

Effect of Taxifolin Glycoside on Atopic Dermatitis-Like Skin Lesions in NC/Nga Mice

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Increased levels of eosinophils, IgE, IL-4, 5, and 13 and pro-inflammatory factors (COX-2, iNOS) are observed in patients with atopic dermatitis (AD). Taxifolin 3-O-β-D-glucopyranoside (TAX) from the roots of *Rhododendron mucronulatum* (RM) was examined to determine whether its immunomodulatory effect was applicable for treating atopic dermatitis.

A total of 7 groups of NC/Nga mice with AD were treated by topical application or intraperitoneal injection of TAX for 4 weeks. Follow-up evaluations were done to assess the changes in clinical observations, eosinophil counts, and levels of IgE, cytokines, COX-2 and iNOS.

In the clinical observation during the experimental period, TAX treatment significantly reduced the severity of AD-like lesions induced in NC/Nga mice. Eosinophil and IgE levels decreased after treatment of the animals with TAX. TAX may thus be associated with improvement of eosinophil-related allergic diseases. The expression of cytokines (IL-4, 5 and 13) was significantly inhibited in the TAX-treated group, suggesting that TAX might play an immunoregulatory role associated with AD. In RT-PCR, iNOS and COX-2 expression levels were reduced in the TAX-treated group. In western blotting, the expression levels of iNOS and COX-2 were also reduced in the TAX-treated group.

These findings suggest that TAX is effective for the treatment of AD by preventing the production of inflammatory cytokines and by reducing skin inflammation. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: *Rhododendron mucronulatum*; flavonoid; taxifolin 3-O-β-D-glucopyranoside; Th2; atopic dermatitis.

INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by hypersensitivity of the skin due to various environmental and genetic factors (Schultz *et al.*, 2002). AD mainly develops in infants and children, although incidences have recently increased in adult patients. Major symptoms of this condition include severe pruritis, dryness, and eczema. Due to the lack of an exact pathogenesis or definitive treatment, studies of AD have been actively conducted worldwide. The currently known treatments, including steroids, antihistamines and immunosuppressants, have various limitations in terms of treatment efficacy; it is therefore imperative that alternative and compensatory agents be developed to treat this condition.

AD induces immune response defects, including increased eosinophil counts and IgE levels, an imbalance in the spectrum of Th1/Th2 responses, and overexpression of iNOS and COX-2 (Thepen *et al.*, 1996; Grewe *et al.*, 1998; Simon *et al.*, 2004; Horney *et al.*, 2006).

To date, several natural substance based anti-atopic agents have been tested as potential therapeutics for AD (Kotani *et al.*, 2000; Kang *et al.*, 2008; Kim *et al.*, 2008; Noh *et al.*, 2008; Choi *et al.*, 2009). *Rhododendron mucronulatum* (RM) is used for treatment of rheumatitis, neuralgia, and inflammation in Korean traditional medicine (Kim *et al.*, 1998). We previously reported that Taxifolin 3-O-β-D-glucopyranoside (TAX) (Fig. 1) inhibited cytokine production, the formation of reactive oxygen species (ROS) and nitric oxide, and changes in intracellular Ca²⁺ levels in dendritic cells of bone marrow and spleen exposed to microbial products and IL-1β *in vitro* (Kim *et al.*, 2008).

This paper describes the effect of TAX against the diphenylcyclopropanone (DPCP)-induced atopic dermatitis-like skin lesions in NC/Nga mice, a model for human atopic dermatitis.

MATERIALS AND METHODS

NC/Nga mice. Female 5-week-old NC/Nga mice were purchased from Charles River Japan (Yokohama, Japan) and were maintained under conventional conditions. All animals were maintained on a 12-h light/12-h dark cycle with food and water available *ad libitum*. The colony room was maintained at 22–23°C and 55 ± 15% humidity (Hajime *et al.*, 1998; Norikazu *et al.*, 2003;

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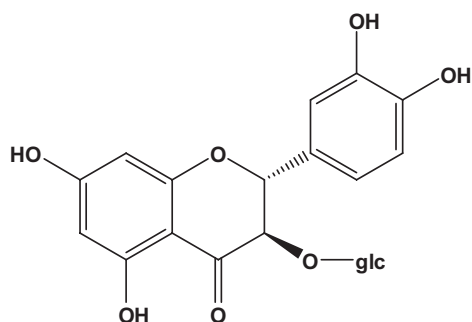


Figure 1. Chemical structure of taxifolin 3-O- β -D-glucopyranoside.

Takano *et al.*, 2006). The animal care, handling, and experimental procedures were performed in accordance with a protocol approved by the Animal Care and Use Committee of Chung Ang University of Korea.

Extraction and isolation of TAX. Taxifolin 3-O- β -D-glucopyranoside (3',4',5',7' tetrahydroxy flavonol glucoside; Fig. 1) was isolated from the root of *R. mucronulatum* (Kim *et al.*, 2008).

