary source of sphingomyelin, and dietary sphingomyelin raises the levels of ceramide in the body. Ceramide plays a role in maintaining the moisture supply of the epidermis and skin barrier function (Parodi, 1997; Graves et al., 2007).

Ceramide accounts for 40 to 60% of the intercellular lipids (Lodén, 2012) and is known to play a crucial

role in skin barrier function and skin moisturization by forming a lamellar structure to support the space between keratinocytes (Boer et al., 2016). Additionally, ceramide levels decrease with age, and the amount also decreases in patients with dry skin or atopic dermatitis (Simpson et al., 2013). Ceramide is recognized as a crucial component in maintaining skin homeostasis.

Skin photoaging caused by UVB exposure over a long period of time causes histological changes, such as irregular changes in keratinocytes, an increase in the number of fibroblasts and inflammatory cells, and an accelerated increase in cross-linked collagen fiber levels in the dermis (Robert et al., 2009). Furthermore, exposure of the skin to UVB also causes ROS production (Ansary et al., 2021; Geng et al., 2021). Exposure to UVB promotes continuous ROS generation in keratinocytes, and ROS are rapidly removed by enzymatic and nonenzymatic methods. However, ROS overgeneration causes skin damage. Free radicals generated from ROS cause deterioration of cell structure and function, which ultimately result in abnormal gene expression in cells. UVB-mediated ROS generation affects mitogen-activated protein kinase signaling. Mitogen-activated protein kinase is known to activate the transcription factor NF- κ B. Nuclear factor- κ B, which mediates the

Figure 4. Effects of milk phospholipids on mRNA expression of *Mmp2* (A), *Mmp3* (B), *Mmp9* (C), *Mmp13* (D), *Timp1* (E), and *Timp2* (F). CON = oral administration of saline with no UVB irradiation; UVB = oral administration of saline with UVB irradiation; ML, MM, and MH = oral administration of low-dose (50 mg/kg of BW), medium dose (100 mg/kg of BW), and high-dose (150 mg/kg of BW) milk phospholipids, respectively, with UVB irradiation. Data are expressed as means $\pm$ standard error ($n = 6$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ for control vs. UVB group without milk phospholipids group using the Tukey's multiple comparison test; ns = not significant.

Figure 5. Effects of milk phospholipids on mRNA expression of *Il4* (A), *Il6* (B), *Il10* (C), and *Tnfa* (D). CON = oral administration of saline with no UVB irradiation; UVB = oral administration of saline with UVB irradiation; ML, MM, and MH = oral administration of low-dose (50 mg/kg of BW), medium dose (100 mg/kg of BW), and high-dose (150 mg/kg of BW) milk phospholipids, respectively, with UVB irradiation. Data are expressed as means $\pm$ standard error ($n = 6$). ** $P < 0.01$ and *** $P < 0.001$ vs. UVB group without milk phospholipids group using the Tukey's multiple comparison test.

inflammatory response, binds to an inhibitor of NF- κ B ($\text{I}\kappa\text{B}$) and is present in the cytoplasm in an inactive form (Yamazaki et al., 2001; Garg and Aggarwal, 2002). When cells are stimulated, such as UV light, $\text{I}\kappa\text{B}$ kinase phosphorylates the inhibitory $\text{I}\kappa\text{B}\alpha$ protein. This phosphorylation results in the dissociation of $\text{I}\kappa\text{B}\alpha$ from NF- κ B. The liberated NF- κ B enters the nucleus and activates the transcription of genes involved in immune and inflammatory responses (Yamazaki et al., 2001; Garg and Aggarwal, 2002).

Keratinocytes that are stimulated by ROS secrete proinflammatory cytokines to stimulate dermal fibroblasts by promoting DNA binding of NF- κ B and AP-1, thereby increasing the expression of MMP. On the other hand, MMP attenuates the synthesis of type-1 procollagen, which in turn inhibits collagen biosynthesis and promotes degradation, forming skin wrinkles and reducing elasticity (Gao et al., 2018; Jin et al., 2021).

Oral administration of milk phospholipids significantly decreased the mRNA expression of *Mmp2*, *Mmp3*, *Mmp13*, and the proinflammatory cytokines *Il4*, *Il6*, *Il10*, and *Tnfa* compared with that of the UVB group.

The decrease in the expression of proinflammatory cytokines suppressed wrinkle formation caused by photoaging. Although there was no significant difference in collagen distribution, there was a considerable decrease when compared with the UVB group. As such, oral administration of milk phospholipids not only decreased the expression of proinflammatory cytokines but also decreased the expression of MMP, thereby inhibiting collagen degradation and suppressing wrinkle formation. Moreover, milk phospholipids exhibited inhibitory effect on UVB irradiation-induced decrease in the skin moisture content and increase in moisture evaporation.

