Effect of *Canavalia gladiata* Extract Fermented with *Aspergillus oryzae* on the Development of Atopic Dermatitis in NC/Nga Mice

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Key Words

Canavalia gladiata · Atopic dermatitis · Fermentation · Immunomodulation · NC/Nga mice

Abstract

Canavalia gladiata has been used as a Chinese traditional folk medicine for its anti-inflammatory properties. However, the use of *C. gladiata* is limited because it contains antinutritional and allergy-causing proteins. We fermented *C. gladiata* with *Aspergillus oryzae* and investigated the effects of fermented *C. gladiata* (FCG) on the development of atopic dermatitis (AD) in mice. The mice were divided into five groups: untreated Balb/c mice; AD control (NC/Nga mice); FCGH (NC/Nga mice fed a dietary supplement of 300 mg/kg fermented *C. gladiata* water extract); FCG30 (NC/Nga mice fed a dietary supplement of 300 mg/kg of fermented *C. gladiata* 30% ethanol extract), and FCG80 (NC/Nga mice fed a dietary supplement of 300 mg/kg of fermented *C. gladiata* 80% ethanol extract). We found increases in the nonessential amino acids and essential amino acid in the FCG compared with the non-FCG. FCG attenuated macroscopic and histopathological changes in dorsal skin of mice when compared with the AD control group. The FCG30 and FCG80 groups, in particular, showed significant decreases in scratching episodes when

compared with the AD control group. FCG improved immune responses, including increases in IgE and histamine for AD, through attenuation of Th1/Th2 cytokine imbalance and the production of proinflammatory cytokines and chemokines. We suggest that FCG may have benefits for improvement of AD function by improving the balance of Th1/Th2 cytokines and by producing anti-inflammatory effects.

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Introduction

Atopic dermatitis (AD), also known as eczema, is a chronic inflammatory skin disease associated with genetic or environmental factors. AD is a chronically relapsing skin disorder characterized by the development of inflammatory skin lesions that range from itchy skin or xerosis to urticarial or chronic eczema [1, 2]. AD usually starts during early infancy or childhood, and can then resolve on its own without treatment. However, it can continue to persist or even emerge in adulthood [3].

AD is difficult to treat and development of treatment drugs is complicated because the principal cause and mechanism of AD pathogenesis remains unknown. This is because a complex mechanism of various body systems,

not a single system, plays a role in AD pathogenesis [4, 5]. Immune cells, immune tissue, and skin tissues all play crucial roles in AD pathogenesis [2, 6].

The epidermis of a patient with AD shows increased production of proinflammatory cytokines and chemokines, such as CCL17/thymus and activation-regulated chemokine (TARC) and CCL11 (eotaxin) following stimulation of keratinocytes [7, 8]. The initiation phase of AD is induced by IL-4 and IL-10, derived from activated Th2-type cells. The Th2-type cytokines, especially IL-4, are inducers of the B-cell isotype class, which triggers a switch from IgM to IgE. IgE binds to receptors for IgE (FcεRI) on the surface of mast cells, and these mast cells then induce the secretion of pharmacologically active molecules, such as histamine, which has a necessary pathogenic role in the development of AD pathogenesis. In addition, Th2-type cytokines can trigger the invasion of macrophages or eosinophils to the skin, which then produce IL-12 that can stimulate activation of Th1-type cells. These immunologic responses can be part of the immunopathogenesis that leads to chronic AD [2, 6, 9, 10].

Topical steroids, antihistamines, and immunomodulators have been used for the treatment of AD, but these drugs have only temporary efficacy and can have adverse side effects [3, 11]. For this reason, several studies have investigated natural plant therapies for the treatment of AD [12, 13]. Fermented natural plants are considered particularly good choices because fermentation breaks down many allergy-causing proteins [14, 15]. In this study, we investigated an extract from *Canavalia gladiata* that had been fermented with *Aspergillus oryzae* (FCG) for its immunomodulatory effects on the development of AD-like skin lesions in NC/Nga mice.

C. gladiata, known as sword bean, belongs to the legume family (Fabaceae) and is mainly distributed in tropical Asia and Africa. This plant has been used as a folk medicine for anti-inflammatory purposes in Chinese herbal medicine [16, 17]. However, the use of *C. gladiata* is limited because it contains anti-nutritional and allergy-causing proteins [18]. Therefore, we fermented *C. gladiata* with *A. oryzae* as a potential method for improving its efficacy in the treatment of AD.

