



Theobroma cacao extract attenuates the development of Dermatophagoides farinae-induced atopic dermatitis-like symptoms in NC/Nga mice



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ABSTRACT

Cacao beans from *Theobroma cacao* are an abundant source of polyphenols, particularly flavonoids. Previous studies demonstrated that cacao flavanols decrease pro-inflammatory cytokines resulting in the alleviation of allergic symptoms. We sought to investigate the effects of cacao extract (CE) on Dermatophagoides farinae extract (DFE)-induced atopic dermatitis (AD)-like symptoms. CE attenuated DFE-induced AD-like symptoms as assessed by skin lesion analyses, dermatitis score, and skin thickness. Histopathological analysis revealed that CE suppressed DFE-induced immune cell infiltration into the skin. These observations occurred concomitantly with the downregulation of inflammatory markers including serum immunoglobulin (Ig) E, chemokine; thymus and activation-regulated chemokine and macrophage-derived chemokine as well as the skin-derived cytokines interleukin (IL)-4, IL-5, and interferon- γ . CE also significantly alleviated transepidermal water loss and increased skin hydration. These results suggest that CE, a natural phytochemical-rich food, has potential therapeutic efficacy for the treatment of AD.

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1. Introduction

Atopic dermatitis (AD) is a chronic skin disorder characterized by relapsing skin inflammation, disturbance of skin barrier function, and immunoglobulin (Ig) E-mediated sensitization to allergens (Grewe et al., 1998). AD has become a significant health problem worldwide, and its prevalence has increased nearly three-fold in industrialized countries over the past three 30 years (Bieber, 2008). A recent analysis has estimated that more than one million children across 97 countries currently suffer from the

disease (Williams & Flohr, 2006). In mechanistic terms, AD is related to several other allergic disorders including asthma, food allergies, and allergic rhinitis (Leung, Boguniewicz, Howell, Nomura, & Hamid, 2004). AD is also generally thought to be one of the initial stages in the atopic March, a procession from AD to allergic rhinitis and asthma (Bantz, Zhu, & Zheng, 2014). AD arises from a complex interaction between the skin as a functional barrier and the immune system. During the progression of AD, the skin barrier function collapses owing to inflammation and itching, resulting in decreased hydration of the stratum corneum and an increase in transepidermal water loss (TEWL) (Mu, Zhao, Liu, Chang, & Zhang, 2014).

The pathogenesis of AD involves T-helper cell (Th) type 2 immune responses together with the dysregulated interleukin 4 (IL-4), IL-5, and IL-13 production (Soumelis et al., 2002). The influx of Th2 cytokines induces isotype class switching from IgM to IgE (Abril-Gil et al., 2012). Following these Th2 responses, eosinophils and mast cells are recruited toward the inflammatory skin lesions (Mochizuki, Bartels, Mallet, Christophers, & Schroder, 1998). The

Abbreviations: CE, cacao extract; AD, atopic dermatitis; DFE, Dermatophagoides farinae extract; TEWL, transepidermal water loss; TARC, thymus and activation-regulated chemokine; MDC, macrophage-derived chemokine.

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expression of CC chemokine receptor (CCR) 4 ligands, such as thymus and activation-regulated chemokine (TARC) and macrophage-derived chemokine (MDC) leads to the further recruitment of Th2 lymphocytes to the inflammatory site (Jahnz-Rozyk, Targowski, Paluchowska, Owczarek, & Kucharczyk, 2005). AD lesions frequently exhibit higher populations of infiltrating Th1 cells and cytokines from Th1 cell including interferon (IFN)- γ induce apoptosis of keratinocytes. It aggravates the symptoms of atopic dermatitis (Ong & Leung, 2006).

Recently, therapeutic ways for the treatment of AD have focused on the use of corticosteroids. However, these agents often elicit undesirable side effects including high blood pressure, sickness, vomiting, kidney injuries, and headaches. There are also complications involved when prescribing such treatments to children. Therefore, a considerable unmet need exists for development of safer and more effective AD treatments (Kang et al., 2015; Park et al., 2005).

Chocolate is one of the most widely recognized foods in the world, of which cacao is the essential ingredient (Kim et al., 2014). Cacao is an abundant source of polyphenols, particularly flavonoids, which primarily exist in the form of monomers (epicatechins and catechins), or oligomers (procyanidins) (Rios et al., 2002). Dark chocolate refers to forms that are at least 54% cacao powder, and typically contains high levels of catechins (107–132 mg/kg) and epicatechins (327–502 mg/kg) (Arts, van de Putte, & Hollman, 2000). The polyphenolic structures of these constituents have been shown to exert beneficial effects toward the prevention of various diseases, including inflammation, cancer, cardiovascular disease, and other chronic diseases (Scalbert, Johnson, & Saltmarsh, 2005). Our previous studies showed that cacao extract (CE) inhibits solar ultraviolet induced wrinkle formation in vivo and regular cocoa flavanol consumption had positive effects on facial wrinkles and elasticity in moderately photo-aged women (Kim et al., 2016; Yoon et al., 2016). However, correlation between consumption of cacao extract and the development of AD has not been reported. Therefore, we sought to investigate whether cacao extract (CE) can suppress the development of AD-like symptoms in NC/Nga mice.

