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Soy milk suppresses cholesterol-induced inflammatory gene expression and improves the fatty acid profile in the skin of SD rats

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ABSTRACT

Recently, an elevation in skin cholesterol level has been implicated in skin inflammation. Given the potential therapeutic effects of soy on low grade inflammatory diseases, we hypothesized that a CHOL diet could promote an inflammatory response in skin and that soy milk (SM) or fermented soy milk (F.SM) could prevent this cholesterol-induced skin inflammation. To test this hypothesis, freeze-dried SM or F.SM was provided as a protein replacement for 20% of the casein in the diets of Sprague–Dawley (SD) rats. The animals were divided into the following groups: (1) control group (CTRL), AIN76A diet without cholesterol, (2) high cholesterol (CHOL) group, AIN76A with 1% (w/w) cholesterol, (3) SM group, CHOL diet with freeze-dried SM, and (4) F.SM group, CHOL diet with F.SM. In the CHOL group, the expression levels of pro-inflammatory genes, including IL-1β, IL-1α, iNOS, and COX-2, were elevated. In comparison, the SM and F.SM groups displayed the lowered expression of IL-1β, COX-2, F4/80, and Cd68, an increase of a *n*-3/*n*-6 ratio, and a reduction in the estimated desaturase activities of delta 5 desaturase (D5D) and steaoryl CoA desaturase (SCD-1). In particular, F.SM significantly increased the proportion of dihomo-γ-linolenic acid (DGLA) in skin fatty acid (FA) composition compared with the CHOL group. Here we present evidence that SM or F.SM could alleviate the inflammatory response in the skin that is triggered by excess dietary cholesterol by reducing the expression of pro-inflammatory genes. This response could be partly associated with a decreased in macrophages in skin and/or by modulation of the skin's FA composition.

1. Introduction

Skin acts as a barrier to protect against water loss and adverse environmental agents [1]. The proper maintenance of the skin layer requires balanced proliferation and differentiation of skin cells through the actions of the epidermis and the dermis [1]. Skin lipid homeostasis is important in the maintenance of the normal barrier function of skin. The outermost epidermis is surrounded by a lipid-rich extracellular matrix where ceramides, cholesterol, and free FAs are abundant [2]. When the tissue barrier is disrupted, the outermost epidermis increases the synthesis of cholesterol and FAs to provide new cell membrane lipids [3]. Though the precise contribution of dietary FAs to the skin lipid pool is not known, the dietary supplementation of *n*-3 polyunsaturated fatty acids (PUFA) was proven to improve the condition of skin disorders [4].

Skin inflammation occurs in the early stages of the normal immune response and provides protection against various foreign molecules. Abnormal regulation of this process can lead to acute and chronic inflammatory diseases such as psoriasis and atopic dermatitis, involving the induction of proinflammatory cytokines and eisocanoids [5,6]. Recently, skin cholesterol deposition was shown to be closely related to skin inflammation [7]. Genetically-engineered double knockout mice of the LDLR and ApoA1 genes showed a significant accumulation of cholesterol in their skin, which exhibited severe lesions as well as the extensive infiltration of macrophages [7]. The role of cholesterol in inflammation was also suggested by the reversible inflammatory response in mice liver upon the removal of cholesterol and cholate from an atherogenic diet [8]. Therefore excessive cholesterol has been implicated as playing a causative role in the induction of inflammation [9].

Soy is widely consumed worldwide. Its beneficial effects on various low-grade inflammation-related diseases are elicited by its high content of *n*-3 FAs and bioactive components including isoflavones and plant sterol [10–13]. Soy and/or soy constituents lowered the risk of allergic reactions and ameliorated dysregulated immune responses [12,13]. The substitution of soy protein for casein dramatically improved inflammation-related markers [14]. The bioactivity of soy isoflavones can be enhanced through fermentation by converting them into aglycones which are easier for intestinal absorption [15]. In particular, equol, a metabolite

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generated by the intestinal gut bacteria, shows a stronger potency than its precursor daidzein [16]. Thus, fermentation with probiotic bacteria can be a potential means to increase the bioavailability of soy constituents and increase the bio-effectiveness of soy products.

We hypothesized that a CHOL diet could initiate the inflammatory response in skin and that SM or F.SM might lessen this inflammation. To this end, we investigated the expression levels of proinflammatory and macrophage marker genes and the FA composition in the skin of rats fed CHOL diets with or without SM or F.SM.