Methods

Fermentation and Preparation of the Extracts

C. gladiata (Mu-an, Korea) was ground and fermented with 3% *A. oryzae* at $25 \pm 2^\circ\text{C}$ and $55 \pm 5\%$ at ambient humidity for 36 h and dried. FCG was extracted with 20 volumes of water at 100°C for 4 h and with 30% or 80% ethanol at 20°C for 12 h in a

reflux apparatus. Extracts were filtered, concentrated in vacuo, and dried in a lyophilizer. Yields of fermented *C. gladiata* water extract (FCGH), 30% ethanol extract (FCG30), and 80% ethanol extract (FCG80) were 10.2, 6.1, and 3.0%, respectively. Each extract was stored in a tight and light-protected container at -20°C until used.

Quantification of Amino Acids in Nonfermented and FCG

Samples were hydrolyzed in 6 N HCl and 1% phenol at 110°C for 24 h in a closed system. The hydrolysis products were neutralized and analyzed by high-performance liquid chromatography (HPLC) (Agilent 1260 Infinity HPLC System; Agilent Technologies, Santa Clara, Calif., USA). The chromatographic separation was performed using a Capcellpak UG120 C18 (250×4.6 mm, $5\ \mu\text{m}$) at 40°C and a UV detector at 450 nm. The mobile phase consisted of: (1) 40 mM NaH_2PO_4 (pH 7.8) and (2) acetonitrile:MeOH:DW (45:45:10). Quantification of the amino acids in the samples was carried out by comparison with the retention times of known standards.

Experimental Animals and Treatment

The experimental protocols described in this study were approved by the Institutional Animal Care and Use Review Committee of Kyung Hee University [KHUASP(SE)-14-007]. NC/Nga mice (22–25 g, 6 weeks, male) and age-matched wild-type Balb/c mice (22–25 g, 6 weeks, male) were purchased from SLC Inc. (Hamamatsu, Japan). The animals were housed in wire mesh-bottomed individual cages, in climate-controlled quarters ($24 \pm 1^\circ\text{C}$, $55 \pm 5\%$ relative humidity), with a 12-hour light:12-hour dark cycle. All mice were acclimatized for an adaptation period of 7 days before the experiment, fed standard pellet chow, and given fresh water ad libitum.

A total of 40 mice were randomly divided into five groups (8 mice per group): untreated Balb/c mice, AD control (NC/Nga mice), FCGH (NC/Nga mice fed a dietary supplement of 300 mg/kg of FCGH), FCG30 (NC/Nga mice fed a dietary supplement of 300 mg/kg of FCG30), and FCG80 (NC/Nga mice fed a dietary supplement of 300 mg/kg of FCG80). Mice in each group were fed their experimental diets, based on the AIN93G diet, for 10 weeks. During the entire 10-week feeding period, all NC/Nga mice were maintained under conventional laboratory conditions, but without air filtration, to raise AD spontaneously [19]. At the end of 10 weeks, all mice were killed by anesthesia. Spleens and dorsal skin tissue of all the mice were dissected, and blood samples were collected for assay.

Evaluation of Scratching Behavior

Scratching behavior was observed at week 10. The mice were placed individually into a clear plastic cage and their behavior was recorded using an automatic video camera (Sony TRV, Japan) for 20 min. The videotapes were reviewed and the scratching behavior of the mice was quantified by counting the number of scratching episodes.

Evaluation of Epidermal Hydration

At 10 weeks, epidermal hydration was measured with a Tewameter CM825 (Courage & Khazaka GmbH, Cologne, Germany) on the dorsal skin. The room conditions were kept at $21\text{--}23^\circ\text{C}$ and a relative humidity of 50–60%.

Morphological and Histopathological Observation

Morphologic changes of mouse dorsal skin surfaces were observed using a digital camera. Histopathological assessment was conducted on dorsal skin tissue fixed in 10% neutral buffered formaldehyde solution, embedded in paraffin, and sectioned into 5- μ m slices. The skin sections were stained with hematoxylin and eosin (HE) and toluidine blue, and observed by light microscopy.

