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Immunosuppressive effects of fisetin against dinitrofluorobenzene-induced atopic dermatitis-like symptoms in NC/Nga mice

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ABSTRACT

Atopic dermatitis (AD) is a multifactorial chronic skin disorder that is increasing in prevalence globally. In NC/Nga mice, repetitive epicutaneous applications of 2,4-dinitrofluorobenzene (DNFB) induces AD-like clinical symptoms. Bioflavonoid fisetin (3,7,3',4'-tetrahydroxyflavone) is a dietary component found in plants, fruits and vegetables. Fisetin has various physiological effects that include anti-oxidation, anti-angiogenesis, anti-carcinogenesis and anti-inflammation. In this study, we investigated whether fisetin relieves AD-like clinical symptoms induced by repeated DNFB treatment in NC/Nga mice. Fisetin significantly inhibited infiltration of inflammatory cells including eosinophils, mast cells and CD4⁺ T and CD8⁺ T cells, and suppressed the expressions of cytokines and chemokines associated with dermal infiltrates in AD-like skin lesions. Total serum immunoglobulin E (IgE) levels and the ratio of phospho-NF-κB p65 to total NF-κB p65 were markedly reduced by fisetin. Fisetin also reduced the production of interferon-γ and interleukin-4 by activated CD4⁺ T cells in a dose-dependent manner, whereas the anti-inflammatory cytokine, interleukin-10 was increased. These results implicate fisetin as a potential therapeutic for AD.

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1. Introduction

Atopic dermatitis (AD) is a chronically relapsing and pruritic inflammatory skin disease that clinically manifests as edema, erythematous, scaly and lichenified lesions that develop by cutaneous hyper-sensitivity, immunoglobulin E (IgE)-mediated sensitization and intense pruritus (Leung and Bieber, 2003). AD affects up to 18% of children and up to 5% of adults worldwide. The prevalence has increased approximately 3-fold over the past 30 years and its incidence is still increasing (Shaw et al., 2011). AD has a multiple and complex pathogenesis including exposure to chemical or environmental agents, and genetic or immunologic abnormality. This complexity has hindered the development of a truly efficacious AD treatment (Ong and Leung, 2006). AD is a biphasic inflamma-

tory skin disease consisting of acute and chronic phases (Jin et al., 2009). In the acute phase, disruption of skin barrier function leads to invasion of external antigens including mites and microorganisms including bacteria, which induce a Th₂-dominant immune response (Nakagawa et al., 2011). In acute AD skin lesions, infiltrated Th₂ cells and the levels of associated cytokines such as interleukin (IL)-4, IL-5, and IL-13 are increased (Grewe et al., 1998). These Th₂-cytokines are associated with eosinophil infiltration, IgE hyper-production and mast cell activation. In contrast, the chronic phase of AD shows delayed-type hypersensitivity (DTH) and Th₁-dominant immune response by an interferon-γ and IL-2 response that induces dermal thickening and tissue remodeling by excessive collagen accumulation (Hamid et al., 1994; Leung, 1999). In a recent study showed that the application of topical antigens and chemical haptens, such as 2,4,6-trinitrochlorobenzene (TNCB) or 2,4-dinitrofluorobenzene (DNFB), on murine skin induce AD-like clinical symptoms (Lee et al., 2007). DNFB binds Lys and Cys residues in epidermal proteins, which evokes enhanced proliferation of effector T cells and disturbed epidermal barrier in murine models of AD

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(Saint-Mezard et al., 2004). NC/Nga mice showed clinical symptoms similar to those of AD in humans and so have been the most extensively studied animal model of AD (Vestergaard et al., 2000b). Under specific pathogen-free (SPF) conditions, the mice remain healthy, whereas in uncontrolled conventional conditions or upon the administration of a chemical hapten in SPF conditions, AD-like clinical symptoms with erythema, scaling, itching and dryness spontaneously develop at 6–7-weeks-of-age (Suto et al., 1999). Furthermore, serum IgE, thymus- and activation-regulated chemokine (TARC) levels are increased, which may play a critical role in recruiting Th₂ cells to the AD-like skin lesions (Vestergaard et al., 2000a). Fisetin (3,7,3',4'-tetrahydroxyflavone) is commonly found in vegetables and fruits including strawberry, apple, persimmon, grapes and onion at concentrations ranging from 2 to 160 µg/g. Fisetin has broad pharmacological effects that include anti-oxidation, anti-inflammation, anti-carcinogenesis and anti-angiogenesis (Khan et al., 2013). Recent studies showed that fisetin suppresses cyclooxygenase (COX)-2 and prostaglandin E₂ (PGE₂) expression in HT29 human colon cancer cells (Suh et al., 2009), inhibits histamine release and IL-4 expression in human mast cells (Park et al., 2008) and down-regulates the production of IL-4, IL-5 and IL-13 by activated basophiles in vitro (Higa et al., 2003). In vivo studies revealed that fisetin ameliorates lipopolysaccharide (LPS)-induced acute pulmonary inflammation (Geraets et al., 2009), streptozotocin-induced diabetes (Prasath and Subramanian, 2011) and ovalbumin-induced asthma. These reactions were related with the modulation of fisetin-mediated anti-inflammatory activity due to the inhibits activation of nuclear factor kappa-light chain enhancer of activated B cells (NF-κB) (Wu et al., 2011). Another recent study showed that fisetin attenuates Th₂-mediated allergic airway inflammation (Goh et al., 2012). However, the therapeutic efficacy of fisetin for AD is unknown. Since in most cases of AD patients have a familial history of allergies and bronchial asthma, the collective data to date support the suggestion that fisetin may be useful in the AD (Rajka, 1986). Presently, we investigated whether fisetin inhibits DNFB-induced AD-like clinical symptoms in a NC/Nga mice model.

