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Original Article

Fermented rice bran prevents atopic dermatitis in DNCB-treated NC/Nga mice

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Abstract

The fermentation of natural plants has a favorable effect on the functional and biological activities of living systems. These include anti-oxidative, anti-inflammatory, and anti-platelet aggregation activities. This is attributed to the chemical conversion of the parent plants to functional constituents, which show more potent biological activity. In our study, rice bran along with oriental medicinal plants (*Angelicae gigantis, Cnidium officinale, Artemisia princeps,* and *Camellia sinensis*) was fermented by *Lactobacillus rhamnosus* and *Pichia deserticola* (FRBE). We evaluated the effects of oral administration of FRBE on atopic dermatitis in 1-chloro-2,4-dinitrobenzene (DNCB)-treated NC/Nga mice. FRBE significantly ameliorated the macroscopic and microscopic appearance of skin lesions in DNCB-induced atopic dermatitis and reduced levels of serum immunoglobulin E and the differential white blood cell count. In addition, it reduced skin thickness compared to that of atopic dermatitis-affected skin. FRBE treatment also reduced mast cell incorporation in skin lesions of atopic dermatitis. The total cell number in dorsal skin tissue and the axillary lymph node increased following DNCB application, and this was normalized by FRBE treatment. Moreover, it decreased the levels of $CD8^+$ helper T cells and $Gr\text{-}1^+/CD11b^+$ B cells in peripheral blood mononuclear cells and skin lesions in DNCB-induced atopic dermatitis. Using real-time polymerase chain reaction analysis, we demonstrated that FRBE significantly inhibited mRNA expression of cytokines (e.g., interleukin-5 and interleukin-13) and cyclooxygenase-2 in AD skin lesions. These results suggest that FRBE could be a valuable herbal remedy for the treatment of atopic dermatitis.

Keywords: fermentation, rice bran, gas chromatography, *Lactobacillus rhamnosus*, *Pichia deserticola*

Introduction

In the extensive globalization of today's world, human predisposition to allergic diseases that are the consequences of advanced industrialization is an inevitable reality. One of the most common allergic diseases is atopic dermatitis (AD). AD originates from multiple sources including those originated from genetic and environmental roots[1]. It is a relapsing and chronic disease that affects almost 20% of children

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worldwide. It is characterized by eczematous pruritic lesions and significantly increased levels of immunoglobulin (Ig) E antibodies in the plasma[2–3]. Two types of hypotheses have been used to explain the pathophysiology underlying the painful and aberrant lesions in AD. Since dermatitis is the most common and obvious manifestation of AD, the immunological hypothesis suggests that dermatitis occurs as a result of an imbalance between two types of T cells, helper T (Th) cells (particularly Th2 cells) and regulatory T cells[2]. The acute eczematic form of dermatitis is an outcome of the differentiation of Th2 cells into naïve CD4 + cells, which causes an aberrant increase in the production of interleukins (ILs), particularly IL-4, IL-5, and IL-13. This rise in the level of cytokines increases the levels of serum IgE. However, the skin barrier hypothesis claims that the pathophysiology of AD is a result of mutational changes in the *filaggrin* gene of affected individuals. This gene is responsible for adhesion of keratinocytes to the stratum corneum and stratum granulosum in the skin layers. If there is a genetic or environmental mutation in this gene, moisture loss occurs due to the dispersion of keratinocytes, leading to eczema and dryness[1,3]. Therefore, the environmental allergens easily penetrate the skin and cause itching, rashes, and later allergic diseases[4].

The severity of AD has been related to increased cytokine levels, particularly ILs, due to an increase in T-helper cell levels. The most important ILs contributing to pathology of AD are IL-4, IL-5, and IL-13. IL-5 has been documented to promote the activation and survival of eosinophils causing eosinophilia and the infiltration of cytokine cells to the skin[5]. IL-4, however, causes the differentiation of naïve T cells into Th cells. It also causes the activation of B cells, which elevates IgE levels, culminating in an allergic reaction[6]. IL-13 is also produced by helper Th2 cells and is a major mediator of allergic inflammation. It is possible that IL-13 could be the central block of inflammatory manifestations in AD[7].

Rice bran, which is the major agro-industrial by-product of rice processing, is vastly underutilized and discarded as waste. It is a valuable source of major dietary fiber, antioxidants, and vitamins. Among the fiber fraction of rice bran, there is an abundance of polysaccharides such as pectins, β-glucans, and arabinoxylans that are mostly unavailable for beneficial use by any mechanism in the body[8–9]. These polysaccharides have been previously reported to possess immunological and anti-cancer properties. However, the major barrier to their appropriate utilization is their presence in the hemicellulose component of the cell wall. Therefore, the best method to make them readily available is through the fermentation of rice bran[10]. In our study, we fermented rice bran with *Lactobacillus rhamnosus* and *Pichia deserticola* supplemented with oriental medicinal plants such as *Angelica gigantis, Cnidium officinale, Artemisia princeps,* and *Camellia sinensis*. The final product was named fermented rice bran extract (FRBE). *A. gigantis radix,* which is also called Korean Angelica, was selected for fermentation because its anti-inflammatory activity has been reported previously in croton oil-induced inflammation models and carrageenan air pouch inflammation[11]. *C. officinale* was used in our study as its anti-inflammatory, anti-cancerous, and anti-oxidant effects were already reported; however, its anti-allergenic effects remained undisturbed[12]. *A. princeps* Pampanini is an herbaceous plant widely used in traditional Chinese, Japanese, and Korean medicine for the treatment of multiple ailments such as diarrhea, colic, menopause problems, and atherosclerosis. Jaceosidin, which is the main biological component of *A. princeps* Pampanini ethanol extract, was reported to suppress inflammation in vitro[13–14]. *C. sinensis* is commonly consumed as green tea throughout the world and is rich in anti-oxidant and anti-inflammatory compounds such as catechin, epicatechin, epicatechin gallate, epigallocatechin, and epigallocatechin gallate[15]. These components play an important role in suppression of allergic conditions.