2. Materials and methods

2.1. Animals

Five-week old SD rats were obtained from Koatech (Pyungtek, Korea). After a one-week adaptation period, the animals were divided into four groups containing eight animals and each fed an experimental diet *ad libitum* for six weeks. The CTRL group was fed a CTRL diet that is AIN76A diet which contains 50% sucrose by weight. The CHOL group was given a CHOL diet, which is an AIN76A diet supplemented with 1% (w/w) cholesterol. The SM group was put on a SM diet, which is a CHOL diet with 20% of its casein and 40% of its corn oil substituted with freeze-dried SM. The F.SM group was fed on a CHOL diet with freeze-dried F.SM replacing 20% of the casein and 40% of the corn oil. At the end of the experimental period, rats underwent a 12-h fast prior to sacrifice. After the animals were euthanized with diethyl ether, their skin tissue was excised, rapidly frozen with liquid nitrogen, and then stored in the freezer at −80 °C. All the experimental procedures were reviewed and approved by the Committee on Animal Experimentation and Ethics of Yonsei University.

2.2. Preparation of diets containing freeze-dried SM and F.SM

SM was provided from Yonsei Milk Corporation (Asan, Korea). SM was mainly composed of the following nutrients: 25 g carbohydrate/L, 35 g protein/L, 17.5 g lipids/L, 10 g dietary fiber/L, and 750 mg sodium/L. To prepare the F.SM, 1 L of SM was mixed with 20 g of galactooligosaccharide (Samyang, Seoul, Korea) and then was fermented using 0.1% (w/v) *Lactobacillus plantarum* (CellBiotech, Seoul, Korea) at 25 °C until the pH reached 5.1. The SM and the F.SM were filtered and the filtrates were dehydrated with a freeze dryer (ILShinBioBase, Dongduchen, Korea).

2.3. RNA extraction and quantitative RT-PCR

Skin tissue samples were homogenized in Trizol (Qiagen, CA) and the total RNA was extracted according to the manufacturer's protocol. cDNA was synthesized from 1 μg of RNA using random hexamers and ImprompII reverse transcriptase (Promega, WI). Expression levels of the genes were measured using EvaGreen qPCR mix plus (Solis BioDyne, Estonia) and CFX96 Touch Real-time PCR Detection System (Biorad, CA). The relative mRNA level was calculated using 18SrRNA as a control and the difference in C_t values among the animals groups was expressed as the fold change.

2.4. Western blot analysis

Skin tissues were homogenized and lysed in RIPA buffer containing protease inhibitors (Sigma–Aldrich, MO). After centrifugation, the skin tissue lysates were boiled in Laemmli buffer. Equal amounts of protein lysates were loaded onto a SDS–polyacrylamide gel. Rabbit anti-COX-2, rabbit anti-IL-1β, and mouse anti-GAPDH (Santa Cruz Biotech., CA) were used to detect the corresponding proteins. Peroxidase-conjugated secondary antibodies (Santa Cruz Biotech., CA) were used to detect the proteins.

2.5. Fatty acids and cholesterol analysis using gas chromatography

Total lipids from the animal skins were extracted according to a modified Folch method [17]. Hexane and 14% boron trifluoride in methanol (Sigma–Aldrich, MO) were used for lipid extraction and methylation. Methylated samples were separated by gas chromatography (Agilent, CA) using capillary columns, with an Omegawax 250 column (Supelco, PA) used for fatty acid methyl esters and an Ultra 1 Methyl Siloxan capillary column (Agilent, CA) used for cholesterol methyl esters. Individual FAs and cholesterols were identified based on their peak retention times compared to those of known standards (PUFA-2, DGLA, and cholesterol, Sigma–Aldrich, MO). Skin FAs were expressed as the percentage of total FAs. The indices of desaturase activities were calculated based on the ratio of product to precursor FAs. SCD-1 activity index was defined by C16:1*n*-7/C16:0. The D6D activity index was C20:3*n*-6/C18:2 *n*-6. D5D activity index was C20:4*n*-6/C20:3*n*-6 [18].

2.6. Statistical analyses

SPSS software was used for statistical analysis (SPSS Ins., IL). The results were presented as means ± SE. The statistical significance was tested using one-way analysis of variance (ANOVA) and Duncan's multiple range test, with $p < 0.05$ as the criterion of significance.