RNA Isolation and Real-Time PCR

Dorsal skin tissue was homogenized using rotor-stator homogenizers in the presence of RLT buffer (lysis buffer; Qiagen, Valencia, Calif., USA), including β -mercaptoethanol. Total RNA was extracted from dorsal skin tissue lysate using the RNeasy Mini Kit (Qiagen). Complementary DNA was synthesized from 1 μ l of purified RNA in 20 μ l of reaction buffer using the iScript^{TM} cDNA Synthesis Kit (BioRad, Hercules, Calif., USA). Real-Time PCR (Applied Biosystems, Foster City, Calif., USA) was performed using the cDNA (1 μ l) with the SYBR Green PCR Master Mix (iQ SYBR Green Supermix; BioRad). The cDNA was amplified for 40 cycles of denaturation (95°C for 30 s), annealing (58°C for 30 s), and extension (72°C for 45 s) using the following primers: GAPDH forward primer 5'-CAT GGC CTT CCG TGT TCC TA-3', reverse primer 5'-GCG GCA CGT CAG ATC CA-3'; eotaxin forward primer 5'-GAC CAG GTT GGG CAA AGA GA-3', reverse primer 5'-GGC ATC CTG GAC CCA CTT CT-3'; TARC forward primer 5'-TCC AGG GCA AGC TCA TCT GT-3', reverse primer 5'-TCT GAT GGC CTT CTT CAC ATGT-3'; TNF- α forward primer 5'-CAC AAG ATG CTG GGA CAG TGA-3', reverse primer 5'-TCC TTG ATG GTG GTG CAT GA-3', and IL-6 forward primer 5'-CCA CGG CCT TCC CTA CTT C-3', reverse primer 5'-TTG GGA GTG GTA TCC TCT GTG A-3'. Data analysis of real-time RT-PCR results and calculations of the relative quantitation were performed using the 7500 System SDS software version 1.3.1 (Applied Biosystems).

T- and B-Cell Proliferation from Primary Splenocytes

Splenic T- and B-cell proliferation was determined by EZ-CyTox (Daeil Lab Service, Seoul, Korea), according to the manufacturer's instructions. The splenocytes were cultured at a concentration of 1×10^6 cells/well in 96-well tissue culture dishes and treated with concanavalin A (Con A; 5 μ g/ml) to induce T-cell proliferation; they were then treated with LPS (5 μ g/ml) to induce B-cell proliferation. After a 48-hour incubation (37°C, 5% CO₂ incubator), 10 μ l of EZ-CyTox were added to each well, followed by incubation for 4 h at 37°C. The absorbance at 340 nm was measured using a Versamax ELISA microplate reader (Molecular Devices, Sunnyvale, Calif., USA).

Cytokine Production from Primary Splenocytes

Spleens were gently teased in a 40 mesh strainer in culture medium (RPMI 1640, 10% fetal bovine serum, 2 mmol/l glutamine, 1/10⁵ units/l penicillin/streptomycin). The released splenocytes were treated by lysing the contaminating red blood cells with red blood cell lysing buffer (Sigma, St. Louis, Mo., USA) at 37°C for 3 min. The splenocytes were washed twice with the culture medium, and cultured at a concentration of 1×10^6 cells/well in 96-well tissue culture dishes. The splenocytes were then stimulated with Con A (5 μ g/ml; Sigma, St. Louis, Mo., USA) to induce IL-2, IFN- γ , IL-4, and IL-10 production; other splenocytes were also stimulated with LPS (5 μ g/ml, Gibco BRL, Grand Island, N.Y., USA) to induce TNF- α and IL-6 production. After 24 h of incubation, the supernatants were collected and analyzed for IL-2, IL-4, IL-10, TNF- α ,