To determine the therapeutic effects of FRBE in AD, we utilized NC/Nga (NC) mice, which are a murine AD model. These mice manifest the typical symptoms of AD such as scratching that terminates as eczematous pruritic lesions. In this study, we reported, for the first time, that the oral administration of FRBE significantly suppressed itching behavior in rats with AD and reduced the levels of keratinization in skin cells. Furthermore, it also decreased the levels of eosinophils and basophils, together with a reduction in the number of CD4 +/CD8 + cells in the serum of AD mice. FRBE also inhibited the levels of IL-5, IL-13, and COX-2, indicating its anti-allergenic activity in AD.

Materials and methods

Sample preparation

Rice bran containing *A. gigantis*, *C.officinale*, *A. princeps*, and *C. sinensis* (at a ratio of 80:8:6:2:2:2, v/v) was injected with enzymes of *L. rhamnosus* (6.2×10^5 CFU/mL) and *P. deserticola* (3.7×10^4 CFU/mL). The hydrated enzymes of *L. rhamnosus* and *P. deserticola* and rice bran mixture were blended and then fermented at room temperature in a dark container. The fermented rice bran mixture was then extracted with 50% ethanol, filtered with Whatman filter paper, and evaporated.

Gas chromatography analysis

Gas chromatography mass spectrometry was carried out using 2.5 mg of FRBE. An Agilent Technology 7890A-Gas Chromatograph system (Agilent Technologies, Santa Clara, CA, USA), coupled to an XLMSD-5975C instrument operating in the electrospray ionization (EI) mode was used. The relative percentage of each component was calculated by comparing its average peak area to the total areas.

Animal experiments

Specific pathogen-free male NC/Nga (NC) mice were procured from the Central Lab Animal Inc. (Seoul, Korea). The study protocol was approved by the local institutional review board at Daejon University Animal Care and Use Committee (DJU-2014-041) and animal studies were carried out in accordance with the established institutional guidelines regarding animal care and use. Animal welfare and the experimental procedures were carried out strictly in accordance with the Guide for Care and Use of Laboratory Animals (National Research Council of USA, 1996). The mice were allowed to acclimatize to their environment for 1 week after the arrival. After 1 week, AD was induced *via* a topical application of 0.2% w/v 2,4-dinitrochlorobenzene (DNCB, Sigma Aldrich, St. Louis, MO, USA) once a week, which was prepared with an olive oil and acetone solution (1:3). On the basis of treatment, mice were divided into three groups (n = 8-10). The control group received vehicle or phosphate buffered saline (PBS) orally. The second group received FRBE at a dose of 300 mg/kg·day orally, and the third group received commercially available dexamethasone (Dex) 3 mg/kg·day treatment for 5 weeks. The severity of dermatitis and the clinical index of dermatitis were assessed by a method previously reported by Park *et al.*[16].

Histological examination of AD-like dermis pathology

The histopathological lesions that developed as a result of AD were visualized using hematoxylin and eosin (H&E) staining and toluidine blue for measuring infiltration of inflammatory cells such as mast cells. Biopsies were taken from the ears of the mice, embedded in paraffin wax, and cut to a thickness of 4 μm. After the biopsy, the mice were sacrificed.

Analysis of white blood cells in the peripheral blood

For the analysis of white blood cells (i.e. neutrophils, eosinophils, basophils, and leukocytes), blood was drawn from the individual sacrificed mice through cardiac puncture. The total cell numbers were then counted using a CELL-DYN® 3200 analyzer (Abbott Laboratories, Santa Clara, CA, USA).

RNA extraction and real-time polymerase chain reaction

Total RNA was extracted using TRIzol® Reagent, and cDNA was synthesized using a PrimeScript™ RT reagent kit (TaKaRa, Shiga, Japan). The mRNA expression levels of IL-5, IL-13, and COX-2 were quantified using the 7500 real-time polymerase chain reaction (PCR) System (Applied Biosystems, CA, USA) using the Power SYBR® Green Master Mix. The sequence of primers used were glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward 5′-TGCACCACCAACTGCTTAGC-3′ and reverse 5′ GGCATGGACTGTGGTCATGAG3′; IL-5, forward 5′-CATTGGAAACATTTAGTTTCACG-3′ and reverse 5′-TGCGTCCCCAGTCAATTTAT-3′; IL-13, forward 5′-GGCCATCCTGCAAAATAGTG-3′ andreverse 5′-ACAGCGTCGGCAAGAACA-3′; and COX-2, forward 5′-GAATCATTCACCAGGCAAATTG-3′ and reverse 5′-TTTCTGTACTGCGGGTGGAAC-3′.