3. Results

3.1. Skin cholesterol accumulation in rats fed high cholesterol diets with and without soy milk substitution

Food intake was not significantly different among the CTRL, SM, and F.SM groups, but the CHOL group showed a lowered food intake compared with the other groups (Fig. 1A). On the other hand, the CHOL, SM, and F.SM groups all displayed a similar elevation in free cholesterol levels in the skin as compared to the CTRL group (Fig. 1B). These data showed that SM or F.SM did not significantly affect the accumulation of cholesterol induced by the CHOL diet in the skin when it was provided *ad libitum*.

3.2. SM and F.SM reduced the expression of IL-1β and COX-2 genes and macrophage marker genes in skin tissues

CHOL significantly elevated IL-1β gene expression in terms of both mRNA and protein levels compared to CTRL (Figs. 1C and 2D) ($p < 0.05$). SM and F.SM decreased IL-1β mRNA to levels comparable to that of CTRL (Fig. 1C). At the protein levels, F.SM significantly reduced while SM showed a tendency for reduction of IL-1β (Fig. 2D). Pro-IL-1β protein showed a similar expression trend to those of IL-1β (Fig. 2C and D). When compared to CTRL, CHOL increased COX-2 mRNA levels but elicited an increased tendency in COX-2 protein levels. CHOL-elevated expression of COX-2 gene were down-regulated by SM and F.SM (Figs. 1D and 2B) ($p < 0.05$). The IL-1α and iNOS mRNA levels were increased by CHOL compared to CTRL ($p < 0.05$); SM and F.SM did not affect the expression of these genes (Fig. 1E and F). The abundance of TNF-α and IL-6 mRNA did not significantly differ among all tested groups (Fig. 1G and H). CHOL did not affect the transcript levels of macrophage marker genes, including F4/80 [19] and Cd68 [20], compared to CTRL (Fig. 2E and F). However, SM and F.SM reduced F4/80 mRNA levels but did not significantly affect Cd68 mRNA levels in the skin as compared to CTRL and CHOL (Fig. 2E and F). These