2. Materials and methods

2.1. Materials

Three-week-old male NC/Nga mice were provided from SLC Japan (Tokyo, Japan). Dermatophagoides farinae extract (DFE) was purchased from Biostir (Hiroshima, Japan). Tacrolimus was purchased from Astellas Pharma (Tokyo, Japan). All ELISA kits were purchased from R&D Systems (Minneapolis, MN). RNA Isolation Kit were purchased from Ambion Ltd (Huntingdon, Cambridgeshire, UK). PrimeScript™ 1st strand cDNA synthesis Kit was purchased from Takara Bio Inc. (Shiga, Japan). IQ SYBR was purchased from Bio-Rad Laboratories, Hercules, CA. Sodium dodecyl sulfate was purchased from Sigma (St. Louis, MO).

2.2. Preparation of CE and animal diets

Cacao extract (CE) was provided by Barry Callebaut (Lebbeke-Wieze, Belgium). *Theobroma cacao* beans were roasted and ground to make cacao liquor, which was separated from cacao butter, to produce cacao cakes. Cacao cakes were ground and 300 kg of ground cacao was extracted using 2400 L of 70% ethanol at 60 °C for 12 h. The extract was filtered and dried (Kim et al., 2016). CE was produced by grinding the cacao cakes. AIN-93G formulation (Harlan, Spain) was used as the standard diet for the animal study. Previous studies showed that this diet does not comprise detectable

concentrations of polyphenols (Ramiro-Puig, Urpi-Sarda et al., 2007). 0.25% and 1% CE diets were made by alteration of the AIN-93G formula by deducting the amount of carbohydrates, proteins, lipids and fiber provided by the related percentage of added CE. Accordingly, the final chow was isoenergetic with the standard diet.

2.3. Characterization of CE

The filtrate (10 μ l) was injected into HPLC (Thermo Fisher Scientific, Waltham, MA). For chromatographic analysis (wavelength: 280 nm, solvent gradient: 40 min), C18 column (4.6 $\times$ 250 mm) was used. Three compounds were analyzed under these HPLC conditions and they did not have any other ingredients (Fig. 1).

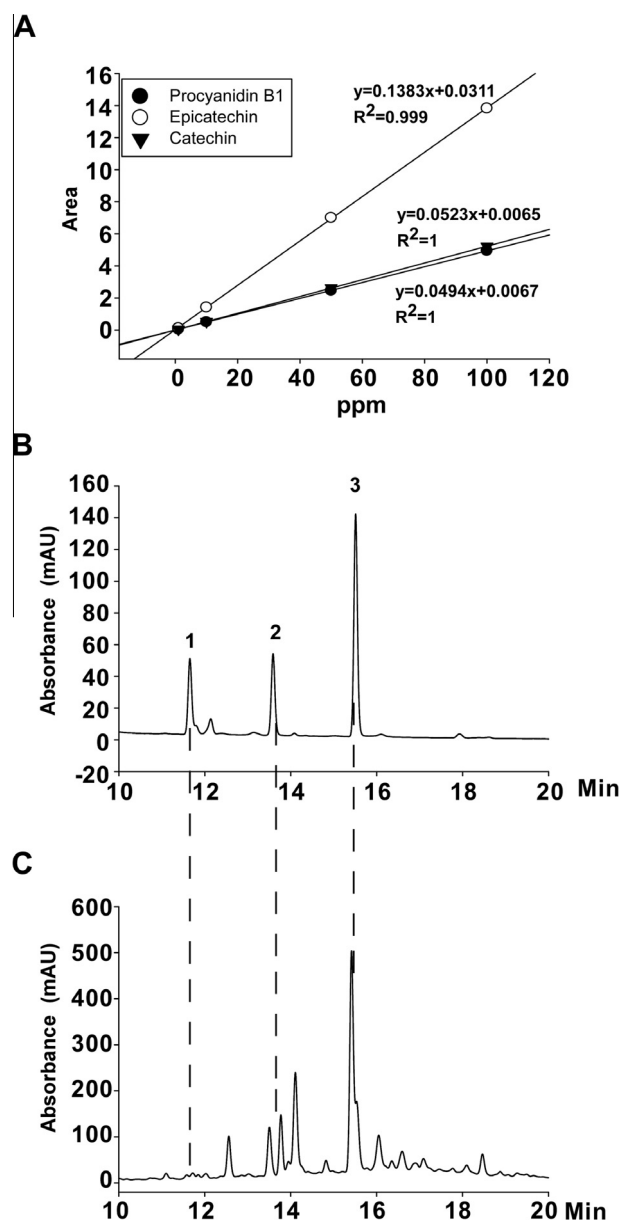
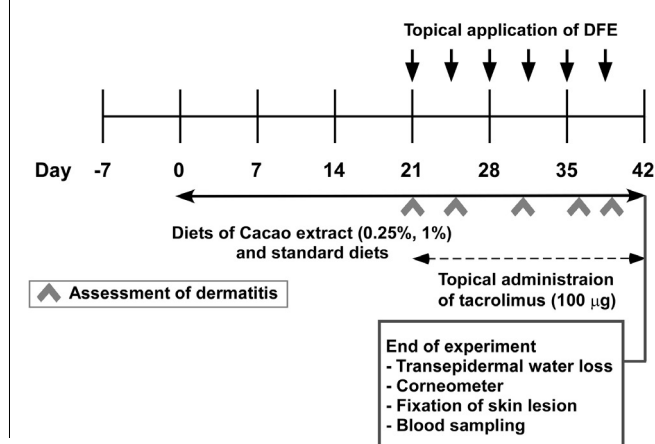


Fig. 1. Characterization of cacao extract (CE) by HPLC. The amount of the active marker (procyanidin B1, catechin, epicatechin) were analyzed using an analytical HPLC with an acetonitrile and water gradient system. The analytes were quantified by UV (280 nm). A, Calibration curves of each chemical. B, The HPLC spectrum of standard. C, The HPLC chromatography of CE. 1: procyanidin B1, 2: catechin, 3: epicatechin.