2. Materials and methods

2.1. Animals

The six-week-old NC/Nga male mice (19–22 g) were purchased from Japan SLC (Shizuoka, Japan) and maintained under SPF conditions. Animals were housed in an air-conditioned animal room at 25 °C ± 1 °C at a relative humidity of 40% ± 5%, and were fed a laboratory diet and distilled water. Animal treatment and maintenance complied with the Principles of Laboratory Animal Care (NIH publication No. #85-23, revised in 1985) and with the guidelines issued by the ethical committee for animal welfare at Kyung Hee University [KHUASP (SE)-09-005]. All procedures were conducted in accordance with the United States National Institute of Health guidelines.

2.2. Drug treatment

Mice were randomly assigned to four treatment groups: DNFB non-treated group, DNFB treated group and DNFB with 20 mg/kg, and DNFB with 50 mg/kg, with five mice per group. Fisetin was dissolved in 20% ethanol with distilled water to a volume of 200 µl. DNFB non-treated group and DNFB group were treated with the same volume of 20% ethanol with distilled water. The fisetin-treated mice received oral fisetin at 20 or 50 mg/kg daily from days 8 to 15.

2.3. Allergen sensitization and challenge

DNFB sensitization were evoked by the repeated application of 25 µl of 0.15% DNFB in acetone/olive oil (3:1) to the outer and inner surfaces of the ears, and 100 µl of the same solution was applied to shaved dorsal skin once on days 3 and 7. On days 10 and 13, sensitized mice were challenged with 0.2% DNFB to the dorsal and ear skin surfaces. Control mice were treated with the same volume of vehicle. Increasing ear thicknesses were measured by a Digimatic Indicator thickness gauge (Mitsutoyo, Tokyo, Japan).

2.4. Dermatitis score

The severity of AD-like symptoms was macroscopically evaluated by the SCORAD method (Oranje et al., 2007). The degree of each symptom was graded from 0 to 3 (0, absence; 1, mild; 2, moderate; 3, severe). This scoring was based on the severity of edema, erythema, oozing, crust, excoriation, and lichenification. Overall dermatitis score was determined from the sum of all individual scores. Assessment was performed by an investigator who was blind to the grouping of the animals.

2.5. Measurement of cytokine production

T lymphocytes were isolated from the lymph nodes of mice, and CD4⁺ T cells were purified using a Biomag separation column (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Isolated CD4⁺ T cells (1 × 10⁶) were cultured in 24-well flat-bottom culture plates in RPMI-1640 medium with 10% heat-inactivated fetal bovine serum (FBS) and 50 µM β-mercaptoethanol, then stimulated with 5 µg/ml of immobilized anti-CD3 and 2 µg/ml of soluble anti-CD28 antibody for 50 h at 37 °C in a 5% CO₂ atmosphere. After incubation, culture supernatants were collected and the productions of IL-4, IL-10 and IFN-γ after T cell activation were quantified by ELISA (Biolegend, CA, USA).

2.6. Measurement of total and DNP-specific IgE

On day 16, total serum samples were prepared and quantified using OptEIA Set Mouse IgE ELISA kits (BD Pharmingen, San Diego, CA) and mouse anti-DNP IgE ELISA kits (Alpha Diagnostic International, San Antonio, TX) according to the manufacturer's instructions. The absorbance was immediately read at 450 nm on an EL 800 ELISA reader (EL 800) (Bio-Tek, Winooski, VT).

2.7. RNA isolation and RT-PCR

Total RNA was extracted from excised dorsal skin using the Trizol method (Jeong et al., 2011). RNA (1 µg) was used as a template for cDNA synthesis. Reverse transcription was performed using 200 U/µL MMLV reverse transcriptase, 10 mM dNTPs, 5× reaction buffer, 0.02 µg/µL Oligo (dT) primers and 40 U/µL RNase inhibitor at 42 °C for 1 h. The reaction was stopped at 72 °C for 7 min, and cDNA products were stored at –20 °C. PCR was performed using the synthesized cDNA as a template and using specific primers for mice IL-5, IL-13, TARC, TNF-α and TSLP or β-actin, which was used as loading control. Primer sequences were as follows; IL-5, sense, 5'-ATGGAGATTCCTCATGAGCAC-3' and antisense, 5'-GTCTCTCTCGCCCACTTCC-3', and IL-13, sense, 5'-CAGCATGGTATGGAGTGTGG-3', and antisense, 5'-ACAGAGCCATGCAATATCC-3', and TARC, sense, 5'-TGCTTCTGGGAGCTTTTCTG-3', and antisense, 5'-CATCCCTGGAACACTCTCACT-3', and TNF-α, sense, 5'-CCGATGGTGTACCTTGTC-3', and antisense, 5'-CGGACTCCGCAAGTCTAAG-3', and TSLP, sense, 5'-CGGATGGGGCTAACTTACAA-3', and antisense, 5'-TCCTCGATTGCTCGCACTT-3', and β-actin, sense, 5'-CGGTTCCGATGCCCTGAGGCTCTT-3', and antisense, 5'-CGTCACACTTCATGATGGAATTGA-3'. Amplified products were separated in 1% agarose gel and analyzed using a SLB MylMager™ gel documentation system (UVP Inc., Upland, CA, USA).

2.8. Western blot analysis

For isolation of protein, the dorsal tissue was excised, and the fat was removed on ice, immediately placed in ice-cold lysis buffer. Lysates were centrifuged at 12,000g for 20 min, and protein concentrations were determined using the Bradford assay. Fifty micrograms of whole-cell lysate protein was separated electrophoretically on 10% Tris-glycine gels and subsequently transferred to PVDF membranes. For immunoblotting, membranes were blocked with 5% non-fat dried milk in Tris-buffered saline for 4 h at room temperature. Primary antibody against p-p65 (1:1000) was applied for 16 h at 4 °C or antibody against p-65 (1:1000) was applied for 6 h at room temperature. After washing, horseradish peroxidase-conjugated anti-rabbit IgG antibody was used as the secondary antibody to detect the aforementioned protein bands by enhanced chemiluminescence (WESTSAVEUPTM; Abfrontier, Seoul, Korea). Intensity quantified using a Labworks image acquisition and analysis software (UVP Inc., Upland, CA, USA).