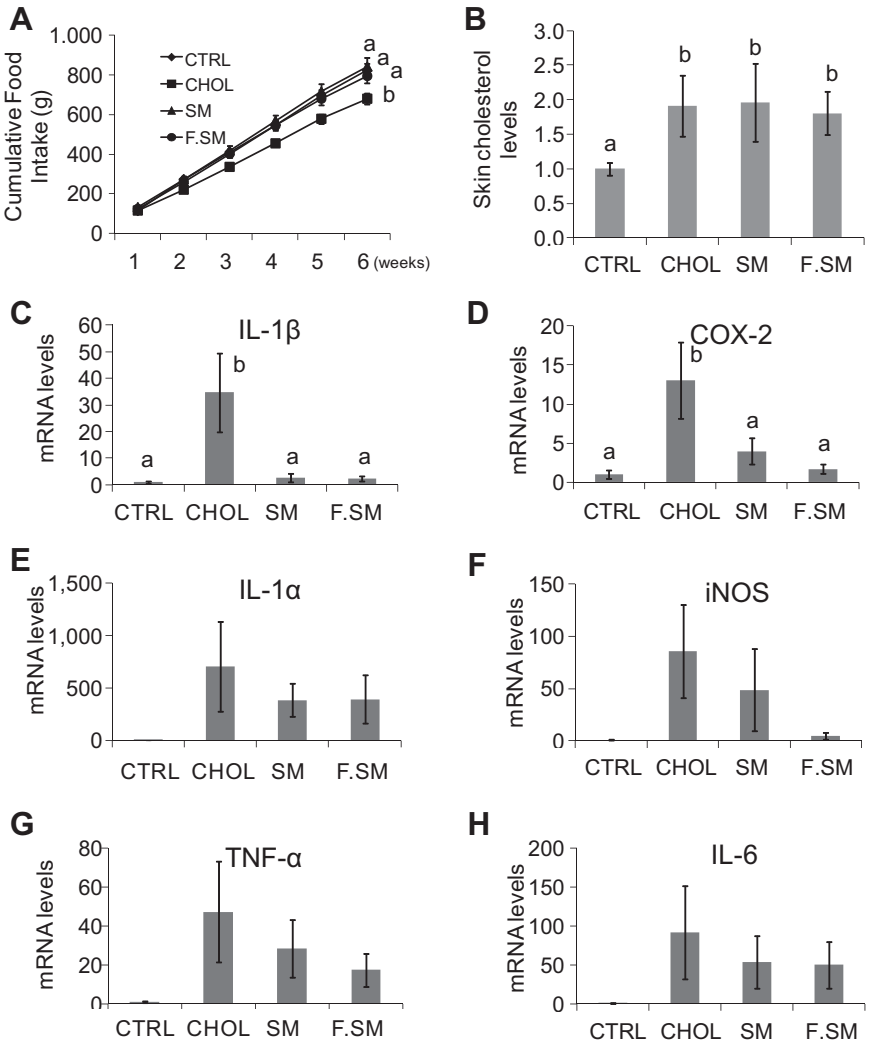

Fig. 1. Food intake, skin cholesterol content, and mRNA levels of proinflammatory genes among rat groups. Cumulative food intake levels (A), skin cholesterol levels (B), skin mRNA levels of IL-1β (C), COX-2 (D), IL-1α (E), iNOS (F), TNF-α (G), and IL-6 (H) genes in rat groups. CTRL : a control group on CTRL diet, CHOL : a group on a CHOL diet, SM : a group on a SM diet, and F.SM : a group on a F.SM diet. The results were expressed as means ± SE. of six to eight animal tissues. Different letters (a,b) within groups represent significant differences ($p < 0.05$).

findings indicate that a CHOL diet unfavorably altered the expression of pro-inflammatory genes, possibly without accompanying macrophage infiltration. In addition the consumption of SM or F.SM alleviated the high cholesterol-induced expression of IL-1β and COX-2 genes in the skin, which may be associated with reduced macrophages in the skin.

3.3. The effects of SM and F.SM on skin FA profile

CHOL, SM, and F.SM decreased the levels of palmitic acid (16:0) compared to CTRL ($p < 0.001$) (Table 1). CHOL increased palmitoleic acid levels (16:1*n*7) in comparison to CTRL ($p < 0.001$), but SM and F.SM decreased palmitoleic acid to a level similar to that of CTRL (Table 1). Both SM and F.SM raised the proportion of α-linolenic acid (18:3*n*3) and linoleic acid (18:2*n*6) compared to that of CTRL and CHOL ($p < 0.001$) and CTRL ($p < 0.01$), respectively (Table 1). A significant reduction in DGLA (20:3*n*6) level was induced by CHOL as compared to CTRL, SM, or F.SM, and was significantly reversed by F.SM ($p < 0.05$) (Table 1). On the other hand, CHOL lowered the ratio of *n*-3/*n*-6 compared to CTRL, but SM or F.SM raised the ratio of *n*-3/*n*-6 to a level comparable to that of CTRL ($p < 0.001$) (Fig. 3A). Together, these results indicated that a CHOL diet altered the FA composition in skin. SM and F.SM also changed skin FA composition in favor of a high *n*-3/*n*-6 ratio and an increase in DGLA. CHOL group demonstrated higher activity indices for SCD1 and D5D when compared to the CTRL, SM, and F.SM groups ($p < 0.05$) (Fig. 3B and C), suggesting that SM and F.SM may recover CHOL-reduced activity indices of SCD-1 and D5D.

4. Discussion

Skin is one of the preferred tissues for the accumulation of cholesterol, which was demonstrated by double knockout mouse models (ABCA1−/−, LDLR−/− and ABCA1−/−, apoE1−/−) [21,22]. Even LDLR−/−, apoA1−/− mice displayed that skin cholesterol accumulated at much higher levels than plasma cholesterol [7]. In addition direct evidence of the role of skin cholesterol on skin inflammation was also provided in this mouse model [7]. Skin cholesterol has previously been well-implicated in low grade inflammation associated with cardiovascular diseases [23]. Our study provides additional evidence to support the hypothesis that skin's cholesterol content, which can be increased by a CHOL diet, is associated with the expression of inflammatory genes including COX-2 and IL-1β in skin. In some cases, we observed skin lesions in the