Skin hydration is closely related to the moisture content of the stratum corneum, which is the outermost layer of the epidermis. The stratum corneum has an organic complex structure of proteins and lipids that forms the skin barrier; a decrease in the moisture content of the stratum corneum results in dryness of the skin and abnormalities in skin barrier function (Nisbet, 2018). Furthermore, the main role of ceramide in the stratum corneum is protecting the epidermal barrier, and a decrease in the ceramide content in the stratum

Figure 6. Effects of milk phospholipids on ceramide (A) and sphingosine-1-phosphate (B) contents. CON = oral administration of saline with no UVB irradiation; UVB = oral administration of saline with UVB irradiation; ML, MM, and MH = oral administration of low-dose (50 mg/kg of BW), medium dose (100 mg/kg of BW), and high-dose (150 mg/kg of BW) milk phospholipids, respectively, with UVB irradiation. Data are expressed as means $\pm$ standard error ($n = 6$). * $P < 0.05$ vs. UVB group without milk phospholipids group using the Tukey's multiple comparison test; ns = not significant differences between groups.

corneum weakens the skin barrier function and acts as a cause of increased water loss between the skin. Milk ceramide used in this experiment was a raw material rich in phospholipids, and sphingomyelin accounts for 20 to 25% of milk ceramide (Lee et al., 2020). Sphingomyelin is the main component of mammalian cell membranes and a basic component of the lamellar structure responsible for the barrier function of the skin epidermis. Dietary sphingolipids, such as sphingomyelin and glucosylceramide, reportedly improve the skin barrier function (Tsuji et al., 2006; Duan et al., 2012; Haruta-Ono et al., 2012a). The content of sphingomyelin, a ceramide precursor, is higher in milk phospholipids than in phospholipids extracted from other foods. Sphingoids

are a structural component of sphingomyelin. Their absorption rate based on their source was evaluated in Caco-2 cells, and it was found that the absorption rate of animal-derived sphingoids was higher than that of plant-derived sphingoids. Therefore, sphingomyelin and glucosylceramide, which are sphingolipids, may be effective in strengthening the barrier function of the skin epidermis (Potočki, 2016; Morifuji, 2019).

Ceramides are an important component of the skin barrier and are involved in the prevention of TEWL. They are also important biological metabolites that regulate cellular processes, such as proliferation, differentiation, and apoptosis (Robert et al., 2009; Simpson et al., 2013). Sphingosine-1-phosphate is a sphingolipid

Figure 7. Effects of milk phospholipids on protein expression of IκBα (A), phosphorylated (p)-IκBα (B), and nuclear factor (NF)-κB (C). CON = oral administration of saline with no UVB irradiation; UVB = oral administration of saline with UVB irradiation; ML, MM, and MH = oral administration of low-dose (50 mg/kg of BW), medium dose (100 mg/kg of BW), and high-dose (150 mg/kg of BW) milk phospholipids, respectively, with UVB irradiation. Data are expressed as means $\pm$ standard error ($n = 6$). ** $P < 0.01$ and *** $P < 0.001$ vs. UVB group using the Tukey's multiple comparison test.

with a phosphate group attached to position 1 and is produced from ceramide by ceramidase and sphingosine kinase. It has a special function in epidermal cells and is involved in keratinocyte proliferation, apoptosis, differentiation, and migration. In keratinocytes, S1P acts as an immune response modulator by activating the NF- κ B pathway to increase the production of cathelicidin antimicrobial peptides as effectors of the innate immune response (Park et al., 2013; Jeong et al., 2015). An increase in the dose of milk phospholipids decreased the S1P content, resulting in decreased *NF- κ B* mRNA and protein expression. Interestingly, NF- κ B is involved in the inflammatory response through various pathways. Therefore, a reduction in *NF- κ B* expression may improve photoaging by reducing the production of proinflammatory cytokines.

CONCLUSIONS

Phospholipids are representative lipids constituting the skin, and milk phospholipids, with a high phospholipid content, may be used as dietary supplements for skin health. In this study, milk phospholipids were found to suppress the expression of MMP and proinflammatory cytokines through the downregulation of NF- κ B and p-I κ B α protein expression, demonstrating its photoprotective effect against UVB irradiation-induced skin damage in a hairless mouse model. In addition, further studies on the effect of milk phospholipids on ROS are needed in the future.

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