2.9. Histological analysis

On day 16, right ears and dorsal skins were removed and fixed with 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4). Fixed tissues were washed in sucrose solution and frozen in OCT compound. Cryostat sections (8 µm) were mounted on slides and dried overnight at room temperature, before being fixed in ice-cold acetone for 5 min. Slides were rehydrated in PBS and blocked with 5% normal goat serum in PBS containing 0.3% Tween 20 (washing buffer) for 1 h at room temperature. After washing, sections were incubated with primary anti-CD4 or anti-CD8 antibody overnight at room temperature then washed and incubated with Alexa-Fluor 488-conjugated goat anti-rat antibody for 4 h at room temperature. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Sections were mounted and images were captured using an Eclipse 50i

fluorescence microscope (Nikon, Tokyo, Japan). Skin sections were also stained with hematoxylin and eosin for inflammatory cells and mast cells were stained using toluidine blue. Numbers of CD4⁺ T cells, CD8⁺ T cells, eosinophils and mast cells were expressed as average total counts in four fields of 100 μm^2 (200 $\times$).

2.10. Statistical analyses

Data are presented as the mean $\pm$ S.E.M. of at least three independent experiments performed in triplicate. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's multiple comparison test. When not shown, error bars lie within symbols. *P*-values of <0.05, <0.01, <0.001 were considered statistically significant.

3. Results

3.1. Fisetin ameliorates DNFB-induced AD-like clinical symptoms in NC/Nga mice

A recent study reported that fisetin has anti-oxidative and anti-inflammatory effects (Maher, 2006). Thus, we investigated the effects of fisetin on the relief of DNFB-induced AD-like symptoms in NC/Nga mice. The repetitive application of DNFB induced AD-like skin lesions involving severe clinical symptoms in NC/Nga mice, whereas oral administration of fisetin to mice at doses of

20 or 50 mg/kg significantly relieved lesion severity (Fig. 1b). Furthermore, scoring AD (SCORAD) including skin erythema, edema, oozing, excoriation, crust and lichenification were dramatically reduced in fisetin treated groups compared with the DNFB-treated group (Fig. 1c). To investigate the therapeutic effects of fisetin for AD, we treated DNFB sensitized mice with fisetin. Every day from 8 to 15 days, DNFB-induced ear swelling was markedly increased after DNFB sensitization. However, oral administration of fisetin at 20 or 50 mg/kg significantly suppressed DNFB-induced ear swelling in a dose-dependent manner (Fig. 2a). DNFB treatment can induce massive infiltration of eosinophils and mononuclear cells, as well as epidermal hyperplasia in AD-like skin lesions (Wood et al., 1992). In this study, DNFB-treated ear lesions showed dermal thickening, hyperkeratosis and infiltration of inflammatory cells in DNFB-treated mice, while the oral administration of fisetin at 20 or 50 mg/kg markedly decreased these reactions (Fig. 2b and c).

3.2. Fisetin suppresses infiltration of inflammatory cells to AD-like skin lesions

In a recent study, in the chronic phase of AD, chemokines and cytokines including CCL5, CCL17, monocyte chemotactic protein

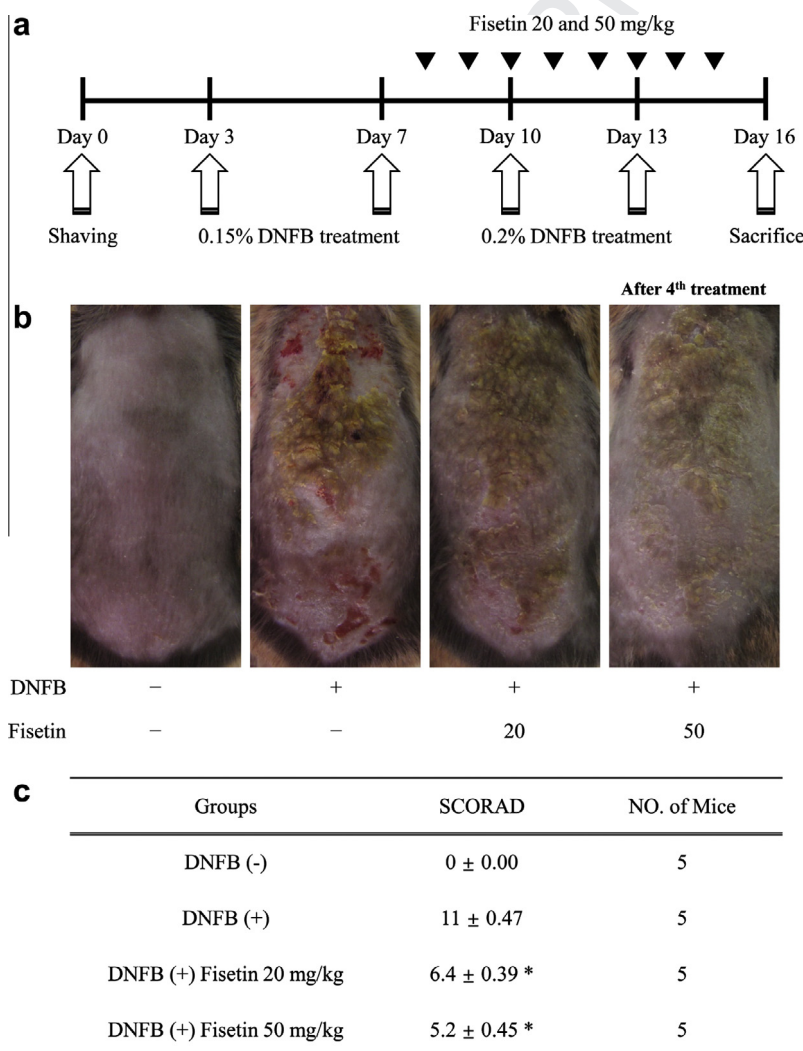


Fig. 1. Effect of fisetin on DNFB-induced AD-like skin lesions. (a) AD-like lesions were evoked by repetitive painting of 0.15% DNFB (acetone:olive oil, 3:1) on dorsal skin of NC/Nga mouse once daily on days 3 and 7, then further challenge with 0.2% DNFB on days 10 and 13. (b) Representative dorsal skin photographs captured after treatment fourth treatment showing comparison of AD-like skin lesions ($n = 5$ in each group). (c) The severity of AD was macroscopically evaluated by SCORAD. Overall dermatitis score was determined from the sum of all individual scores. Data are presented as mean $\pm$ SEM of five determinations. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. DNFB (+) groups.