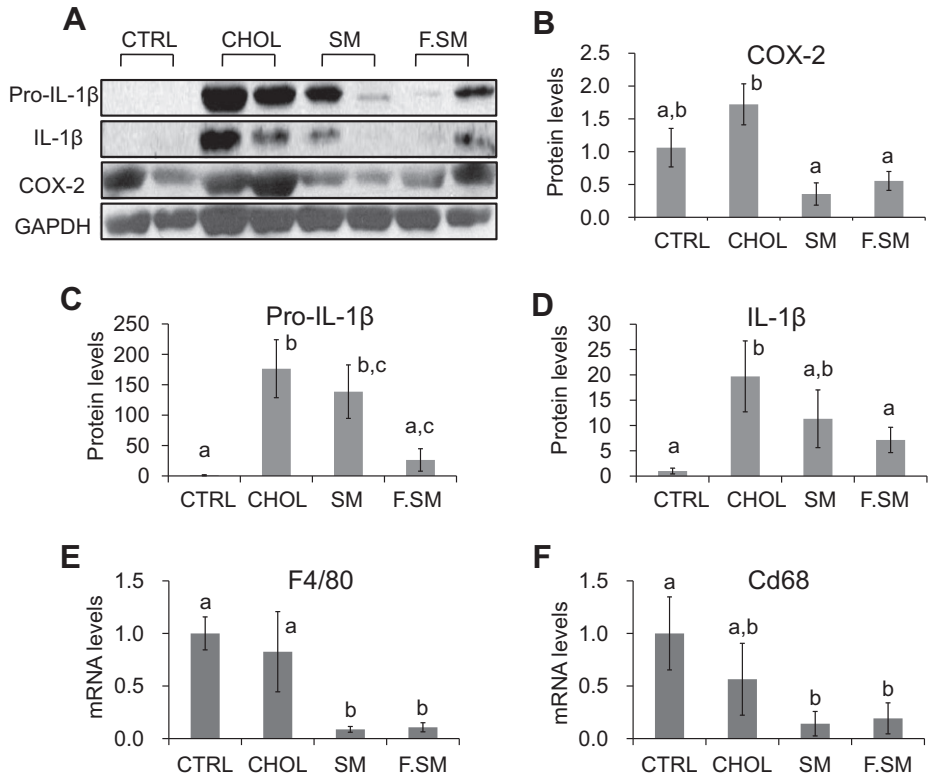

Fig. 2. The skin protein levels of IL-1β and COX-2 and transcript levels of F4/80 and Cd68 in rat groups. (A) Pro-IL-1β, IL-1β, COX-2, and GAPDH protein levels (representative images are shown). The densities of corresponding bands were normalized using GAPDH: COX-2 (B), Pro-IL-1β (C), and IL-1β (D). Skin mRNA levels of F4/80 (E) and Cd68 (F). The results were expressed as means ± SE. of eight animal tissues. Different letters (a–c) within groups represent significant differences ($p < 0.05$).

animals fed a CHOL diet, something we did not observe in the rats fed SM or F.SM diet. On the other hand, the cholesterol-induced expression of inflammatory genes does not appear to be related to enhanced macrophage infiltration based on the lack of change in transcript levels of macrophage marker genes, such as F4/80 and Cd68, in the CHOL group as compared to the CTRL group. This observation is inconsistent with a previous report showing increased macrophage infiltration in the skin of LDLR−/−, ApoA1−/− double knockout mice [7]. This discrepancy could be due to a different degree of skin cholesterol accumulation caused by different conditions such as genetic defects or high cholesterol diets.

To investigate the possible anti-inflammatory effects of soy products in cholesterol-induced skin inflammation, we utilized SM and F.SM made from whole soy. Previously soy as a whole was demonstrated to be more effective on inflammation than soy isolates [24]. This idea was further supported by a study using whole soy nut compared with isolated soy protein [3]. We further fermented SM using *L. plantarum* to increase the proportion of bioavailable soy isoflavones by facilitating the hydrolysis of isoflavone glucosides into aglycones. Our unpublished data indicated that the fermentation transformed part of the soy isoflavones into simplified forms lacking modifications such as acetylation and malonylation, as well as increased the levels of genistein and daidzein.

The modulation of skin cholesterol deposition is not likely to play a significant role in the anti-inflammatory mechanisms of SM or F.SM because skin cholesterol levels were not different among the CHOL, SM, or F.SM groups. Instead, we observed that soy milk decreased the cholesterol-induced expression of proinflammatory genes such as IL-1β and COX-2 regardless of whether it was fermented. IL-1 plays a crucial role in inflammation and skin irritation [5]. The activation of IL-1 was detected in hyperproliferative inflammatory skin lesions [25,26]. Furthermore, the upregulation of IL-1β by dysregulated metabolism such as in cases of hyperglycemia indicates that IL-1β may mediate the activation of the immune response to metabolic stress [27]. Even if soy milk was effective in lowering IL-1β expression, F.SM was more effective in the reduction of IL-1β protein and the production of DGLA than SM, which might be attributable to the differential isoflavone profiles caused by fermentation. In comparison to CHOL, SM and F.SM also downregulated COX-2, which is an inducible enzyme that

Table 1
Fatty acid composition in the skin of rats fed on a high cholesterol diet with SM or F.SM. The results were expressed as means ± SE. Different letters (a,b) within groups represent significant differences.