Fluorescence-activated cell sorting

Fluorescence-activated cell sorting (FACS) was performed by mincing the dorsal skin of experimental mice followed by incubation in PBS containing 1 mg/mL collagenase IV and 2 mg/mL dispase for 40 minutes at 37°C. Later, the cells were stained for 30 minutes on ice with phycoerythrin (PE)-, fluorescein isothiocyanate (FITC)-, orperidinin chlorophyll protein complex (PerCP)-conjugated antibodies specific for CD4 +, CD8 +, and Gr-1 +/CD11b + (BDBiosciences) in staining buffer (PBS containing 1% v/v fetal bovine serum and 0.01% w/v sodium azide). They were then analyzed by a FACS analyzer using Cell-Quest software (BD Biosciences).

Enzyme linked immunosorbent assay

For the assessment of IgE levels in the plasma, an enzyme linked immunosorbent assay (ELISA) assay was performed using commercially available kits (Shibayagi, Gunma, Japan).

Statistical analysis

Data are expressed as mean±standard deviation (SD) and are representative of three independent experiments. One way analysis of variance (ANOVA) and Duncan's test were applied using IBM SPSS Statistics for Windows, Version (IBM Corp., Chicago, IL, USA); $P < 0.05$ was considered significant.

Results

Effects of FRBE on AD macroscopic lesions

Since AD lesions have the most prominent effect on the skin i.e. pruritus and itching, our first goal was to determine the effects of orally administered FRBE (300 mg/kg) on AD skin lesions. As shown in ***Fig. 1A***, areas that were not induced with AD showed normal dorsal skin patches. However, in ***Fig. 1B***, the mice skin showed maximum skin damage as a result of AD-triggered pruritus. In ***Fig. 1C***, there was amelioration of pruritic lesion after treatment with 300 mg/kg of FRBE that is comparable to the healing effect observed with Dex treatment, a synthetic corticosteroid (***Fig. 1D***). From these visual results, we inferred that FRBE efficiently reverses debilitating AD skin lesions.

FRBE improves the histological manifestations of AD dermis lesions

The histological manifestations of AD are reflected by outrageous visible lesions that are eczematous pruritus. Hyperkeratosis, infiltration of inflammatory and allergenic cells, and secondary bacterial infections change the histology of the dermis in AD. In this study, we investigated the effect of FRBE on histological lesions in AD. As shown in ***Fig. 2A*** (***1-4***), FRBE was able to recover dermal thickness to almost normal levels when compared to the control group, indicating that it reduces the hyperkeratinization of skin. As indicated by staining of the dorsal skin with toluidine blue in ***Fig. 2B*** and ***2C*** (***1-4***), a reduction in the infiltration of mast cells, which are the major marker for the production of IgE, in the FRBE-treated group was observed. These visible improvements in AD lesions from a histological viewpoint unravel FRBE's antiallergic activity.

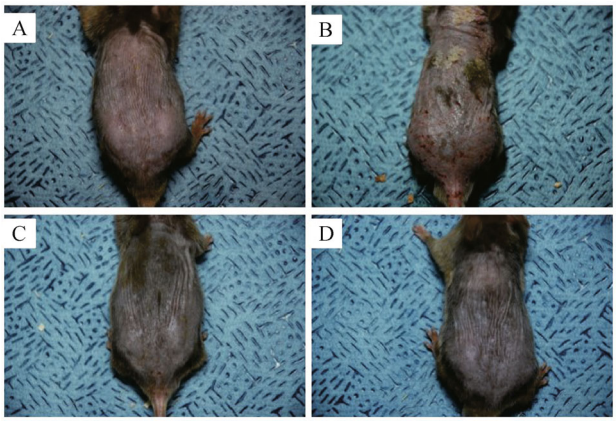

Fig. 1 **Macroscopic lesions manifested by atopic dermatitis in NC/Nga mice induced *via* 2,4-dinitrochlorobenzene (DNCB) topical application.** A: Normal mice with no DNCB application. B: The control mice with DNCB application but no treatment. C: DNCB application and fermented rice bran extract (FRBE) 300 mg/kg treatment. D: DNCB application and dexamethasone 3 mg/kg treatment.

Suppression of blood cell population levels by FRBE

White blood cells play a major role in innate and adaptive immunity in the living body. Any intrusion into the body by foreign substances triggers their production, which leads to the recruitment of function specific white blood cells[17]. Thus, we checked the levels of white blood cells in the serum of AD-induced mice. As shown in ***Fig. 3A***, there was only a minimal effect observed on the total number of white blood cells. However, the differential white blood cell count showed a remarkable suppression in the levels of neutrophils, eosinophils, and basophils (***Fig. 3B***). The basophils, in particular, showed a drastic decrease, which could be due to that they are primarily responsible for the allergic responses in the body. Similarly, the total cell number in the cases of skin tissue and the axillary lymph node also statistically decreased following FRBE treatment, as shown in ***Fig. 3C-D***.