Schedule of experiment



Groups and treatment

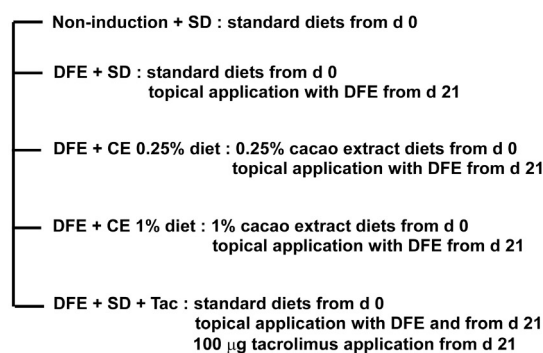


Fig. 2. Experimental design overview.

2.4. Animals

All experimental protocols were followed the regulation of the Institutional Animal Care and Use Committee (IACUC) of Seoul National University, Korea (Case Number: SNU-131119-2). The overall experimental design is depicted in Fig. 2. After 1 week of acclimation, mice were divided into the following five groups ($n = 8$ per group): (1) Non-induction + standard diet, (2) DFE + standard diet (3) DFE + CE 0.25% standard diet, (4) DFE + CE 1% standard diet, (5) DFE + tacrolimus + standard diet. Mice were fed on their designated diets for 6 weeks. On the third week, DFE was topically applied onto the mice twice per week for 3 weeks. For positive control, Tacrolimus (0.1% Protopic Ointment, 100 µg/mouse/day) was topically applied onto back skins of the mice five times a week for 3 weeks (Curry Martins et al., 2015).

2.5. Induction of AD-like symptoms

For shaving, NC/Nga mice were anesthetized with 2% isoflurane (HanaPharm, Seoul, Korea) and an electric clipper (Daito Thrive, Tokyo, Japan) and shaving cream (Reckitt Benckiser, Cedex, France) was used for shaving. For disruption of the skin barrier, 150 ml of 4% sodium dodecyl sulfate was topically applied. To induced AD-like symptoms, 100 mg Biostir cream including DFE was applied to the dorsal skin, the face and both ears of the mice, twice per week for 3 weeks (Yamamoto et al., 2007).

2.6. Assessment of skin lesions and dermatitis score

NC/Nga mice were anesthetized with 2% isoflurane, and pictures of the dorsal skin were taken using a digital camera (Canon SX 40 HS, Tokyo, Japan) in the last week of the experiment (Yamamoto et al., 2007). The dermatitis scores were evaluated five times in three weeks. Based on four symptoms (erythema/hemorrhage, scaling/dryness, edema and excoriation/erosion), scores of 0 (none), 1 (mild), 2 (moderate), and 3 (severe) were given. A total dermatitis score was specified as the sum of all scores (maximum: 12) (Jin, He, Oyoshi, & Geha, 2009).

2.7. Histological examination

For evaluation of epidermal thickness, the dorsal skin of each mouse was collected on the final day of the experiment, followed by fixing with 10% neutral-buffered formalin, and embedded in paraffin. 4 mm thick sections were sliced and moved onto slides. Hematoxylin and eosin reagent was used for deparaffinized skin staining and finally analyzed at 100× magnification. For the detection of eosinophils and mast cells infiltration, same paraffin materials were used (4 µm thick). These deparaffinized skin sections were stained with Congo red and Toluidine blue, severally. The number of each immune cells per 0.025 mm² skin was determined at 400× magnification. Tissue sections were analyzed using an Olympus AX70 light microscope (Tokyo, Japan). Epidermal thickness was measured by calculating the area-length ratio in single sections of skin (Yamamoto et al., 2007).

2.8. Measurement of serum IgE, TARC and MDC

Blood samples were obtained from anesthetized mice by cardiac puncture on the last day of the experiment, and serum collected from the whole blood was kept at −80 °C until further use. The total levels of IgE, and chemokines in the serum were measured using an ELISA kit (R&D Systems, Minneapolis, MN), according to the manufacturer's manual.

2.9. Measurement of Th2 and Th1 cytokines mRNA levels

For frozen skin tissue, total RNA was collected using an Ambion RNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA). RNA quantity was measured using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific). PrimeScript™ 1st strand cDNA synthesis Kit was used for cDNA synthesis. Real-time quantitative RT-PCR was conducted using IQ SYBR and 2 µl of cDNA in triplicate with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a quantitative control. The primers were denatured at 95 °C for 3 min before PCR amplification. The amplification program was consisted of 44 cycles at 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s. PCR was conducted with a CFX Connect™ Real-Time PCR Detection System. cDNA was probed with the following primers: IL-4 forward (5'-TCA TCG GCA TTT TGA ACG AG-3'); IL-4 reverse (5'-CGT TTG GCA CAT CCA TCT CC-3'); IL-5 forward (5'-AAA GAG AAG TGT GGC GAG GAG A-3'); IL-5 reverse (5'-CAC CAA GCA ACT CTT GCA GGT AA-3'); IFN-γ forward (5'-AAC GCT ACA CAC TGC ATC TTG G-3'); IFN-γ reverse (5'-GCC GTG GC AGTA ACA GCC-3'); GAPDH forward (5'-CAA GGA GTA AGA AAC CCT GGA CC-3'); GAPDH reverse (5'-GCC ATG GTA TTT GGA GCA CT-3').