	CTRL ($n = 8$)	CHOL ($n = 8$)	SM ($n = 8$)	F.SM ($n = 8$)	p
Saturated fatty acids					
14:0	2.12 ± 0.25	1.86 ± 0.06	1.50 ± 0.17	1.57 ± 0.16	NS
16:0	39.45 ± 2.47[a]	30.04 ± 2.32[b]	25.73 ± 2.30[b]	29.13 ± 1.05[b]	<0.01
18:0	11.49 ± 2.88	7.58 ± 1.10	6.61 ± 0.25	9.01 ± 1.29	NS
Monounsaturated fatty acids					
16:1*n*7	3.18 ± 0.62[a]	5.16 ± 0.51[b]	2.80 ± 0.21[a]	2.41 ± 0.37[a]	<0.01
18:1*n*7	3.34 ± 0.39	3.80 ± 0.19	3.66 ± 0.16	3.74 ± 0.21	NS
18:1*n*9	20.20 ± 3.21	22.18 ± 0.98	21.99 ± 1.36	22.09 ± 2.29	NS
20:1*n*9	1.71 ± 0.46[a]	0.37 ± 0.11[b]	0.99 ± 0.20[a,b]	0.99 ± 0.18[a,b]	<0.05
n3-Polyunsaturated fatty acids					
18:3*n*3	0.28 ± 0.03[a]	0.21 ± 0.02[a]	0.94 ± 0.11[b]	0.87 ± 0.05[b]	<0.01
20:5*n*3	0.11 ± 0.06	0.07 ± 0.02	0.18 ± 0.05	0.09 ± 0.06	NS
22:6*n*3	0.14 ± 0.06	0.07 ± 0.02	0.18 ± 0.05	0.09 ± 0.06	NS
n6-Polyunsaturated fatty acids					
18:2*n*6	11.36 ± 2.68[a]	17.43 ± 1.48[a,b]	20.66 ± 1.47[b]	21.35 ± 2.16[b]	<0.01
18:3*n*6	4.24 ± 2.34	7.41 ± 2.31	12.06 ± 3.35	4.20 ± 2.62	NS
20:3*n*6	1.22 ± 0.18[a,b]	0.77 ± 0.32[a]	1.08 ± 0.12[a,b]	1.96 ± 0.40[b]	<0.05
20:4*n*6	0.98 ± 0.37	2.32 ± 0.63	1.04 ± 0.24	1.93 ± 1.19	NS
22:4*n*6	0.18 ± 0.07	0.43 ± 0.10	0.30 ± 0.06	0.58 ± 0.15	NS

Mean ± SD.
Expressed as percentage of total fatty acids.

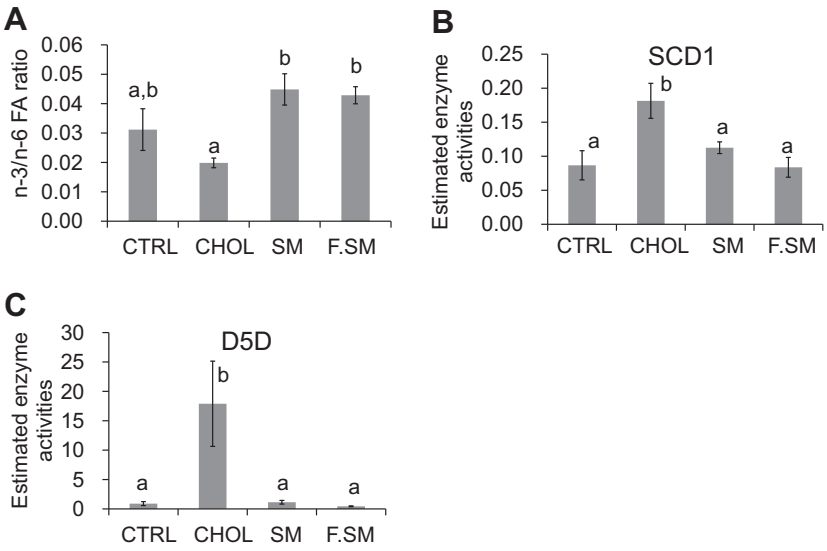

Fig. 3. Skin FA analysis from rats provided with SM or F.SM. The *n*-3:*n*-6 FA ratio (A), the estimated index of enzyme activity of SCD1 (B), and D5D (C) in the skin tissue of rat groups. The results were expressed as means ± SE. of eight animal tissues. Different letters (a,b) within groups represent significant differences ($p < 0.05$).