Table 1. NEAA and EAA composition of *C. gladiata* and FCG

	<i>C. gladiata</i>	FCG
NEAA, mg/g		
Asp	25.94 $\pm$ 0.19	25.43 $\pm$ 0.22*
Glu	30.38 $\pm$ 0.12	31.40 $\pm$ 0.55*
Ser	13.89 $\pm$ 0.36	14.66 $\pm$ 0.14*
Gly	9.34 $\pm$ 0.23	9.63 $\pm$ 0.21
Thr	11.97 $\pm$ 0.27	12.34 $\pm$ 0.42
Ala	10.14 $\pm$ 0.19	10.31 $\pm$ 0.3
Tyr	4.66 $\pm$ 0.29	4.85 $\pm$ 0.13
Pro	11.49 $\pm$ 0.38	10.87 $\pm$ 0.38
Cys	6.11 $\pm$ 0.43	6.88 $\pm$ 0.19*
Total	123.92 $\pm$ 0.12	126.37 $\pm$ 0.97*
EAA, mg/g		
His	7.86 $\pm$ 0.15	8.20 $\pm$ 0.10*
Arg	9.95 $\pm$ 0.15	11.01 $\pm$ 0.36*
Val	10.34 $\pm$ 0.36	10.71 $\pm$ 0.26
Met	9.85 $\pm$ 0.38	8.80 $\pm$ 0.29*
Phe	10.45 $\pm$ 0.21	11.18 $\pm$ 0.21*
Ile	8.70 $\pm$ 0.16	9.12 $\pm$ 0.31
Leu	17.35 $\pm$ 0.23	18.04 $\pm$ 0.09*
Lys	13.13 $\pm$ 0.16	14.88 $\pm$ 0.14*
Trp	2.22 $\pm$ 0.24	1.49 $\pm$ 0.33*
Total	89.85 $\pm$ 1.24	93.42 $\pm$ 1.16*

Data represent means $\pm$ SD (n = 3, triplicate determination of one sample). Asterisks indicate statistically significant differences from the *C. gladiata* group at p < 0.05.

and IL-6 concentrations. After 72 h of incubation, the supernatants were collected and analyzed for IFN- γ concentration. Concentrations of cytokines were analyzed using the R&D DuoSet ELISA development kit (R&D Systems, Minneapolis, Minn., USA) according to the manufacturer's instructions.

Determination of Immunoglobulin Concentration

Serum samples were collected after centrifugation (1,500 g at 4°C for 20 min) of arterial whole blood. IgG serum levels were measured using a mouse immunoglobulin E (IgE) ELISA kit, mouse IgG1 ELISA kit, and mouse IgG2a ELISA kit (Abcam, Cambridge, Mass., USA), according to the manufacturer's instructions.

Determination of Histamine Concentration

Serum samples were collected after centrifugation (1,500 g at 4°C for 20 min) of the arterial whole blood. Levels of histamine were measured using a mouse histamine ELISA kit (LDN, Nordhorn, Germany).

Statistical Analysis

All data were expressed as means $\pm$ SD. All data were analyzed by Student's t test or one-way ANOVA using SPSS statistical software for Windows (SPSS PASW Statistic 20.0; SPSS Inc., Chicago, Ill., USA). Duncan's multiple range test was used for examination of differences among groups. Statistical significance was considered to be a value of p < 0.05.

Fig. 1. Representative images of macroscopic morphological and histological observations following HE staining of the skin of mice after a 10-week experiment to evaluate the inhibitory effects of FCG.

Table 2. Effect of FCG extracts on FER and organ weights of NC/Nga mice

	Untreated Balb/c mice	NC/Nga mice			
		AD control	FCGH	FCG30	FCG80
FER ¹	3.67±0.91 ^{n.s.}	3.51±0.56	3.60±0.35	3.35±0.18	3.26±0.53
Spleen, g	0.13±0.03 ^b	0.18±0.01 ^a	0.16±0.05 ^{a, b}	0.12±0.03 ^b	0.13±0.03 ^b
Kidney, g	0.49±0.04 ^{n.s.}	0.48±0.09	0.52±0.05	0.53±0.08	0.49±0.04
Liver, g	1.33±0.09 ^c	1.62±0.11 ^a	1.49±0.06 ^{a, b}	1.47±0.11 ^{b, c}	1.49±0.09 ^{a, b}

Data represent means ± SD (n = 8). Different letters indicate (a > b > c) a significant difference at p < 0.05, as determined by Duncan's multiple range test. n.s. = Not significant. ¹ FER = Weight gain (g)/food intake consumption (g) × 100.