Diphenylcyclopropenone (DPCP). Diphenylcyclopropenone (DPCP, Sigma, St Louis, MO, USA), a well-known contact allergen, was used to induce atopic-like skin lesions.

DPCP stimulation. The dorsal hair of the mice was removed and the skin was then stimulated for 10 weeks with DPCP under conventional conditions. NC/Nga mice were sensitized with DPCP (0.05%, 0.1%) on the back, once a week, as previously described (Tomomi *et al.*, 2001; Shahin, 2005; Kang *et al.*, 2008).

Clinical observation of AD. The backs of NC/Nga mice were clinically observed for 4 weeks. The challenged sections of mouse skin were photographed. The clinical severity of the dermatitis was scored using previously described macroscopic diagnostic criteria that are normally used for human AD. The severity of dermatitis was evaluated once a week. The development of (1) erythema/haemorrhage, (2) scarring/dryness, (3) oedema, and (4) excoriation/erosion was individually scored as 0 (none), 1 (mild), 2 (moderate) and 3 (severe), respectively. The sum of the individual scores was taken as the dermatitis score (Yamamoto *et al.*, 2007; Noh *et al.*, 2008).

TAX treatment. Solutions of PBS, 0.1% TAX, or 1% TAX (Kim *et al.*, 2008) were injected intraperitoneally twice a week, and base cream or 1% TAX topical cream was applied to exposed skin on the backs of the mice for 4 weeks as indicated.

Measurement of eosinophil counts. Blood samples were collected at 20 weeks of age. Whole blood cell (WBC) counts were measured in 30 μ L of capillary blood diluted 6-fold with 150 μ L of saline. Microscopic differential counts were measured using Wright-Giemsa-stained blood smears. Eosinophil counts were calculated from differential counts.

Measurement of serum IgE. The amount of total IgE in the serum was determined by sandwich ELISA as previously described (Hiroshi *et al.*, 1997; Eishin *et al.*, 1999; Luca and Perretti, 1999). In brief, 96-well plates were coated with monoclonal anti-mouse IgE overnight at 4°C and then further treated with 2% (w/v) BSA (bovine serum albumin) dissolved in phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBS-T) for 2 h at room temperature (RT) to block nonspecific binding. Next, serial dilutions of the serum samples were incubated in the wells, in duplicate, for 1 h at RT. After 3 rinses with PBS-T, biotin-conjugated rat anti-mouse IgE Ab (LO-ME-2) was added to the wells, followed by the addition of streptavidin-peroxidase (Seikagaku Corp., Tokyo, Japan). After 3 rinses, the plates were developed using a substrate solution containing 0.04% *O*-phenylenediamine dissolved in phosphate citrate buffer (pH 5.0). The reactions were terminated by the addition of sulfuric acid. The absorbance was read using a microplate reader at 490 nm, and the amount of IgE isotype was calculated by comparison with the mouse IgE standard (Pharmingen, San Diego, CA, USA) (Hiroshi *et al.*, 1997; Nobuyuki *et al.*, 2001).

Splenocyte culture. Splenocytes were isolated from spleen tissue. Red blood cells (RBC) were lysed in RBC lysis buffer (Sigma, St Louis, MO, USA). The lysate was centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was removed and the cells were seeded at 1×10^6 /well, which were then cultured in 24-well plates. The supernatant containing cultured splenocytes was harvested and cytokines were measured.

Cytokine measurement. Blood samples were collected at 20 weeks of age. The supernatants of cultured lymphocytes were analyzed for cytokine levels using mouse cytokine enzyme immunoassay kits for IL-4, IL-5, and IL-13 (R&D Systems, Minneapolis, USA) (Yoshiki *et al.*, 2001).

Tissue sampling. Tissues from the AD-like skin lesions were collected in order to measure iNOS and COX-2 expression.

Real-time PCR – preparation of primers. We synthesized the PCR primers (Genotech, YG, KOR) using data from GeneBank. Primers were chemically synthesized using a DNA synthesizer (Pharmacia, Björkgatan, Uppsala, Sweden). Their sequences were as follows:

iNOS (65bp)
 5'-CTG ATG CCT CTT CCA GGT GT-3' (sense)
 5'-GAG GGA GCC CTT TCT GAA TC-3' (anti-sense)
 COX-2 (80bp)
 5'-CCA CCC ATG GCA AAT TCC ATG GCA-3' (sense)
 5'-GGT GCT GCT TGT TAG GAG GTC AAG TAA AGG GC-3' (anti-sense)
 GAPDH (93bp)
 5'-CCA CCC ATG GCA AAT TCC ATG GCA-3' (sense)
 5'-CCC TGT TGC TGT AGC CGT AT-3' (anti-sense)

Total RNA was isolated from tissue using 1 ml of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). After

5 min at room temperature, 0.2 ml of chloroform per ml of TRIzol reagent was added and the tubes were shaken vigorously by hand for 15 s. These samples were then incubated for 3 min at 15°C to 30°C. The mixture was centrifuged at 12,000 rpm for 15 min at 4°C, and then the upper aqueous phase was transferred to a fresh tube into which the same amount of 2-propanol was added. After incubation at 4°C for 15 min, each sample was centrifuged at 12,000 rpm for 15 min (4°C). The supernatant was removed, washed with 500 µL of 70% ethanol and centrifuged at 12,000 rpm (4°C) for 5 min. The resulting RNA pellet was briefly dried. Purified RNA was dissolved in diethyl pyrocarbonate-distilled water (DEPC-DW). Total cellular RNA was reverse transcribed at 42°C for 30 min using reverse transcriptase (TaKaRa, Shiga, Japan), 10X buffer, 10 mM dNTP (dNTP mix), oligo dT primer, RNase inhibitor, and 25 mM MgCl₂.