Decrease in the levels of peripheral blood mononuclear cells by FRBE

Peripheral blood mononuclear cells (PBMCs) include monocytes, lymphocytes, and macrophages and are critical to the immune system of body. The various types of lymphocytes are dominant players in the immune response to allergenic bodies. In this regard, we checked the levels of $CD4^+$, $CD8^+$, and $Gr\text{-}1^+/CD11b^+$ cells in PBMCs and axillary lymph nodes (ALN). As shown in ***Fig. 4A-C***, FRBE significantly reduced the levels of these cells, indicating that it reduces the inflammatory and allergic cell numbers.

Inhibition in the levels of IgE as determined by ELISA

IgE is specific to the body's response to any antigen, especially allergic pathogens. It plays a key role in signaling the body towards immunostimulatory mechanisms, which leads to increased levels of lymphocytes. However, an elevated IgE level is also deleterious to the body due to it causes more severe pruritus. ***Fig. 5*** shows that FRBE efficiently and significantly decreased the levels of IgE, as determined by an ELISA experiment.

Depression in the levels of $CD4^+$, $CD8^+$, and $Gr\text{-}1^+/CD11b^+$ cells in the skin of AD mice

Previously, we found suppression in PBMC cell levels obtained from the plasma of AD mice. We next checked the same cells in the skin lesions of mice with

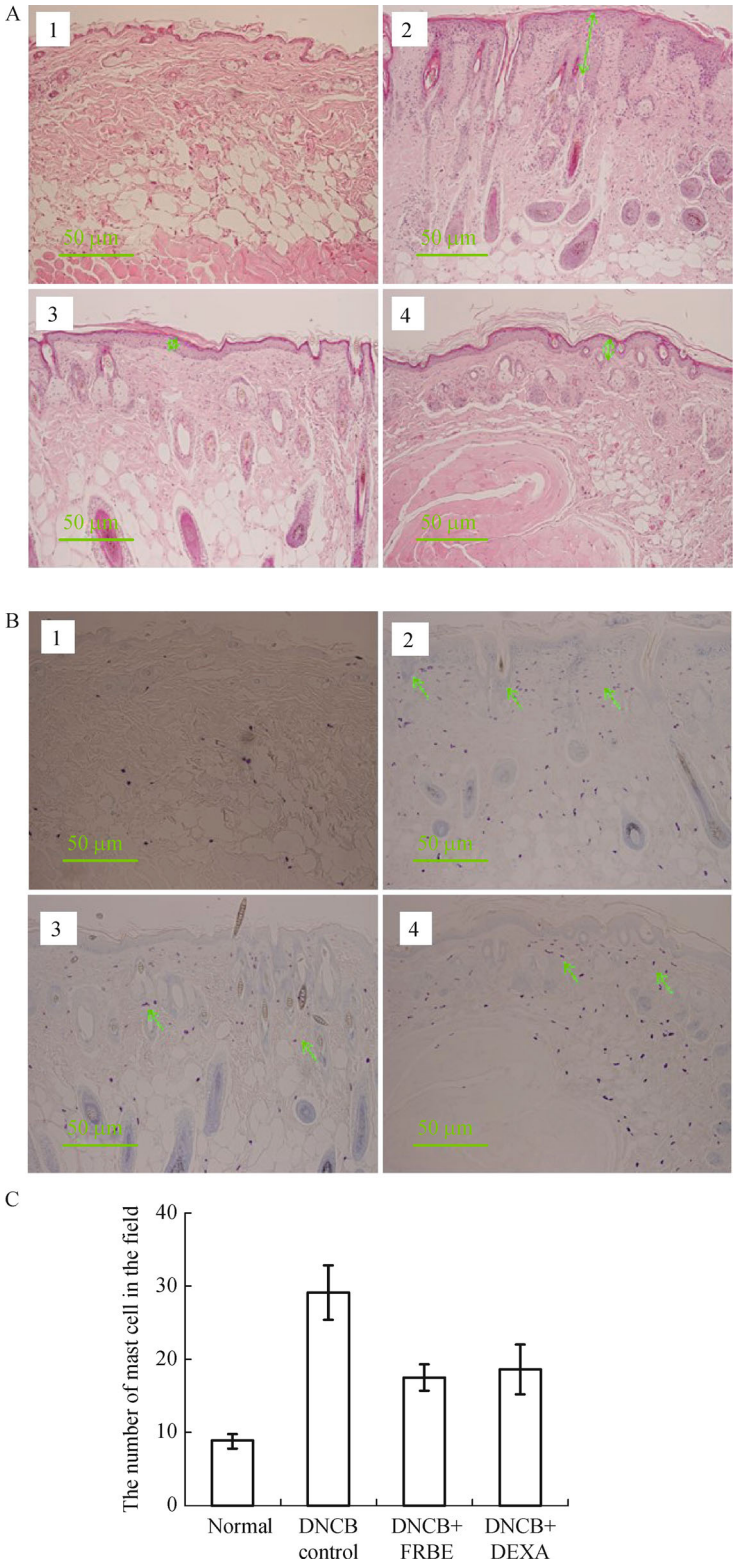

Fig. 2 **Effect of FRBE on the histological manifestations of AD dermis lesions.** A: Dermal skin cells stained with H&E staining. The dermal thickness and infiltration of cells were decreased by fermented rice bran extract (FRBE) treatment. 1 = normal mice skin, 2 = 2,4-dinitrochlorobenzene (DNCB) atopic dermatitis (AD)-induced mice skin, 3 = DNCB- and FRBE-treated mice skin, and 4 = DNCB- and dexamethasone-treated mice skin. B: Skin cells stained with toluidine blue for mast cell infiltration. 1 = normal mice skin, 2 = DNCB AD-affected mice skin, 3 = DNCB- and FRBE-treated mice skin, and 4 = DNCB- and dexamethasone-treated mice skin. Cells were observed at a magnification of 200× under a visible light microscope**.** C: Total number of mast bells in different fields. The data are shown as mean±SEM (n = 8).