Results

Quantification of Amino Acids in Nonfermented and Fermented *C. gladiata*

The amino acid profiles of non-fermented and fermented *C. gladiata* are shown in table 1. The amounts of nonessential amino acids (NEAAs) were increased in FCG (126.37 ± 0.97 mg/g) when compared with nonfermented *C. gladiata* (123.92 ± 0.12 mg/g). The amounts of essential amino acids (EAAs) in FCG (93.42 ± 1.16 mg/g) were also increased compared with nonfermented *C. gladiata* (89.85 ± 1.24 mg/g).

Food Efficiency Rate and Organ Weights

No statistically significant differences were found among any of the groups in terms of food efficiency rate and kidney weight. Mice that developed AD subsequent-

ly showed a significant increase in weights of the spleen and liver, compared with the untreated Balb/c mice. In the FCG30 group, weights of the spleen and liver decreased significantly compared with those of the AD control. In the FCG80 group, only the spleen weight decreased significantly compared with the AD control. However, no marked difference was observed between the FCGH group and the AD control group in terms of weights of the spleen and liver (p < 0.05; table 2).

Morphological and Histological Observations

The dorsal skin of the AD control group showed hemorrhage, erythema, and excoriation when compared with the untreated Balb/c mice. The HE-stained sections showed an increase in epidermal thickness of the dorsal skin and the epidermal cells assumed an irregular shape in the AD control group. Dietary supplementation with

Fig. 2. Effect of FCG extracts on frequency of scratching episodes in mice. Data represent means $\pm$ SD ($n = 8$). Different letters show statistically significant differences ($a > b > c$) at $p < 0.05$, as determined by Duncan's multiple range test.

FCG extracts resulted in attenuation of the morphological and histological changes induced by the development of AD. The effects of FCG30 and FCG80 were more pronounced than the effects of FCGH (fig. 1).

Scratching Behavior

The number of scratching episodes over a 20-min period increased significantly in the AD control group (57.0 ± 12.5) when compared with the untreated Balb/c mice. The FCG30 (33.0 ± 10.4) and the FCG80 (30.0 ± 10.7) groups showed a significant reduction in the number of scratching episodes when compared with the AD control group, but the FCGH group (58.5 ± 4.5) showed no significant differences when compared to the AD control group ($p < 0.05$; fig. 2).

Epidermal Hydration of Dorsal Skin

The epidermal hydration of the dorsal skin was significantly decreased in the AD control group (25.34 ± 0.35 AU) when compared to the untreated Balb/c mice (39.23 ± 2.80 AU). This hydration was significantly increased in the FCGH (28.23 ± 0.35 AU), FCG30 (34.00 ± 2.00 AU), and FCG80 (36.29 ± 0.75 AU) groups when compared with the AD control group ($p < 0.05$; fig. 3).

mRNA Expression of Chemokines and Proinflammatory Cytokines in Dorsal Skin of Mice

The mRNA expression of chemokines (eotaxin and TARC) was significantly increased in the AD control group when compared with the untreated Balb/c mice.

Fig. 3. Effect of FCG extracts on epidermal hydration of dorsal skin in mice. Data represent means $\pm$ SD ($n = 8$). Different letters show statistically significant differences ($a > b > c > d$) at $p < 0.05$, as determined by Duncan's multiple range test.

Supplementation of the NC/Nga mouse diets with FCG30 and FCG80 significantly decreased the mRNA expression of chemokines. Dietary supplementation with FCGH caused significant decreases in mRNA expression of eotaxin, but mRNA expression of TARC showed no statistically significant difference ($p < 0.05$; fig. 4a).

The AD control group showed a significant increase in the mRNA expression of proinflammatory cytokines when compared with the untreated Balb/c mice. By contrast, the groups receiving dietary supplementation of FCGH, FCG30, and FCG80 showed significant decreases in the mRNA expression of proinflammatory cytokines when compared with the AD control group ($p < 0.05$; fig. 4b).

T- and B-Cell Proliferation from Primary Splenocytes

Mitogen-stimulated primary splenocytes from the AD group showed significantly decreased T-cell proliferation when compared with the untreated Balb/c mice. The dietary supplementation with FCG extracts caused no significant changes in the levels of T-cell proliferation. However, the mitogen-stimulated primary splenocytes from the AD group showed significantly increased B-cell proliferation when compared with the untreated Balb/c mice. The FCGH and FCG80 groups showed no significant differences in the level of B-cell proliferation when compared with the AD control. By contrast, the dietary supplementation of FCG30 significantly decreased the level of B-cell proliferation when compared with the AD control ($p < 0.05$; fig. 5).