2.10. Assessment of TEWL and corneometer units

TEWL measures the volume of water that across from inside the body into the atmosphere through the epidermal layer (skin) through diffusion and evaporation (Kelleher et al., 2015). TEWL was determined in mouse dorsal skin on the last day of the

experiment under precise conditions at 21–22 °C and 50–55% humidity, using a skin evaporation recorder Tewameter TM300 (Courage and Khazaka, Cologne, Germany). Skin hydration was measured by using Corneometer CM 825 (Courage and Khazaka). This device assesses the skin moisture level by electrical capacitance (Clarys, Clijsen, Taeymans, & Barel, 2012). These measurements were repeated 10 times for the same area and same pressure.

2.11. Statistical analysis

Statistical analysis was processed with SPSS Statistics Software (SPSS Inc., Chicago, IL). The data are shown as mean $\pm$ standard error (SEM). One-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test was applied to

check significance between multiple groups. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Phytochemical analysis of CE by HPLC

In our previous study, CE has 71.5 mg/g of flavanol (Kim et al., 2016). Procyanidin B1, catechin, and epicatechin are the well-known functional components of cacao and used as markers for quality control (Calderon, Wright, Hurst, & van Breemen, 2009). Therefore, we analyzed these chemicals in this study using HPLC (Fig. 1). Comparison of the peaks was conducted on the basis of their UV absorption spectra and retention times, which presented

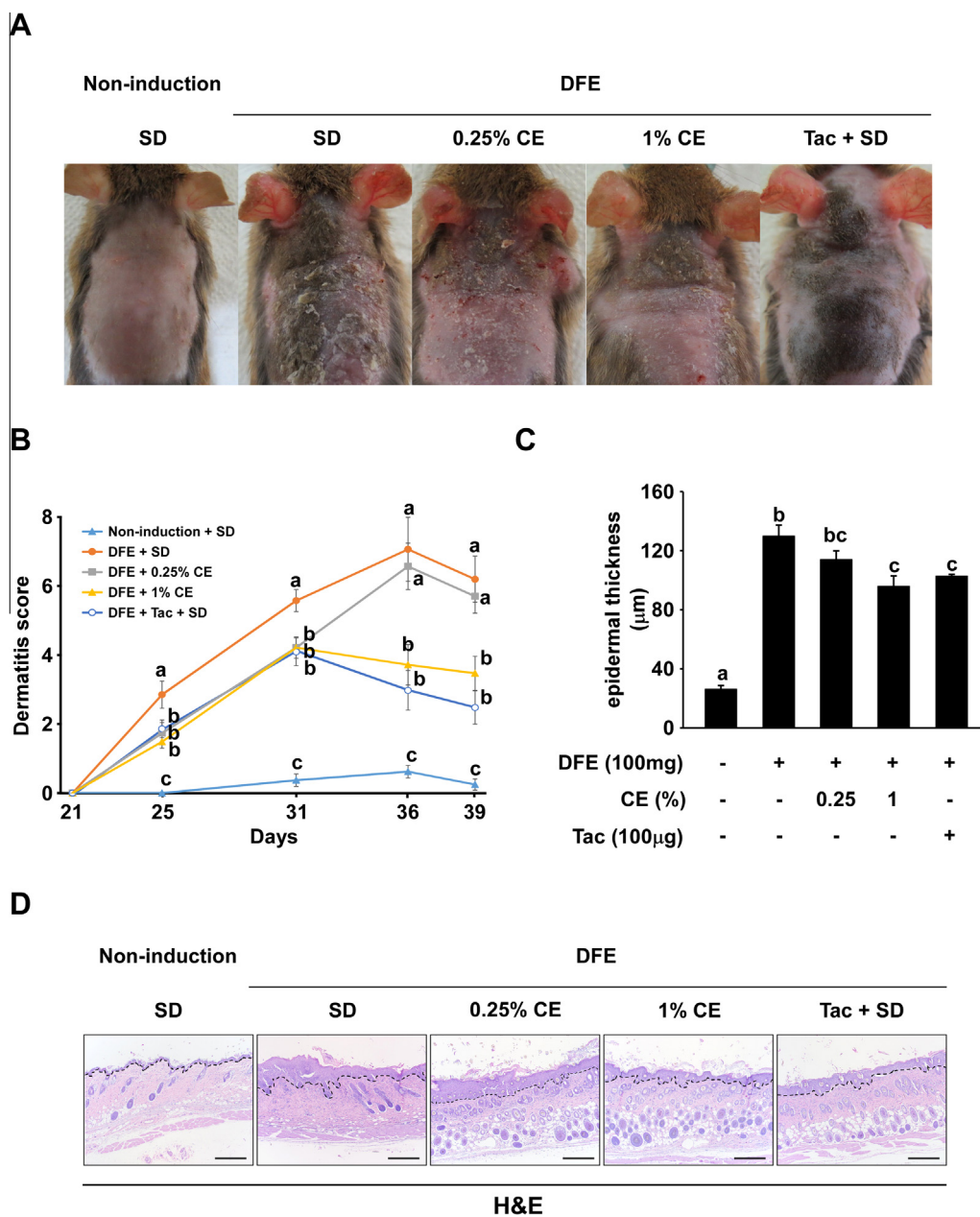


Fig. 3. Effect of CE on DFE-induced AD-like symptoms in NC/Nga mice. **A**, Skin lesions typical of AD. Images of skin lesions were taken in the sixth week. **B**, Dermatitis scores. The scoring represents a clinical index that was evaluated each week from Day 21–39. **C**, Epidermal thicknesses of skin lesions was measured until the final week. **D**, H&E stained skin recovered in the sixth week (100 $\times$). Dotted lines indicate epidermal hypertrophy. Scale bar is 200 μm . Data represent mean values $\pm$ SEM ($n = 6$). Means with letters (a–c) within a graph are significantly different from each other at $p < 0.05$.

that the peaks for CE corresponded to procyanidin B1 (1, 11.65 min, 0.2 mg/g), catechin (2, 13.59 min, 2 mg/g) and epicatechin (3, 15.51 min, 3 mg/g).