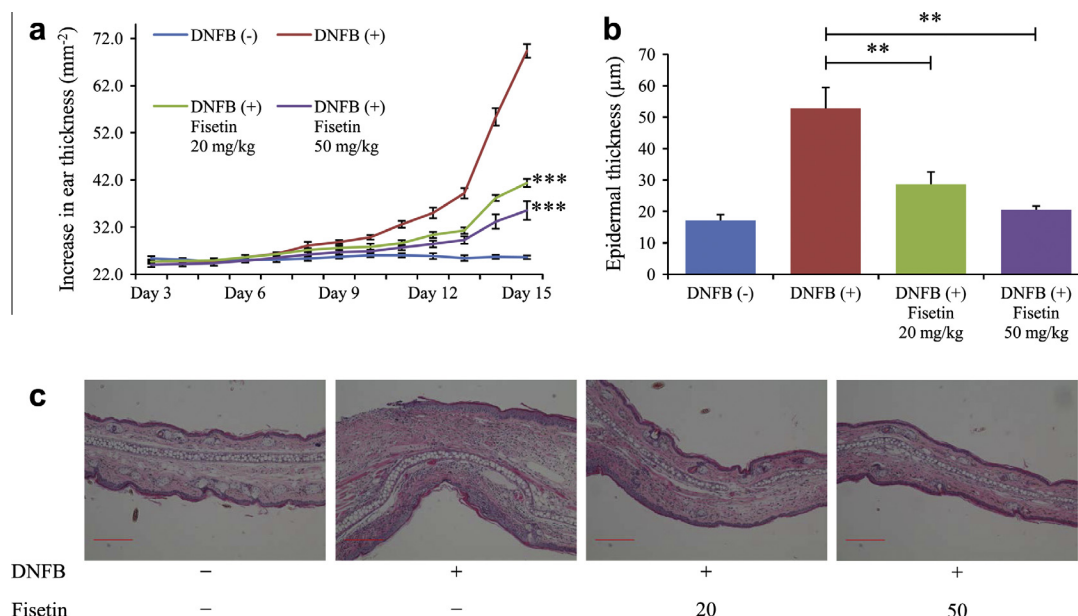


Fig. 2. Fisetin reduces increasing ear swelling and epidermal thickness in skin-lesions. (a) Ear swelling was measured with a thickness gauge every day. Fisetin was orally administered at doses of 20 or 50 mg/kg. Five mice were placed into each of four groups: DNFB (-) (acetone 3: olive oil 1); DNFB (+) (acetone 3: olive oil 1 with 0.15–2% DNFB); DNFB with fisetin, 20 mg/kg; and DNFB with fisetin, 50 mg/kg. (b) Skin sections were H&E stained ($n = 20$). (c) Scale bar = 100 μ m. Data are presented as mean $\pm$ SEM of five determinations. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. DNFB (+) groups.

(MCP)-4 and IL-16 were increased in AD-like skin lesions that induced chemotaxis of eosinophils, mast cells and T lymphocytes (Kaser et al., 1999; Onoue et al., 2009). In AD, eosinophils have an important role in promoting tissue inflammation and inducing tissue damage by the release of toxic granule proteins, lipid mediators and cytokines including major basic protein (MBP) and eosinophil peroxidase (EPO) (Wakita et al., 1994). In this study, repetitive treatments of DNFB induced enormous infiltration of eosinophils and dermal thickness in dorsal skin lesions of NC/Nga mice. However, fisetin at 20 or 50 mg/kg suppressed infiltration of eosinophils in a dose-dependent manner (Fig. 3). Mast cells activated by allergen-sensitized IgE through the Fc ϵ RI receptor and enhances development of Th₂ cells and induces the release of chemokines, cytokines and granular mediators such as eotaxin, IL-13, leukotrienes, prostaglandins, platelet activating factor (PAF) that correlated with the AD-like skin lesion development (Amon et al., 1994; Harvima et al., 1994). In the present study, we examined the effect of fisetin on dermal infiltration and degranulation of mast cells. DNFB treatment induced numbers of infiltrating mast cells and degranulation. However, fisetin administration markedly reduced these observations in dorsal skin lesions (Fig. 4). T lymphocytes are as a important source of Th₂ cytokines including IL-4, IL-5 and IL-13, which are critical to IgE synthesis and eosinophil accumulation in AD-like skin lesions (Novak et al., 2004). We examined whether fisetin suppressed infiltration of T lymphocytes in skin lesions using immunofluorescence. In this study, fisetin significantly inhibited infiltration of CD4⁺ T and CD8⁺ T cell numbers in dorsal skin lesions in a dose-dependent manner (Fig. 5).

3.3. Fisetin inhibits inflammatory cytokine synthesis by DNFB-induced AD-like skin lesions and activated CD4⁺ T cells

Recent studies conducted in murine models showed that dermal dendritic cells (DCs) or epidermal langerhans cells (LC) migrate into draining lymph nodes (DLN) where they bind IgE or induce T lymphocytes activation in AD-like skin lesion (Kaplan, 2010; Kissenpfennig et al., 2005). In this study, we found that