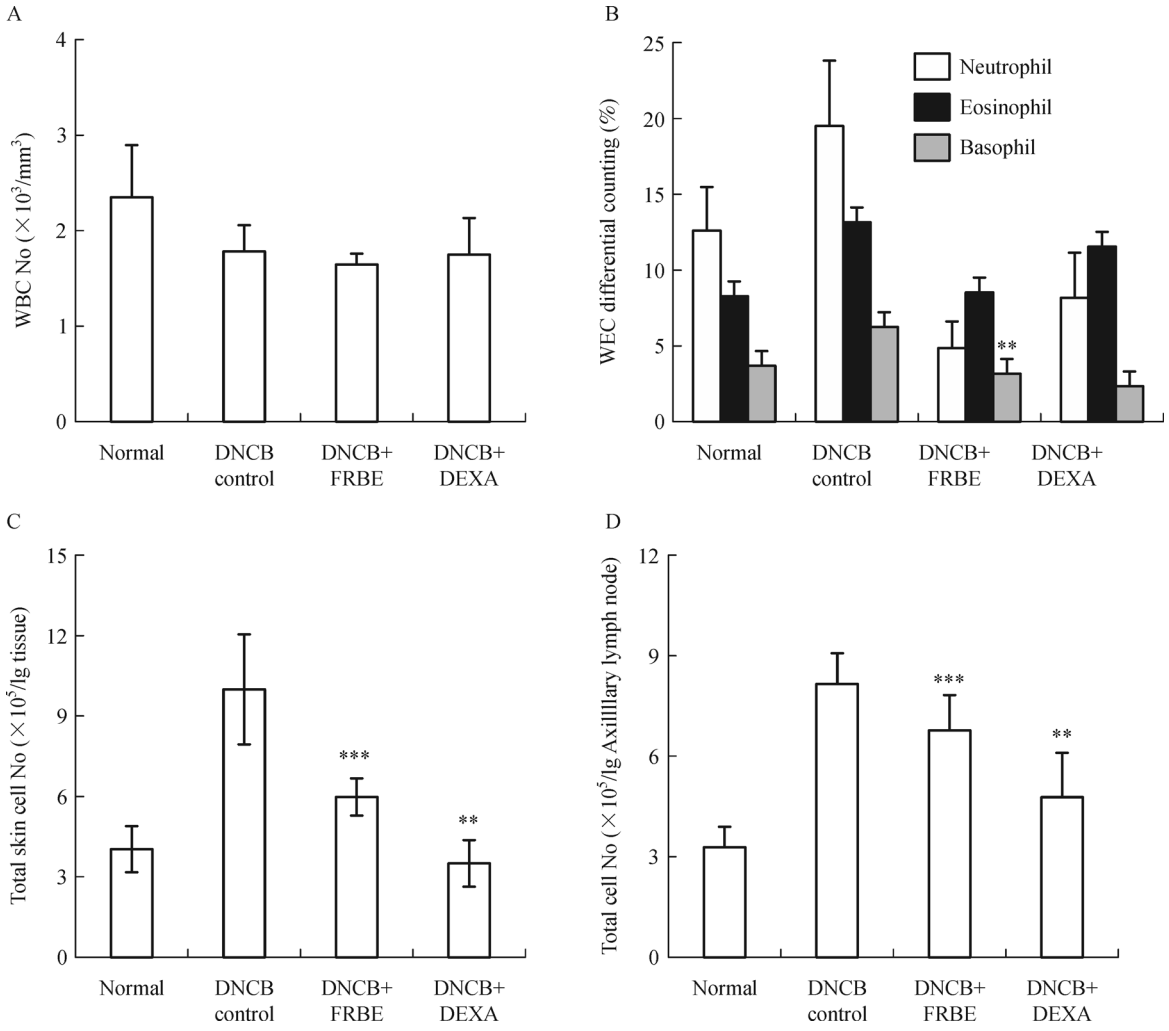

Fig. 3 **Effect of FRBE on suppression of blood cell population levels.** Blood samples were collected from each group of mice at 11 weeks of age after atopic dermatitis (AD) induction using 2,4-dinitrochlorobenzene, and subsequent treatment with fermented rice bran extract (FRBE) or dexamethasone. The total number of neutrophils, eosinophils, and basophils were counted using a hemocytometer. Bars correspond to the mean±standard error of the mean of 10 mice. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs control was considered statistically significant. A: white blood cell count as whole. B: differential white blood cell count. C: total skin cells/g of skin tissue. D: total skin cells/g of axillary lymph node.