3.2. CE supplementation attenuates DFE-induced AD-like skin symptoms in NC/Nga mice

To investigate the effects of CE on DFE-induced AD-like symptoms in NC/Nga mice, pictures of dorsal skin lesions were used to determine the dermatitis score, and ear thickness was measured. In the last week, the dorsal lesions of DFE-treated mice shown to exhibit severe AD-like symptoms including erythema, erosion, and dryness. In contrast, mice that were supplemented with CE or topical applications of tacrolimus exhibited markedly reduced severity in their skin lesions (Fig. 3A). In DFE-treated mice, the dermatitis score was significantly increased (6.25 ± 0.67) compared with the non-induced + SD group (0.25 ± 0.16) (Fig. 3B). Intake of 1% CE (3.5 ± 0.50) or tacrolimus (2.50 ± 0.49) significantly reduced the DFE-induced increases in dermatitis score (Fig. 3B). The epidermal thickness was significantly higher in DFE-treated NC/Nga mice ($129.64 \pm 7.64 \mu\text{m}$) than the non-induced + SD group ($26.01 \pm 2.70 \mu\text{m}$) (Fig. 3C). Intake of 1% CE ($95.70 \pm 7.29 \mu\text{m}$) or topical application of tacrolimus ($102.48 \pm 1.49 \mu\text{m}$) also markedly reduced the DFE-induced increases in epidermal thickness (Fig. 3C). In the sixth week, the skin of each NC/Nga mouse was examined. The skins of DFE-treated NC/Nga mice exhibited a swollen epidermis and epidermal hypertrophy. Intake of CE or topical application of tacrolimus was observed to attenuate this epidermal hypertrophy in comparison to DFE-treated NC/Nga mice (Fig. 3D).

3.3. CE supplementation improves skin hydration by an attenuation of DFE-induced TEWL increase in NC/Nga mice

To investigate the effect of CE on DFE-induced loss of skin hydration through the epidermis, TEWL was measured in the final week. DFE-treated NC/Nga mice ($63.78 \pm 4.81 \text{ g/m}^2/\text{h}$) exhibited higher levels of TEWL than the non-induced + SD group ($5.06 \pm 1.43 \text{ g/m}^2/\text{h}$). Intake of 0.25% ($37.06 \pm 2.67 \text{ g/m}^2/\text{h}$), 1% CE ($38.16 \pm 2.84 \text{ g/m}^2/\text{h}$), or topical application of tacrolimus ($35.34 \pm 3.71 \text{ g/m}^2/\text{h}$) significantly reduced TEWL in DFE-treated NC/Nga mice (Fig. 4A). DFE-treated NC/Nga mice (3.71 ± 0.64) also exhibited lower levels of skin hydration when measured with a corneometer unit, in comparison to the non-induced + SD group (24.58 ± 4.93). Intake of 0.25% CE (11.04 ± 1.19) significantly increased skin hydration in DFE-treated NC/Nga mice (Fig. 4B).

3.4. CE supplementation suppresses DFE-induced infiltration of eosinophils and mast cells into skin lesions in NC/Nga mice

Congo red (CR) was used to identify eosinophils in the skin of NC/Nga mice (Fig. 5A and B), while toluidine blue (TB) was used to identify mast cells (Fig. 5C and D). The number of eosinophils stained by CR per mm^2 in skin lesions of DFE-treated mice ($886.67 \pm 71.12 \text{ cells/mm}^2$) was significantly increased compared with the non-induced + SD group ($80.00 \pm 14.61 \text{ cells/mm}^2$). Intake of 0.25% ($146.67 \pm 8.43 \text{ cells/mm}^2$), 1% CE ($126.67 \pm 16.06 \text{ cells/mm}^2$), or topical application of tacrolimus ($120.00 \pm 17.89 \text{ cells/mm}^2$) significantly reduced the number of eosinophils in the skin lesion areas (Fig. 5A and B).

Similarly, the number of mast cells stained by TB per mm^2 in skin lesions of DFE-treated mice ($273.33 \pm 19.10 \text{ cells/mm}^2$) was significantly increased compared with the non-induced + SD group ($20.00 \pm 8.94 \text{ cells/mm}^2$). Intake of 0.25% ($113.33 \pm 12.29 \text{ cells/mm}^2$), 1% CE ($66.67 \pm 7.30 \text{ cells/mm}^2$), or topical application of tacrolimus ($53.33 \pm 8.43 \text{ cells/mm}^2$) significantly reduced the number of mast cells in the skin lesions of DFE-treated mice (Fig. 5C and D).