Fig. 4. Effect of FCG extracts on mRNA expression of chemokines (eotaxin and TARC; **a**) and proinflammatory-type cytokines (IL-6 and TNF- α ; **b**) in dorsal skin of mice. Data represent means $\pm$ SD ($n = 8$). Different letters show statistically significant differences ($a > b > c$) at $p < 0.05$, as determined by Duncan's multiple range test.

Fig. 5. Effect of FCG extracts on T- and B-cell proliferation from primary splenocytes. Data represent means $\pm$ SD ($n = 8$). Different letters show statistically significant differences ($a > b > c$) at $p < 0.05$, as determined by Duncan's multiple range test.

Th1-Type and Th2-Type Cytokine Production from Primary Splenocytes

The production of Th1-type cytokines (IL-2 and IFN- γ) from Con A-induced primary splenocytes in the AD control group was significantly decreased when compared with the untreated Balb/c mice. The FCG30 and FCG80 groups showed significant increases in the levels of Th1-type cytokines when compared with the AD control group. However, no significant differences were noted between the FCGH group and the AD control group in terms of Th1-type cytokine production ($p < 0.05$; fig. 6a).

The AD control group showed a significant increase in the levels of Th2-type cytokines (IL-4 and IL-10) in Con A-induced primary splenocytes when compared with the untreated Balb/c mice. The FCG30 and FCG80 groups showed significant decreases in levels of both IL-4 and IL-10 when compared with the AD control group. The FCGH group showed significant decreases in the level of IL-10, but the levels of IL-4 were not significantly different from those of the AD control group ($p < 0.05$; fig. 6b).

Proinflammatory Cytokine Production from Primary Splenocytes

Proinflammatory cytokine (TNF- α and IL-6) production from Con A-induced primary splenocytes was significantly increased in the AD control group when compared with the untreated Balb/c mice. However, the dietary supplementation of FCG extracts (FCGH, FCG30, and FCG80) caused a significant decrease in proinflammatory cytokine production ($p < 0.05$; fig. 7).

Serum Immunoglobulin Production

The AD control group showed a marked decrease in serum IgG2a production when compared with the untreated Balb/c mice. The dietary supplementation of FCG80 caused a significant increase in serum IgG2a production when compared with the AD control group. The production of IgG1 and IgE in the AD control group increased significantly when compared with the untreated Balb/c mice. No significant differences were noted in serum IgG1 production following dietary supplementation with FCG extracts when compared with the AD control

Fig. 6. Effect of FCG extracts on Th1-type cytokines (IL-2 and IFN-γ; **a**) and Th2-type cytokine (IL-4 and IL-10; **b**) production from Con A-stimulated primary splenocytes. Data represent means $\pm$ SD ($n = 8$). Different letters show statistically significant differences ($a > b > c$) at $p < 0.05$, as determined by Duncan's multiple range test.

Fig. 7. Effect of FCG extracts on proinflammatory type cytokine (TNF-α and IL-6) production from Con A-stimulated primary splenocytes. Data represent means $\pm$ SD ($n = 8$). Different letters show statistically significant differences ($a > b > c$) at $p < 0.05$, as determined by Duncan's multiple range test.

Fig. 8. Effect of FCG extracts on serum IgE, IgG1, and IgG2a production in mice. Data represent means $\pm$ SD ($n = 8$). Different letters show statistically significant differences ($a > b > c$) at $p < 0.05$, as determined by Duncan's multiple range test.

group. However, serum IgE production in the FCG30 and FCG80 groups decreased significantly when compared with the AD control group ($p < 0.05$; fig. 8).

Infiltration of Mast Cells

Infiltration of mast cells into the skin was analyzed by toluidine blue staining, as shown in figure 9. The AD control group showed enhanced the infiltration of mast cells in the epithelial tissue. By contrast, the infiltration of mast cells was reduced by dietary supplementation with FCG when compared with the AD control group ($p < 0.05$).