AD. By using FACS, we found that FRBE significantly suppressed the levels of $CD4^+$, $CD8^+$, and $Gr\text{-}1^+/CD11b^+$ cells in the skin area (***Fig. 6A-B***).

Effect of FRBE on the cytokine expression in AD-induced mice

IL-5 and IL-13 are major cytokines whose levels are elevated in people suffering from AD. Similarly, COX-2, which is one of the pro-inflammatory cytokines in the body, is also raised in the inflammatory insult elicited as a result of mechanical itching leading to pruritus. Proper management with drugs or other exogenous medications is critically required to keep their levels under control as continuously elevated levels lead to autoimmune diseases and systemic inflammatory response syndrome (SIRS). As shown in ***Fig. 7A-B***, FRBE significantly decreased mRNA expression levels of these cytokines in the mice with AD.

Discussion

Topical corticosteroids have been the mainstay for treatment of AD as they are considered excellent anti-inflammatory agents and possess remarkable anti-allergic activity[18]. However, side effects posed by these steroids are irreversible, include permanent mutations in immune system functioning leading to immune suppression, and increase the susceptibility to infection with common pathogens. The current global environment is contaminated with different kinds of pathogens that can easily enter the body if proper care is not ensured. If steroidal treatment is prolonged, patients

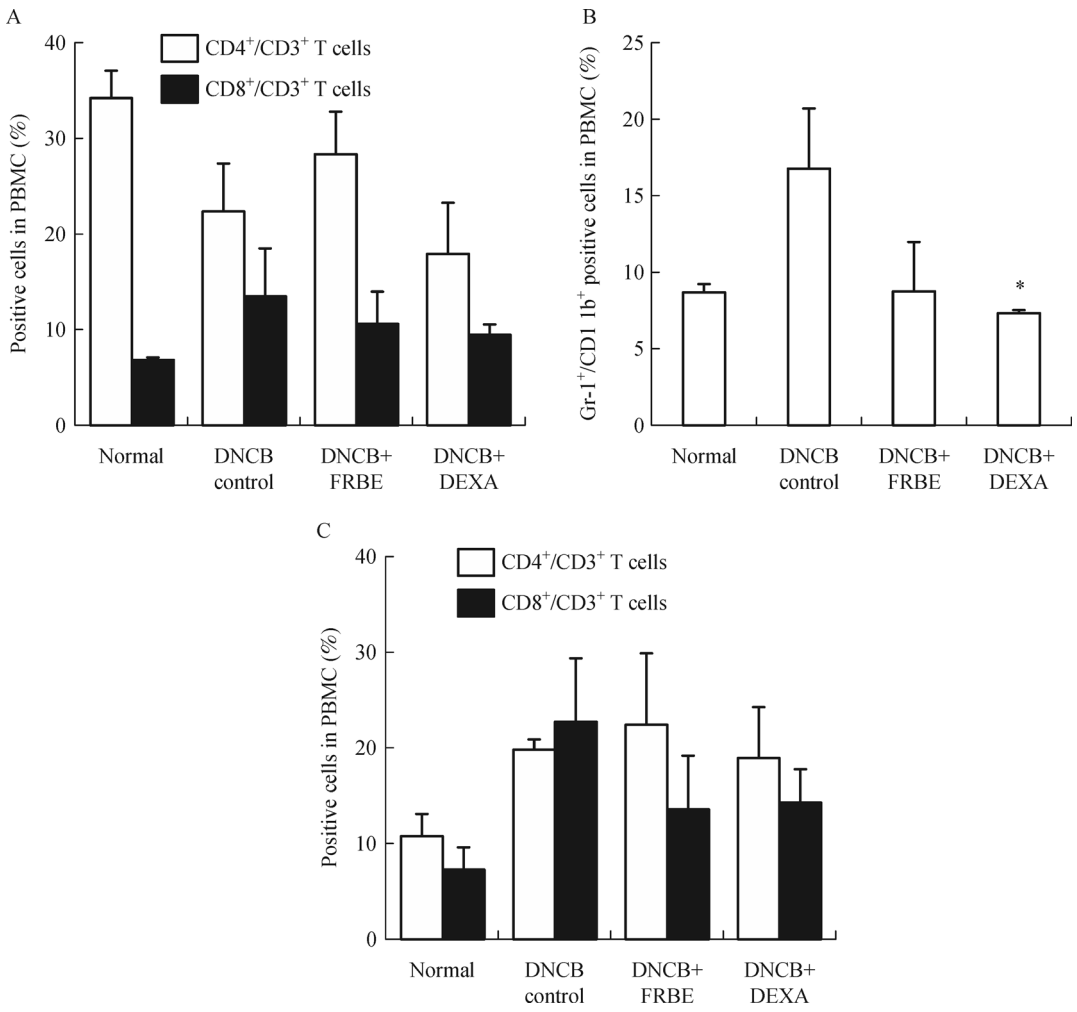

Fig. 4 **Effect of FRBE on CD4 +, CD8 +, and Gr-1 +/CD11b + cells in peripheral blood mononuclear cells (PBMCs) and axillary lymph nodes (ALN) as observed by fluorescence-activated cell sorting (FACS) analysis.** A: CD4 + and CD8 + positive cells in PBMCs decreased following FRBE treatment. B: Gr-1 +/CD11b + positive cells in PBMCs. C: Absolute number of cells in ALN tissue as analyzed by FACS. Bars represent the mean±standard error of the mean of at least 3 independent experiments. *$P < 0.05$ *vs.* control was considered statistically significant.