DNFB-induced increased DLN weight was reduced by the administration of fisetin (Fig. 6a). AD has two distinct phase with Th₁ or Th₂ dominant inflammation. Th₁ cells produce IFN- γ and IL-2, whereas Th₂ cells produce IL-4, IL-5 and IL-13 (Spergel et al., 1999). Furthermore, innate immune cytokines, such as IL-1 α , IL-1 β and tumor necrosis factor (TNF)- α , synergize with Th₂ cytokines including IL-4, IL-5, IL-13 and IL-10 to induce thymic stromal lymphopoietin (TSLP) and TARC production by keratinocytes (Nosbaum et al., 2009), which induces the migration of LCs to DLN, where naive T cells develop to proallergic cells that home to the AD-like skin lesions and secrete Th₂ cytokines (Dubrac et al., 2010). To investigate whether fisetin regulates the Th₁ and Th₂ immune responses of CD4⁺ T cells in DLN and secretion of inflammatory cytokines in dermal skin lesions, we performed RT-PCR and an enzyme-linked immunosorbent assay (ELISA). The expression of IL-5, IL-13, TNF- α , TARC and TSLP mRNA levels markedly increased in DNFB-induced dorsal skin lesions. However, fisetin at a concentration of 20 or 50 mg/kg suppressed mRNA expression in a dose-dependent manner (Fig. 6b and c). Furthermore, fisetin suppressed IL-4 and IFN- γ production in activated CD4⁺ T cells in a dose-dependent manner, whereas the anti-inflammatory cytokine IL-10 was increased (Fig. 6d). A recent study reported that immunosuppressive effects of fisetin due to inhibition of NF- κ B activity (Wu et al., 2011). To investigate whether the anti-inflammatory effects of fisetin in the DNFB-induced AD-like model were mediated by the inhibition of NF- κ B, we examined the phosphorylation of the p65 subunit of NF- κ B (p-p65) from dorsal tissues prepared 24 h after the last DNFB challenge. DNFB challenge significantly increased the level of p-p65 subunit in the dorsal skin compared with vehicle control, while fisetin at 20 or 50 mg/kg significantly reduced the p-p65 level (Fig. 6f).

3.4. Fisetin suppresses DNFB-induced serum IgE level

AD is frequently associated with elevation of total serum IgE levels, and markedly increasing plasma levels of the total IgE with continuously suffering from AD-like skin lesions are reported common features of all AD-like mice models (Tanaka et al.,

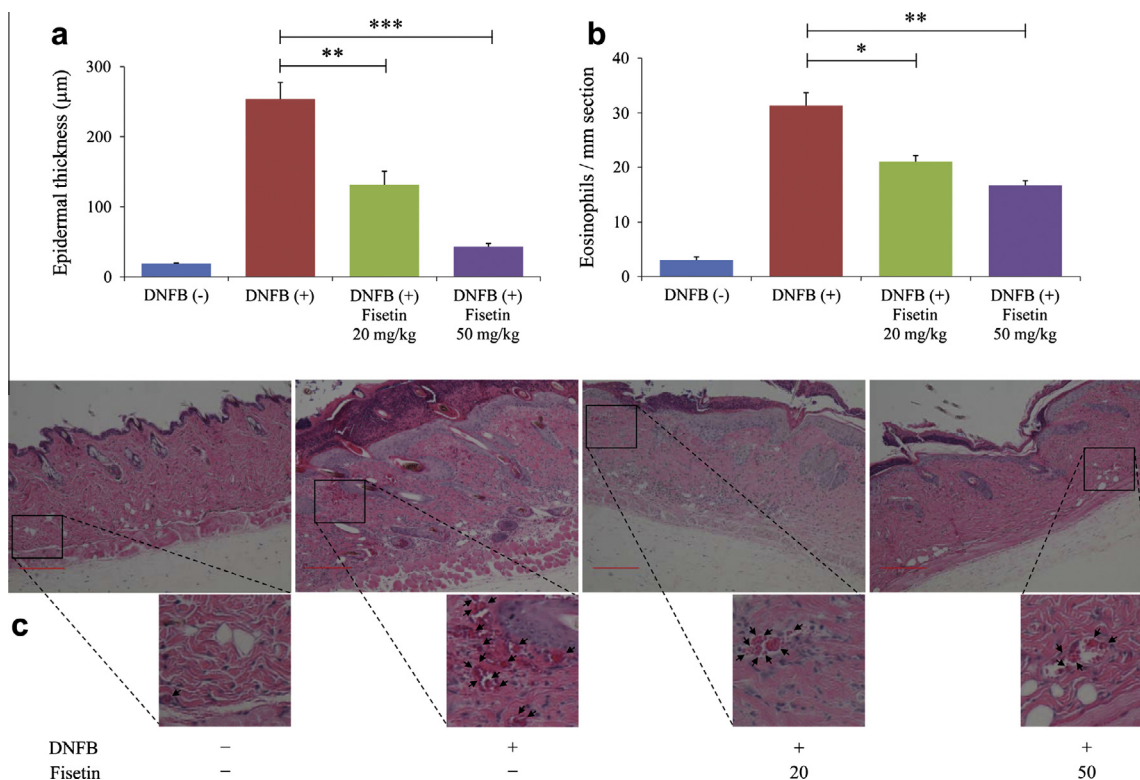


Fig. 3. Fisetin inhibits infiltration of eosinophils in dorsal skin lesions. (a) Skin sections were H&E stained ($n = 20$). (b) Eosinophils in H&E stained sections were counted under a microscope and the numbers of infiltrated eosinophils were expressed as average total counts in four fields of $100 \mu\text{m}^2$. (c) Scale bar = $100 \mu\text{m}$, the number of cells is expressed as the mean $\pm$ SEM of five sections. Arrows indicate eosinophils. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs. DNFB (+) groups.