Serum Histamine Production

Serum histamine production was increased significantly in the AD control group when compared with the untreated Balb/c mice. The dietary supplementation with FCG30 and FCG80 resulted in significant decreases in the serum histamine production when compared with the AD control group ($p < 0.05$; fig. 10).

Fig. 9. Effect of FCG extracts on the observation of mast cell infiltration by toluidine blue staining on the skin of mice. Data represent means $\pm$ SD ($n = 8$). Different letters show statistically significant differences ($a > b > c > d > e$) at $p < 0.05$, as determined by Duncan's multiple range test.

Discussion

Legumes are good sources of plant protein, but commercialization of legume products is limited because many legume proteins are associated with allergic reactions. Therefore, Asian cultures have traditionally developed various fermented legume foods for consumption, including miso, natto, and doenjang [20]. Fermentation of food breaks down nutrients, including proteins associated with allergic reactions, making them easily digestible, and it also creates beneficial nutrients, including amino acids, enzymes, vitamins, and fatty acids [21, 22]. The present study confirmed increases in several NEAAs and EAAs (but not Asp, Pro, Met, and Trp) in the FCG, when compared with the non-fermented *C. gladiata*. Frias et al. [23] also documented that fermentation of soybean with *A. oryzae*, a fermentation microbe used in various traditional Asian legume foods, resulted in significantly increased amounts of NEAAs and EAAs when compared with raw soybeans. In addition, they found that degradation of soybean protein into amino acids by fermentation reduced immunoreactivity and eliminated antigenic soybean proteins.

Fig. 10. Effect of FCG extracts on serum histamine production in mice. Data represent means $\pm$ SD ($n = 8$). Different letters show statistically significant differences ($a > b > c$) at $p < 0.05$, as determined by Duncan's multiple range test.

In the present study, we investigated the immunomodulatory effects of extracts from the *C. gladiata* fermented with *A. oryzae* on the development of AD-like skin lesions in NC/Nga mice. NC/Nga mice spontane-

ously develop AD-like skin lesions in response to environmental factors under conventional laboratory conditions without air filtration. NC/Nga mice with AD pathogenesis are characterized by morphological symptoms such as itching, erythema, hemorrhage, hyperkeratosis, and xerosis, and have several similarities to patients with AD [19]. We found that the dorsal skin of NC/Nga mice developed erythema, hemorrhage, hyperkeratosis, and showed reduced hydration in the epidermis and a significantly increased frequency of scratching when compared with the untreated Balb/c mice. An increase in skin hydration, which plays an important role in maintaining the water balance of the skin, can improve AD, while a disruption of the balance and increased dryness of skin can aggravate AD [24]. We also showed that NC/Nga mice showed significant increases in spleen and liver weights when compared with the untreated Balb/c mice ($p < 0.05$). Enlargement of the spleen and liver can be caused by infectious conditions and disease [25].

Taken together, these results indicate that the NC/Nga mice in this study developed normal AD pathogenesis. However, the dietary supplementation of FCG attenuated macroscopic and histopathological changes and alleviated the reduced hydration in the dorsal skin of NC/Nga mice when compared with the AD control group. The FCG30 and FCG80 groups, in particular, showed a significant decrease in scratching episodes when compared with the AD control group ($p < 0.05$).

Eotaxin and TARC are chemokines responsible for the selective recruitment of eosinophils and Th2 cells to the dermis. The induction of chemotaxis of these cells in the early stages of skin disease is implicated in inflammation and allergic responses. Therefore, increased amounts of eotaxin and TARC are found in the dermis of skin with AD [7, 8]. Yasukochi et al. [7] found a link between a reduction in serum TARC levels and topical anti-inflammatory treatments. They showed a significant correlation between TARC and eosinophil numbers, whereas the application of the topical agents caused a reduction in serum TARC levels.

Like chemokines, proinflammatory cytokines induce the recruitment of pathogenic leukocytes, as well as support the proliferation of lymphocytes in the early stages of skin disease [26]. Homey et al. [9] reported that chemokines and proinflammatory cytokines act to control innate and adaptive immunity at sites of atopic skin injury, which can lead to the development of AD. The present study revealed that dietary supplementation with FCG attenuated mRNA expression of chemokines and proinflammatory cytokines in the skin of NC/Nga mice.