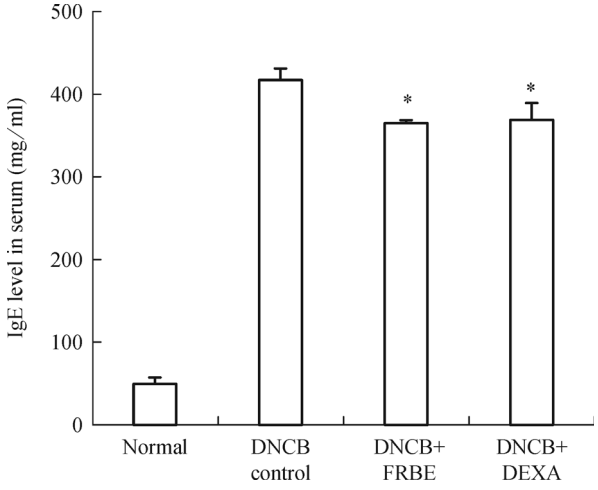

Fig. 5 **Suppressive effect of FRBE on serum immunoglobulin E levels as determined by the enzyme linked immunosorbent assay.** Bars represent mean±standard error of the mean of 3 independent experiments. *$P < 0.05$ *vs.* control was considered statistically significant.

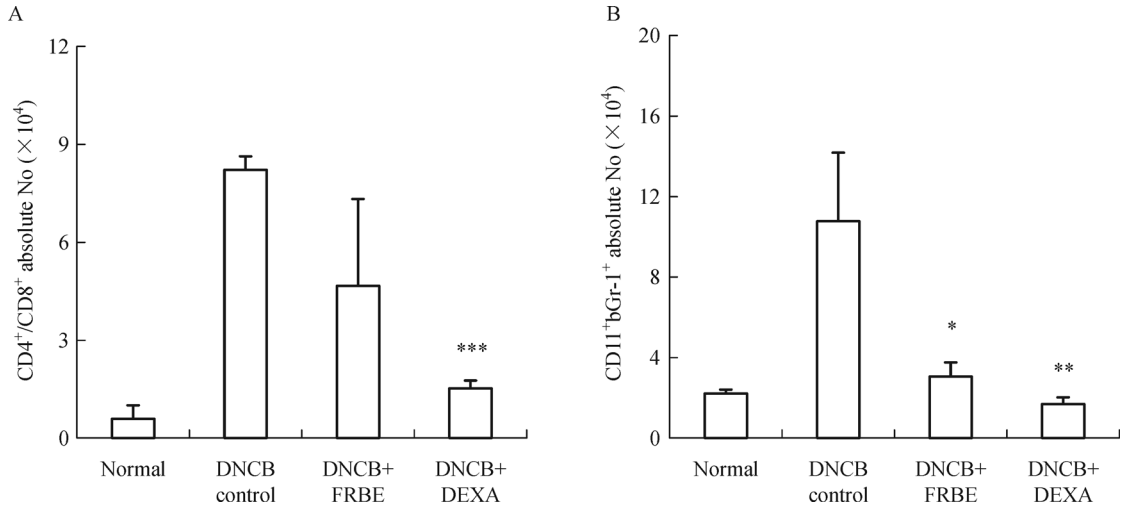

Fig. 6 **Decrease in the levels of CD4 +, CD8 +, and Gr-1 +/CD11b + in skin lesions after FRBE treatment as observed by fluorescence-activated cell sorting.** A: CD4 + and CD8 + cells in skin lesions. B: Gr-1 +/CD11b + in skin lesions. Bars are representative of mean±standard error of the mean of 3 independent experiments. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ *vs.* control was considered statistically significant.