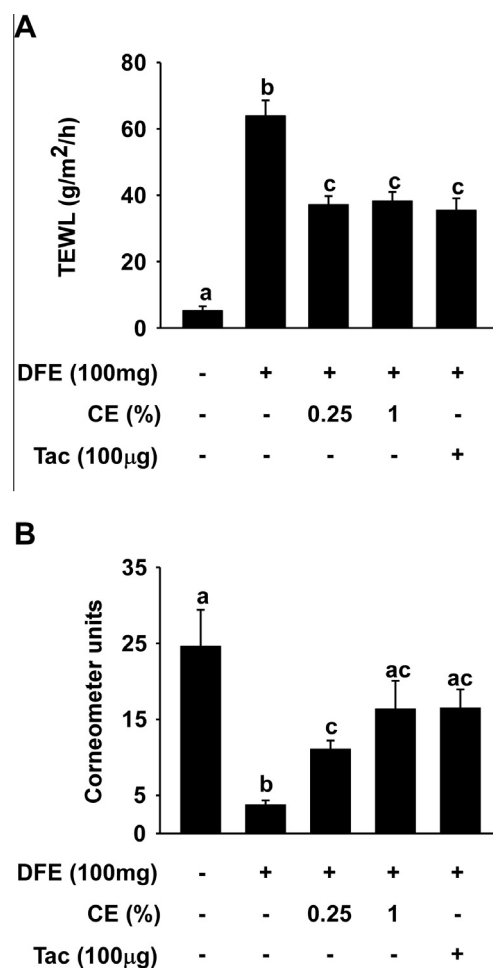


Fig. 4. Effect of CE on DFE-induced decreases in skin hydration. A, TEWL in mouse dorsal skin was measured using a skin evaporative water recorder in the final week of the experiment. B, The level of skin hydration in mouse dorsal skin was also measured using a skin corneometer recorder in the final week. Data represent the mean values $\pm$ SEM ($n = 6$). Means with letters (a–c) within a graph are significantly different from each other at $p < 0.05$.

mm²), 1% CE ($66.67 \pm 7.30 \text{ cells/mm}^2$), or topical application of tacrolimus ($53.33 \pm 8.43 \text{ cells/mm}^2$) significantly reduced the number of mast cells in the skin lesions of DFE-treated mice (Fig. 5C and D).

3.5. CE supplementation attenuates DFE-induced increases in IgE, TARC, and MDC levels in NC/Nga mice

To investigate the effect of CE on DFE induction of IgE, TARC, and MDC in NC/Nga mice, blood samples were collected in the sixth week. DFE-treated NC/Nga mice ($178.91 \pm 28.53 \text{ ng/ml}$) exhibited higher levels of IgE than the non-induced + SD group ($8.00 \pm 0 \text{ ng/ml}$) (Fig. 6A). Intake of 1% CE ($68.89 \pm 8.94 \text{ ng/ml}$) significantly reduced levels of IgE in serum (Fig. 6A). TARC is induced in DFE-treated NC/Nga mice ($55.15 \pm 4.47 \text{ pg/ml}$) than the non-induced + SD group ($33.45 \pm 5.15 \text{ pg/ml}$) (Fig. 6B). Intake of 0.25% ($4.72 \pm 4.31 \text{ pg/ml}$), 1% CE ($4.39 \pm 1.68 \text{ pg/ml}$) or topical application of tacrolimus ($10.20 \pm 5.48 \text{ pg/ml}$) significantly reduced levels of TARC (Fig. 6B). DFE-treated NC/Nga mice ($136.57 \pm 13.59 \text{ pg/ml}$) also exhibited higher levels of MDC than the non-induced + SD group ($68.10 \pm 4.27 \text{ pg/ml}$) (Fig. 6C). Intake of 0.25% ($17.26 \pm 2.77 \text{ pg/ml}$), 1% CE ($16.34 \pm 2.15 \text{ pg/ml}$) or topical application of tacrolimus ($36.75 \pm 7.62 \text{ pg/ml}$) significantly reduced levels of MDC in serum (Fig. 6C).