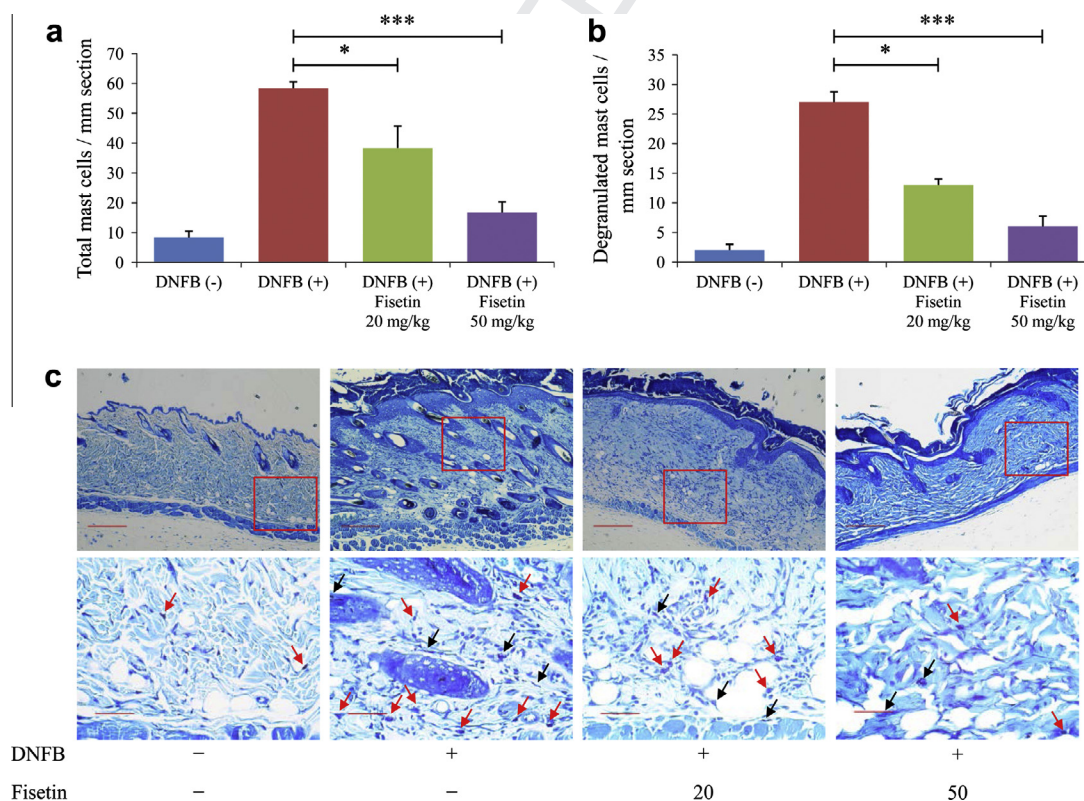


Fig. 4. Fisetin suppresses infiltration of mast cells and mast cell degranulation in AD-like skin lesions. (a) DNFB (-), DNFB (+), DNFB + fisetin (20 mg/kg) and DNFB + fisetin (50 mg/kg) treated. Mast cells in toluidine blue stained sections were counted under a microscope. (b) Degranulated mast cells were expressed as average total counts in three fields of $100 \mu\text{m}^2$ ($n = 5$ sections each). (c) Scale bar = $100 \mu\text{m}$, the number of cells is expressed as the mean $\pm$ SEM of five sections. Both red and black arrows indicate mast cells and degranulated mast cells. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs. DNFB (+) groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

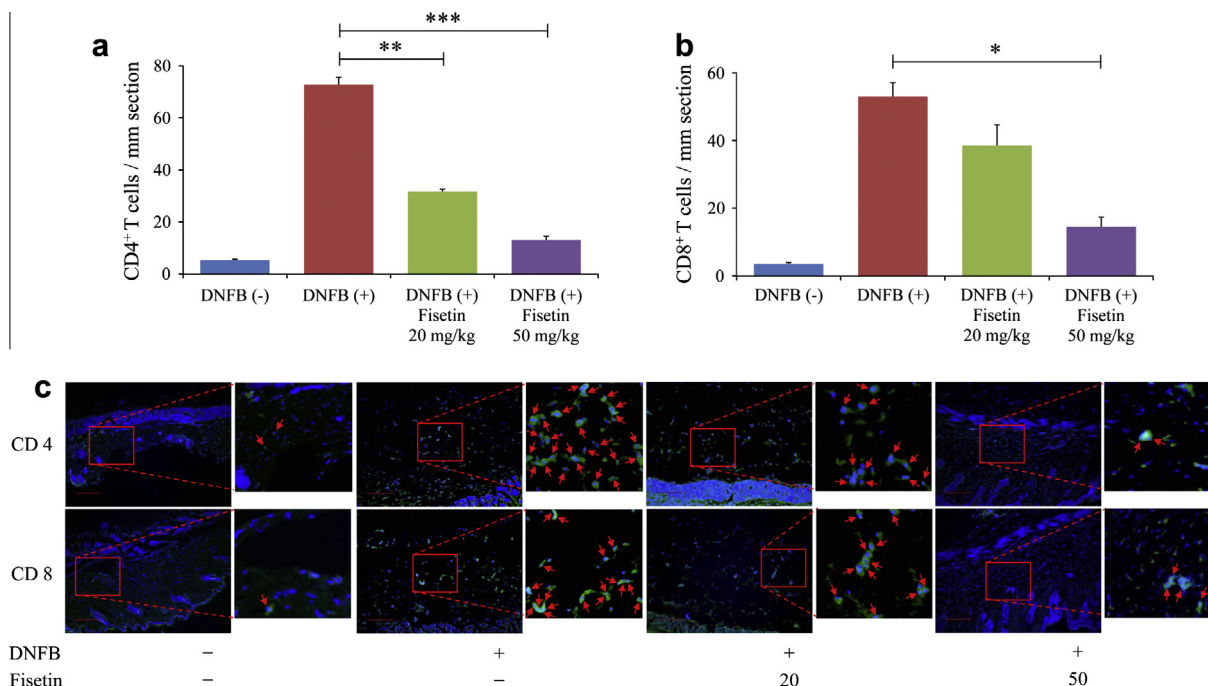


Fig. 5. Fisetin inhibits infiltration of CD4⁺ T cells and CD8⁺ T cells in dorsal skin lesions. NC/Nga mouse were treated with 0.15% DNFB (acetone:olive oil, 3:1) on shaved back skins on days 3 and 7, and further challenged with 0.2% DNFB on days 10 and 13. Fisetin was oral administrated daily from the day of first DNFB challenge (day 8). On day 16, immunofluorescence staining was done with the cryosections of dorsal skin samples with fluorescein isothiocyanate. Representative pictures showing activation of CD4⁺ T cells (a) and CD8⁺ T cells (b) After DNFB treatment, which was inhibited by fisetin. Merged CD4⁺ T cells and CD8⁺ T cells (c) were counted. The number of cells is expressed as the mean $\pm$ SEM of five sections. Arrows indicate merged cells. * p < 0.05, ** p < 0.01, *** p < 0.001 vs. DNFB (+) groups; scale bar = 100 μ m.