These data suggest that FCG may prevent the development of skin inflammation or serve as a treatment for AD by recruiting fewer immune cells to the dermis.

CD4⁺ T-helper cells are divided into Th1 cells and Th2 cells, based on the nature of their cytokine production. Th1 cells produce IFN- γ and IL-2 (Th1-type cytokines), while Th2 cells produce IL-4, IL-10, and IL-13 (Th2-type cytokines) [27]. Th2-type cytokines are responsible for B-cell responses and antibody development, while Th1-type cytokines are responsible for macrophage activation, cytotoxic T-cell development, and delayed-type hypersensitivity reactions [27, 28]. It has been reported that Th2-type cytokines play a key pathogenic role in the initiation phase of AD [28]. The increase of IL-4, one of the Th2-type cytokines, in the blood is linked to AD as shown by the correlation with serum IgG1 and IgE levels, which activate mast cells [2, 10]. Mast cells have a high-affinity receptor (Fc ϵ RI) for the Fc region of IgE. When IgE binds to mast cells, it releases histamine and pharmacological molecules, triggering allergic responses, such as the surrounding itchy skin, characteristic of AD [27–29]. Matsuda et al. [29] found that plasma levels of IgE were significantly elevated and IL-4 was released by CD4⁺ T-helper cells in the spleen of NC/Nga mice.

The Th2-type cytokines can also contribute to recruitment of macrophage eosinophils to the skin; these eosinophils produce IL-12. IL-12, in turn, is characterized by production of the effector IFN- γ and IL-2 cytokines from predominantly Th1 cells. The action of Th1-type cytokines in AD is unclear, as some animal studies have shown IFN- γ therapy can improve AD [30]. Other studies have suggested that IFN- γ can trigger the immunopathogenesis of chronic AD [31].

The data from the present study show a marked increase in the production of serum IgG1, IgE, histamine, and Th2-type cytokines in splenocytes, and an enhancement of mast cell infiltration in the AD control group. However, Th1-type cytokine production by splenocytes in the AD control group were significantly decreased when compared with the untreated Balb/c mice ($p < 0.05$). In addition, T-cell proliferation decreased and B-cell proliferation increased in the AD group. The results suggested that NC/Nga mice developed the immunopathogenesis of AD, but not chronic AD, as indicated by the decrease in Th1-type cytokines and the increase in B-cell proliferation that induced the response of Th2 cytokines. Werfel and Wittmann [28] suggested that the production of cytokines from CD4⁺ T-helper cells and skin-infiltrating immune cells could play a key role in the regulation of pathologic skin reactions associated with

AD. Therefore, the balance of Th1-/Th2-type cytokines plays an important role in the immune response and improvement of AD.

In the present study, the dietary supplement FCG, and particularly FCG30 and FCG80, attenuated Th1-/Th2-type cytokine imbalances, mast cell infiltration, and production of serum IgE and histamine. The results suggest that FCG30 and FCG80, more so than FCGH, can attenuate the activation and infiltration of mast cells in the skin by inhibition of IgE production in B cells induced by Th2-type cytokines. Reports by Kim et al. [32] and Cho et al. [33] documented that *C. gladiata* has radical-scavenging abilities and antioxidant effects. Apart from those findings, the study of the biological activity of *C. gladiata* has been insufficient.

C. gladiata has been used in herbal medicine in Asia, but it is rarely consumed as a food because of its association with allergic reactions and antinutritional factors [17, 18]. Yeh et al. [34] found that a legume product fermented by *Saccharomyces cerevisiae* attenuated the inflammatory responses in the skin and suggested potential applications for the prevention and treatment of AD. A

report by Sung et al. [35] also indicated that fermented soy curd relieved the phenotypes of AD by reduction of IgE concentration and dermal thickness. These results and our data suggest that fermented legumes may represent potential candidates for the treatment of AD.

In conclusion, the present study demonstrated that NEAAs and EAAs (except Asp, Pro, Met, and Trp) increased following fermentation of *C. gladiata*. In addition, extracts of FCG improved immune responses in NC/Nga mice, including increases in IgE and histamine for AD through attenuation of Th1/Th2 cytokine imbalance, and production of proinflammatory cytokines and chemokines. FCG may therefore have benefits for improvement of AD. The active compound in FCG has not yet been identified, so further work is required.

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