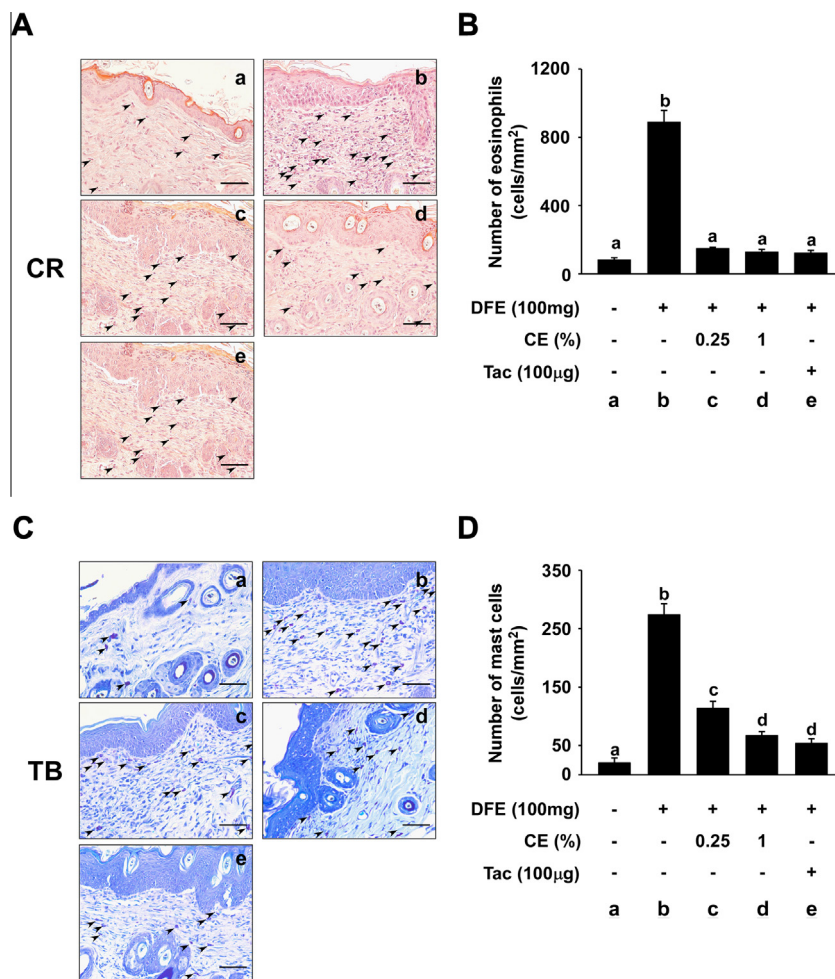


Fig. 5. Effect of CE on DFE-induced eosinophil and mast cell infiltration into the skin lesions. **A**, Representative images depicting the histological features of skin lesions are shown. Skin lesions were stained with Congo red (CR) to identify eosinophils. The arrows indicate the CR-stained eosinophils. Eosinophils were counted under a microscope at 400× magnification. Scale bar is 50 µm. **B**, The numbers of eosinophils in 1 mm² sections of skin lesion were measured and graphed. Data represent the mean values ± SEM (n = 6). **C**, Representative images depicting the histological features of skin lesions. Skin lesions were stained with toluidine blue (TB) to identify mast cells. The arrows indicate the TB-stained mast cells. Mast cells were counted under a microscope at 400× magnification. Scale bar is 50 µm. **D**, The numbers of mast cells in 1 mm² sections of skin lesion were measured and graphed. Data represent the mean values ± SEM (n = 6). Means with letters (a–c) within a graph are significantly different from each other at $p < 0.05$.

3.6. Intake of CE attenuates DFE-induced increases in cytokine mRNA in NC/Nga mice

To investigate the effect of CE on DFE-induced increases in cytokine mRNA, skin tissues were collected on the sixth week. The mRNA levels of the Th2 cytokines IL-4 and IL-5 were significantly increased in skin lesions of DFE-treated NC/Nga (28.69 ± 7.18 and 9.84 ± 3.28) mice compared to the non-induced + SD group (2.03 ± 1.02 and 0.84 ± 0.27) (Fig. 6D and E). Intake of 0.25% CE (5.32 ± 0.64) significantly reduced the mRNA levels of IL-4 (Fig. 6D), while intake of 1% CE (1.84 ± 0.70) significantly reduced the mRNA levels of IL-5 (Fig. 6E). The mRNA levels of the Th1 cytokine IFN- γ were significantly increased in skin lesions of DFE-treated NC/Nga (11.27 ± 0.60) mice compared with the non-induced + SD group (1.34 ± 0.63). Intake of 1% CE (2.41 ± 2.03) also significantly reduced the mRNA levels of IFN- γ (Fig. 6F).

4. Discussion

Previous studies have demonstrated that *Theobroma cacao* extract can modify the activity of lymphoid cell lines (Ramiro, Franch, Castellote, Andres-Lacueva et al., 2005). Also its diet

decreased spleen cell IgG, IgM secretion and total IgA secreting cells (Ramiro-Puig, Perez-Cano et al., 2007). In addition, a cacao diet in rats has been shown to induce ovalbumin (OVA) alleviated anti-OVA IgG1 (the main isotype of the Th2 immune response) and IgE (Abril-Gil et al., 2012). These reports led us to hypothesize that CE might have immunomodulatory properties applicable to the treatment of AD.

The NC/Nga mice used in these experiments are currently available as an animal model of human clinical AD case (Tanaka, Amagai, Oida, & Matsuda, 2012). NC/Nga mice spontaneously develop AD-like skin lesions with elevation in total IgE levels of plasma (Matsumoto et al., 1999). House dust mite allergens are associated with human AD. Therefore, DFE induced NC/Nga mice is relevant to human AD (Tanaka & Matsuda, 2011; Tanaka et al., 2012). The skin lesions of these mice are distinguished by edema, hemorrhage, erosion, dryness and express lymphocyte infiltration with a high CD4/CD8 ratio, as well as mast cell and eosinophil degranulation. Th2 cytokines such as IL-4 and IL-5 are both produced by mast cells in these lesions, and CD4+ T cells also produce IFN- γ at later stages (Vestergaard et al., 1999). Therefore, our results propose that CE supplementation may relieve the symptoms of atopic dermatitis.