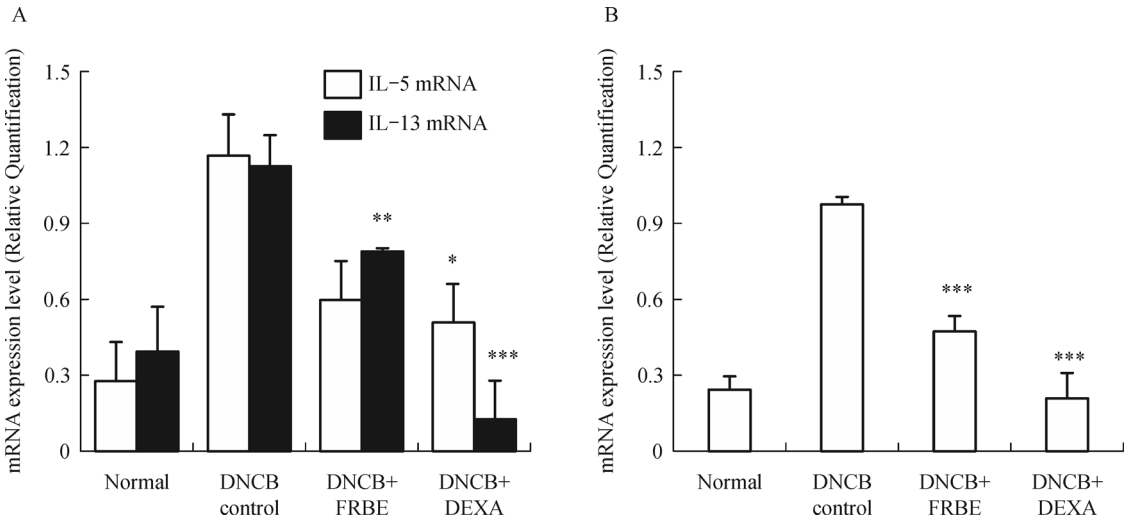

Fig. 7 **Inhibitory effects of FRBE on cytokine expressions of interleukin (IL)-5, IL-13, and COX-2 as measured using Real-time PCR.** A: mRNA expression levels of IL-5 and IL-13 compared with the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). B: mRNA expression of COX-2 compared to GAPDH. Bars represent mean±standard error of the mean values of at least 3 independent experiments. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ *vs.* control was considered statistically significant.

will have an increased susceptibility to diseases. For this reason, emphasis is being placed on natural products for the treatment of the most common recurrent diseases. These natural products include various herbs, plants, fungi, mushrooms, yeasts, and flowers. The biggest advantage of natural products is that they benefit body without any side effects, even though the healing process may be prolonged[19–20]. Therefore, we prepared a natural herbal remedy for AD, a disease that is hard to cure. FRBE is a novel composition of various plants combined with rice bran fermented by bacteria. The fermentation process in preparation of this extract contributes to the enhanced anti-allergic properties of FRBE.

FRBE is a unique formula, which has not been tested for any ailment to date. However, the constituents of this formula individually possess anti-inflammatory, anti-platelet aggregation and other medicinal properties[21]. In our study, we found that FRBE potently suppressed the visible dermal lesions induced by AD. As shown by H&E staining of dorsal skin patches, the hyperkeratosis or thickening of the stratum corneum, which is the reaction of skin cells to a manual insult such as itching, was ameliorated by the FRBE extract. The infiltration of

white blood cells, particularly lymphocytes of various kinds, is elevated in AD eczematous lesions. This is particularly due to mast cell infiltration into the diseased site, which leads to increased production of IgE and subsequent pruritus and allergic manifestations. The toluidine blue-stained skin patches showed a decrease in the number of these cells in the FRBE-treated patches when compared to skin patches of the control mice. It is known that whenever there is an exogenous pathogen invasion or endogenous insult signal, the prime cells of defense are white blood cells[16]. Our results showed that FRBE strongly decreased the amount of basophils. Basophils aggravate inflammation and allergies as their numbers are increased in allergic syndromes. They store an abundant quantity of histamine that provokes severe allergic sensations. Controlling their number and production is the key to minimizing the intensity of an allergic reaction[22].

IgE is an important component of allergic diseases as it binds itself to mast cells to sensitize or activate them. When a foreign allergen enters the body, it binds to sensitized mast cells that ultimately leads to the secretion of various allergic mediators such as histamine and cytokines[23]. Therefore, the lower the IgE level, the lesser the allergic responses and subsequent levels of lethal cytokines. As shown in ***Fig. 5***, FRBE potently ameliorated the levels of IgE, indicating its usefulness in the treatment of AD.

$CD4^+$ Th lymphocytes play a pivotal role in orchestrating immune responses. On the basis of their differentiation, they can be divided into two types. The first type is Th1 lymphocytes, which secrete interferon (IFN)-γ, tumor necrosis factor (TNF), and IL-2. The second type is Th2 lymphocytes that secrete IL-4, IL-5, IL-10, and IL-13. Both types of lymphocytes are involved in the promotion of cell-mediated and humoral immunity[24]. On the other hand, $CD8^+$ (cytotoxic) T cells, such as $CD4^+$ Th cells, are produced in the thymus and express T cell receptor. $CD8^+$ T cells are crucial for immune defense against intracellular pathogens and tumor surveillance[25]. Like $CD4^+$ cells, these cells are also involved in the secretion of various cytokines that kill the invading pathogen[26]. The problem, however, arises when their levels are highly elevated due to the continuous presence of pathogens or allergens in the body. In this case, to keep their levels in check, an exogenous agent with anti-allergic capabilities is required. $CD11^+bGr\text{-}1^+$, myeloid-derived suppressor cells, also work together with T-lymphocytes and cause allergic response if they are excessively stimulated[27].***Fig. 4A-C*** show that FRBE lowered the levels of these cells in the PBMC, axillary lymph node, and the skin.

IL-5 and IL-13 are secreted by Th2 cells that are responsible for attracting and activating eosinophils, in addition to activating mast cells to produce more IgE[5,28–29]. In our study, the FRBE extract strongly suppressed the levels of these two cytokines, indicating that it prevents the excessive secretion of IgE that leads to the extreme allergic pathology. Similarly, FRBE also ameliorated the levels of COX-2, which causes inflammation by stimulating prostaglandin and prostacyclin production.

In conclusion, FRBE was shown to have marked suppressive activity on all the basic components involved in the exacerbation of allergy. The herbal components used in this extract require further study and validation of the specific biologically active components that contribute to the anti-allergic activity. With further study, we speculate that FRBE could be a novel herbal remedy for the treatment of AD.

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