1994). IgE synthesized by B cells and induced activation of mast cells through Fc ϵ RI evokes the release of histamine and cytokines including IL-4, IL-5 and IL-13 (Kim et al., 2012). To investigate whether fisetin inhibits IgE production, we prepared serum samples after the final DNFB challenge and total serum IgE and DNP-specific IgE levels were measured by ELISA. Repetitive DNFB treatment significantly induced total serum IgE and DNP-specific IgE levels compared with control. However, daily oral administration with 20 or 50 mg/kg fisetin significantly suppressed total serum IgE and DNP-specific IgE levels as compared with DNFB alone (Fig. 6e).

4. Discussion

AD is a chronic inflammatory skin disease characterized by pruritus, edema, erythema and scaling that severely impairs the quality of life (Galli et al., 2003). Topical steroid therapy is an effective treatment for AD. However, repetitive long-term application of steroids produces side effects that include skin atrophy or thinning, necessitating new kinds of treatments (Rudikoff and Lebwohl, 1998). Flavonoids are abundantly found in most plants, vegetables, fruits and teas. They have various biological activities including anti-oxidant, anti-viral, anti-carcinogenic, anti-allergic and anti-inflammatory activities. Therefore, we hypothesized that suitable intake of flavonoids may be therapeutic for AD (Kawai et al., 2007; Yao et al., 2004). Fisetin is a bioactive flavonoid that has multiple biological activities. The anti-oxidative effects of fisetin include increased intracellular glutathione (GSH) levels in mouse hippocampal HT-22 cells (Ishige et al., 2001), suppressed human low density lipoprotein (LDL) oxidation (Lian et al., 2008) and up-regulation of heme oxygenase-1 (HO-1) expression against oxidative stress in human umbilical vein endothelial cells (HUVECs) (Lee et al., 2011). Furthermore, fisetin has immunomodulatory effects including suppressed production of IL-6, IL-8, TNF- α and

vascular endothelial growth factor (VEGF) in a mice rheumatoid arthritis (RA) model (Lee et al., 2009) and inhibited expression of TNF- α , IL-1 β , IL-6, IL-8 and histamine in human mast cells (Park et al., 2007). These results suggest that fisetin may be useful in the treatment of allergic disorders like asthma, psoriasis and AD. We investigated the effect of fisetin on the severity of AD-like clinical symptoms in DNFB-treated NC/Nga mice. A recent study showed that the flavonoids tannic acid and quercetin suppressed AD-like symptoms through angiogenesis and TARC expression in murine models (Jung et al., 2010). Also, fisetin inhibits infiltration of inflammatory cells in the ischemic hemisphere (Gelderblom et al., 2012). These observations might be correlated with hyper-permeability of blood vessels and Th₂ immune response. The observations support a new therapeutic strategy for AD. In this study, treatment of mice with fisetin at doses of 20 or 50 mg/kg significantly relieved AD-like clinical symptoms, such as erythema, edema, oozing and excoriation (Fig. 1b and c). Furthermore, ear swelling was partially inhibited (Fig. 2). Continual DNFB application disturbed epidermal barrier and induced adhesion molecules, such as intracellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 (Deane and Hickey, 2009) and Fc ϵ RI on eosinophils or mast cells, and secretion of immune mediators including IL-5, IL-13, TARC and TSLP. The secreted compounds lead to a cytokine cascade and an increase in epidermal LC density that provoke leukocyte rolling or infiltration of inflammatory cells (Barker et al., 1991; Proksch and Brasch, 1997). In the present study, application of DNFB increased epidermal thickness and infiltration of eosinophils in ear and dorsal skin in NC/Nga mice (Figs. 2 and 3). However, fisetin at 20 or 50 mg/kg reduced these reactions. Furthermore, treatment of fisetin suppressed mast cell degranulation and the number of infiltrating mast cells (Fig. 3), CD4⁺ T and CD8⁺ T cells (Fig. 4) in a dose-dependent manner. These results suggested that fisetin ameliorates AD-like clinical symptoms and infiltration of inflammatory cells through the inhibition of Th₂ cytokines and chemokines like TARC and TSLP, which have roles