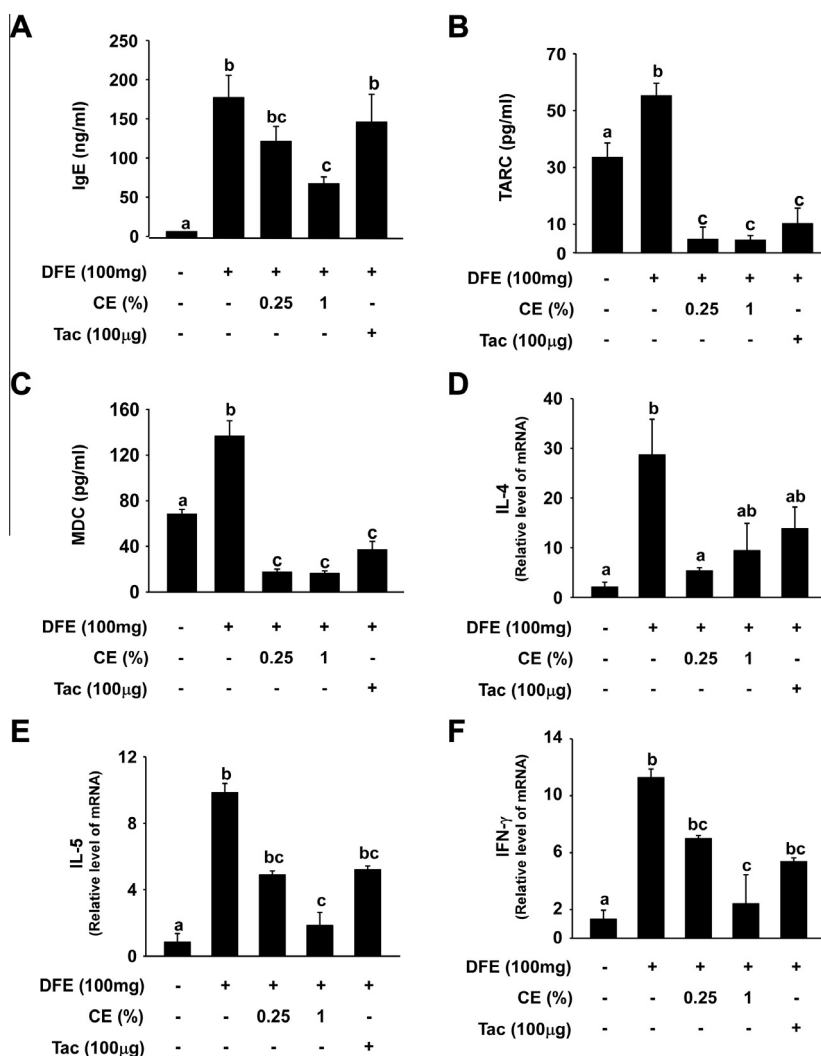


Fig. 6. Effect of CE on DFE-induced increases in serum IgE, TARC, MDC levels in the skin lesions and cytokine mRNA. Levels of serum IgE (A, $n = 7$), TARC (B, $n = 7-8$), and MDC (C, $n = 7-8$), were measured by ELISA. IL-4 (D), IL-5 (E), and IFN- γ (F) mRNA was analyzed by real-time quantitative RT-PCR. Data represent the mean values $\pm$ SEM ($n = 6-7$). Means with letters (a-c) within a graph are significantly different from each other at $p < 0.05$.

The chemokines TARC and MDC in serum trigger increases in Th2 cytokines (IL-4 and IL-5). In this study, CE inhibits DFE-induced TARC and MDC expression. CE decreased the level of these cytokines even below those of non-induced animals. NC/Nga mice have elevated level of TARC and MDC and show atopic dermatitis symptoms spontaneously (Tanaka & Matsuda, 2011; Tanaka et al., 2012). CE could inhibit basal TARC and MDC expression in NC/Nga mice. These Th2 cytokines are known to infiltrate immune cells, with IL-4 activating mast cells (Yamaguchi et al., 1999), and IL-5 triggering the differentiation of eosinophils (Dent, Strath, Mellor, & Sanderson, 1990). Th1 cytokines are also involved in AD severity. IFN- γ (a Th1 cytokine) activates Fas (CD95) which in turn activates keratinocytes, triggering the formation of eczematous lesions (Trautmann et al., 2000). AD patients have defective skin barriers that increase water loss through the epidermis. TEWL is well correlated with AD severity and itch intensity. It is also important to recover skin hydration in AD patients by reducing TEWL for protection against allergens and pathogens (Berardesca, Fideli, Borroni, Rabbiosi, & Maibach, 1990). Through our result, we demonstrated that CE diet could down-regulate inflammatory chemokines while protecting water loss from skin surface.

Polyphenols such as catechin, epicatechin, and procyanidin are shown to regulate several inflammatory mediators (Ramiro,

Franch, Castellote, Perez-Cano et al., 2005). To analyze the contents of effective polyphenols in CE, we conducted HPLC analysis and the result showed that CE contains high level of procyanidins and epicatechins. Therefore, we can speculate that cacao polyphenols inhibit production and secretion of pro-inflammatory molecules resulting in the alleviation of AD. In a previous study, rat allergy model was used to examine the effects of cocoa diet and the highest cocoa diet used contained 22 mg/g of polyphenols (Abril-Gil et al., 2012). In our study, we used 83 mg/g polyphenol-enriched cacao extract and polyphenol content was at least 4 times higher than the cocoa diet used in the previous study. Therefore, we selected 1% cacao diet as the highest group which can be translated into 156.25 mg/kg/day polyphenols in human.

In summary, we observed that cacao extract suppressed the development of AD-like symptoms in NC/Nga mice. These results suggest that the intake of sufficient quantities of CE may be useful for the treatment of AD by modifying the balance of Th1 and Th2 cytokine production. Our observations included decreases in epidermal thickness, attenuation of immune cell infiltration into lesion areas, as well as reductions in TEWL score and inflammatory chemokines. Further experiments are necessary to determine the precise compounds and molecular mechanisms responsible for these effects.

Conflict of interest

The authors declare that there is no conflict of interest.

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