converts arachidonic acid (AA, 20:4 *n*6) to eicosanoids [28], and thereby possibly lowered the production of proinflammatory mediators. The levels of AA, a COX-2 substrate, could also influence inflammation risk [29]. LDLR −/− and apoA1−/− mice showed elevated levels of AA along with inflammation in their skin [7]. We did not observed significant changes in AA levels in the skin of the CHOL group compared to the CTRL, SM and F.SM groups. Thus, at least in our study, the possible effects of soy milk in lowering levels of proinflammatory mediators appear to be mediated through a decrease in the expression of COX-2. Considering that SM and F.SM decreased expression of the macrophage marker gene F4/60 compared to CHOL, it is possible that the reduction of macrophage infiltration into the skin by SM or F.SM partly contributes to the reduction of proinflammatory gene expression.

Interestingly, we observed an increase in the levels of DGLA as well as a significant reduction of the estimated activity index of D5D in the SM and F.SM groups compared to the CHOL group. DGLA competes with AA for the action of COX-2 and reduces the production of AA-derived inflammatory eicosanoids [30]. The SM-induced reduction in the estimated D5D activity might have contributed to an accumulation of the precursor DGLA. The accumulated DGLA in SM and F.SM could act as an competitive inhibitor of AA to COX-2 and prevent the synthesis of proinflammatory mediators. Similarly, a marked increase in DGLA was shown to be associated with a reduction of PGE_2, an inflammatory metabolite in a study of sesame oil [30]. DGLA detected at higher levels in F.SM than SM, could indicate that F.SM exert a stronger anti-inflammatory effect than SM via elevation in DGLA. On the other hand, the estimated activity of SCD-1, which has been positively associated with C-reactive peptide, a low-grade inflammatory marker in humans [31], was elevated by high cholesterol but reversed in the SM and F.SM groups, providing another indicator of the anti-inflammatory actions of SM and F.SM.

Soy contains a high proportion of *n*-3 FAs. However, the *n*-3/*n*-6 ratio in the skin of animals from the SM or F.SM groups was not significantly higher than that of the CTRL group. Instead, a CHOL diet unexpectedly lowered the *n*-3/*n*-6 ratio in the skin compared to the CTRL group, which was first proven in our study. This cholesterol-driven reduction of the *n*-3/*n*-6 ratio was reverted by SM or F.SM. This effect is important because *n*-3 FAs are intimately related to inflammation and repress the production of proinflammatory cytokines [29,32]. People who consumed large quantities of *n*-3 FAs showed low levels of proinflammatory markers including IL-6 and TNF-α and high levels of anti-inflammatory markers such as IL-10 and TGF-β [33]. In addition, a high proportion of *n*-3 FAs was associated with a decreased risk of atopy [34]. Therefore, the high *n*-3 FA contents of SM or F.SM could in part mediate their anti-inflammatory effects in skin by reversing the cholesterol-lowered *n*-3/*n*-6 ratio.

In summary, our data provides novel evidence to support the hypothesis that dietary cholesterol may be a risk factor for skin inflammation and that the consumption of SM or F.SM may prevent dietary cholesterol-induced skin inflammation when provided as a substitution of 20% of total animal protein. Although its effects are not fully understood, we demonstrated that the anti-inflammatory actions of soy milk could involve the down-regulation of the expression of pro-inflammatory genes such as COX-2 and IL-1β and the increased levels of DGLA and the *n*-3/*n*-6 ratio in the skin. Finally, we propose that whole soy provided as SM or F.SM may be an effective measure to prevent skin inflammation exacerbated by a CHOL diet. Further investigation are warranted to uncover the therapeutic components of SM and F.SM and to elucidate the roles and the mechanism of actions of individual soy components in skin inflammation.

5. Conflict of interest

None.

Acknowledgments

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