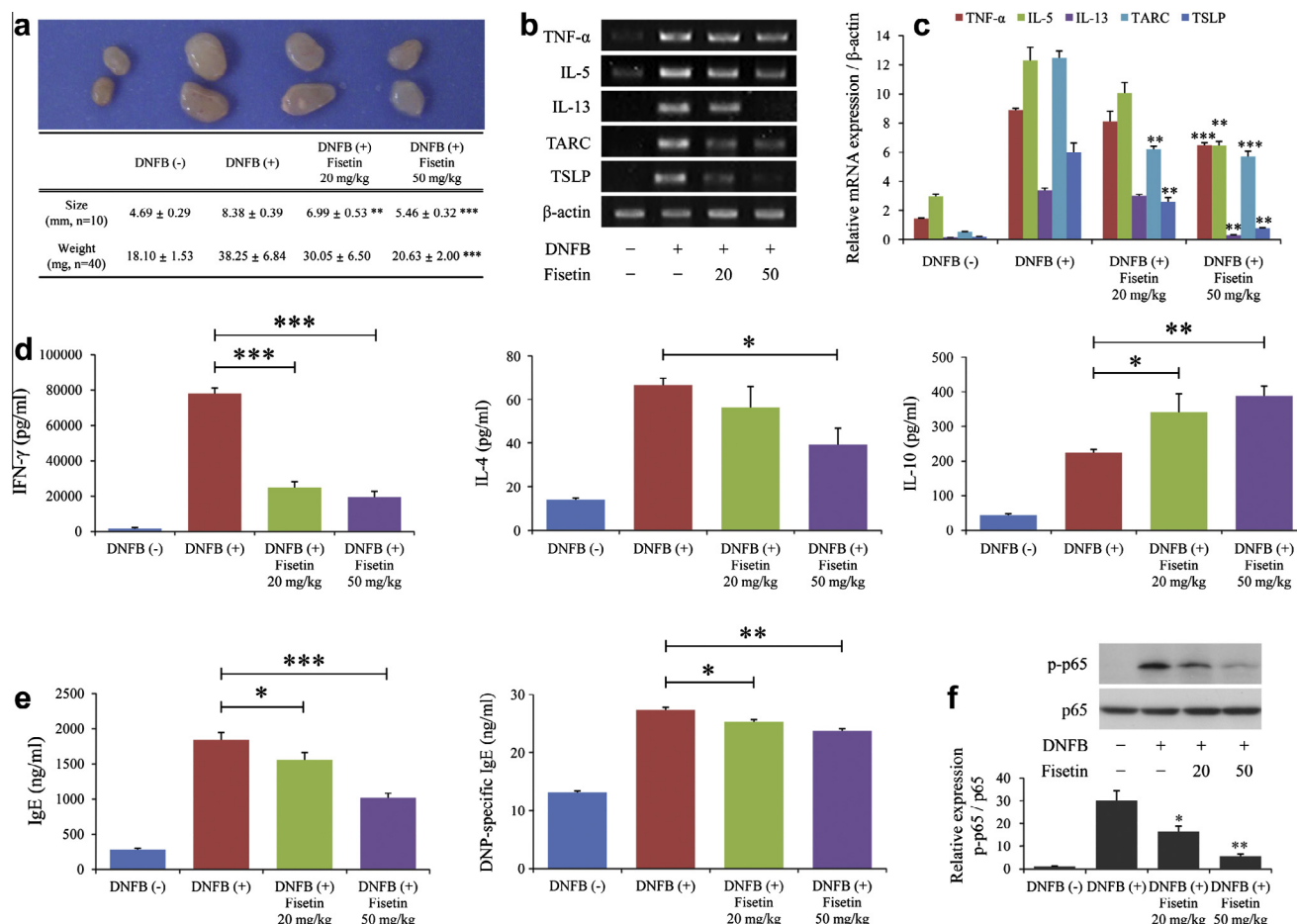


Fig. 6. Fisetin inhibits cytokine and chemokine production of both dorsal skin and activated CD4⁺ T cells and reducing total serum IgE levels. (a) On day 16, DLN were excised then evaluated of weights and CD4⁺ T cells were purified. (b) Dorsal skins excised for RT-PCR and evaluated mRNA expression of Th₂ cytokine and chemokines, β-actin was used as loading control. (c) Relative mRNA expression that normalized by β-actin. (d) The purified CD4⁺ T cells (1×10^6 cells/ml) were stimulated with anti-CD3 antibody and anti-mouse CD28 antibody for 50 h. The production of IL-4, IL-10 and IFN-γ after T cell activation was quantified by ELISA. (e) Total IgE and DNP-specific IgE in the indicated groups was measured 24 h after final administration. DNFB (-), DNFB (+), DNFB + fisetin (20 mg/kg) and DNFB + fisetin (50 mg/kg) treated. (f) Results are based on triplicate experiments. Detection of p-p65 or p65as shown by western immunoblotting (50 μg per lane). Data are mean ± SEM (n = 5). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. DNFB (+) groups.

as a transporter of Th₂ cells. We explored whether the Th₂ immune mediators TARC, TSLP, IL-5 and IL-13 correlated with the therapeutic effect of fisetin in AD. DNFB-induced expression of IL-5, IL-13, TNF-α, TARC and TSLP mRNA levels were reduced by fisetin in a dose-dependent manner (Fig. 6b and c). These observations demonstrated that fisetin inhibits inflammatory mediators produced by dermal LCs and keratinocytes. The expression of CCR7 allows dermal DCs or epidermal LCs to migrate towards DLN where they induce the activation of CD4⁺ T cells and antigen-specific CD8⁺ T cells in AD-like skin lesions. The activated CD4⁺ T cells induce the differentiation of Th₁ cells that produce IFN-γ and Th₂ cells that secrete IL-4, IL-5 and IL-13 (Nosbaum et al., 2009; Ohl et al., 2004). Presently, fisetin reduced the DNFB-induced increase in DLN weight (Fig. 6a) and suppressed IFN-γ and IL-4 production by the activated lymph node CD4⁺ T cells in a dose-dependent manner (Fig. 6d). However, IL-10 production was increased. IL-10 inhibits the proliferation of human T lymphocytes and suppresses cytokine synthesis in human monocytes (de Waal Malefyt et al., 1991). Thus, IL-10 may act in synergy with the anti-inflammatory effects of fisetin in AD. Since NF-κB activation has been correlated with the development of allergic airway inflammation (Yang et al., 1998), we investigated whether the anti-inflammatory effects of fisetin by the inhibition of NF-κB activation. DNFB-induced p-p65 level was markedly increased. However, fisetin at 20 or 50 mg/kg reduced p-p65 level in a dose-dependent manner (Fig. 6f). These

observations suggested that the anti-inflammatory effects of the fisetin in our murine model are possibly due to inhibition of NF-κB activation. Th₂ cytokine IL-4 induces further rearrangement of variable diversity by joining (VDJ) to Cγ₄ or Cε in the B cell for IgE synthesis. IgE then mediates mast cell degranulation, which induces infiltration of eosinophils and lymphocytes into the AD-like skin lesions (Bacharier and Geha, 2000; Kim et al., 2011). In this study, total serum IgE levels and DNP-specific IgE were increased by the repeated application of DNFB in NC/Nga mice, whereas fisetin treated mice displayed significantly reduced production of these molecules (Fig. 6e). Further research is necessary to investigate the molecular mechanisms of fisetin that inhibits production of inflammatory mediators in LC or keratinocytes associated with T lymphocytes in the NC/Nga AD model. In summary, fisetin relieves the clinical symptoms of AD-like skin lesions caused by repeated DNFB application in NC/Nga mice by suppressing Th₂ cytokine and chemokines, IFN-γ and IL-4 production from activated CD4⁺ T cells and reducing total serum IgE levels and infiltration of inflammatory cells involving eosinophils, mast cells and CD4⁺ T and CD8⁺ T cells.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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