Two µL of each cDNA sample from the RT reaction was amplified by PCR in 25 µL containing 2.5 µL of 10X buffer, 2.5 µL of 25 mM MgCl₂, and 10 pmol (0.75 µL) of primer. PCRs were conducted with 10X Buffer for Taq polymerase [100 mM Tris-Cl (pH 8.5), 400 mM KCl], 1 mM dNTPs, 50 mM MgCl₂, upstream primer (1 µL), downstream primer (1 µL), DNA template (less than 200 ng) and SyBr Green I.

Western blotting. Tissue was lysed in buffer containing 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 100 µg/ml phenylmethanesulphonyl fluoride (PMSF), 1 µg/ml aprotinin, and 1% Triton X100, and centrifuged at 12,000 rpm (4°C) for 30 min. The supernatant was transferred into a new tube, and 30 µg of soluble protein was loaded in a 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) system with sample buffer containing 1 M Tris and 50% glycerol. The samples were heated at 95°C for 5 min prior to gel loading.

For iNOS and COX-2 detection, the electrophoretically resolved proteins were transferred onto nitrocellulose membranes (Osmonics, Milwaukee, WI, USA) at 0.16 A for 1 h. The membranes were washed 3 times with Tris-buffered saline-Tween 20 (TBS-T) and blocked with 5% skim milk for 1 h at room tempera-

ture. The membranes were then incubated overnight at 4°C with goat antihuman iNOS and COX-2 polyclonal antibodies (1:1000 in 5% bovine serum albumin, Chemicon, Temecula, CA, USA) and then washed 3 times with TBS-T. The membrane was then incubated with secondary mouse anti-rabbit peroxidase conjugated antibody (1:2000 in blocking solution, Chemicon, CA, USA) for 1 h at room temperature.

Quantitative analysis. A densitometer was used to measure iNOS, COX-2, and Actin signals on DIG chemiluminescent film after western blotting (volume of iNOS, COX-2/volume of Actin ×100).

Statistical analysis. All data are expressed as the mean ± SE. Statistical differences between groups were determined by a factorial analysis of variance (ANOVA) followed by an unpaired *t*-test.

RESULTS

AD-like skin lesions were induced by DPCP

We treated mouse skin with DPCP for 16 weeks to induce AD-like skin lesions. The time schedule for DPCP provocation is included in Fig. 2. DPCP treatment significantly induced AD-like lesions in NC/Nga mice.

AD-like skin lesions were reduced by TAX

To investigate the effect of TAX on AD-like lesions, we examined the anti-inflammatory effects of TAX in this AD model. Figure 3 shows that TAX treatment significantly reduced AD-like lesions in NC/Nga mice.

Clinical severity score was reduced by TAX

TAX treatment significantly reduced the severity of AD-like lesions induced in NC/Nga mice. The severity

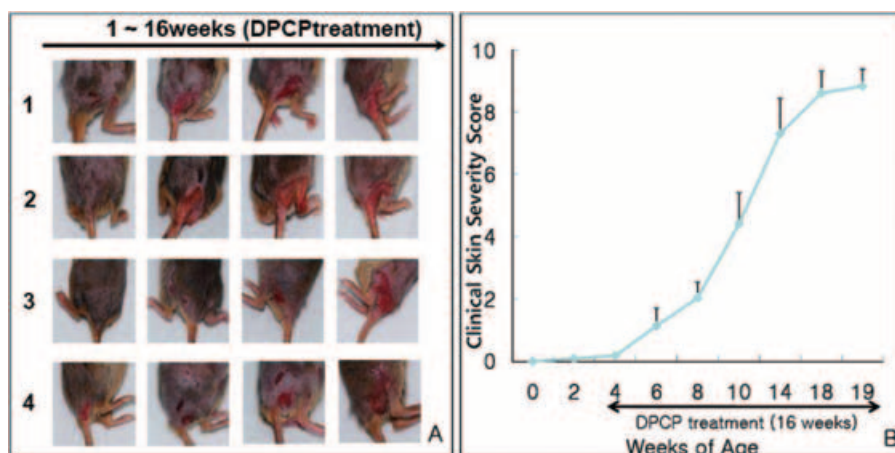


Figure 2. AD-like skin lesions in NC/Nga mice induced by DPCP. (A) Photographs of AD-like skin lesion induced in NC/Nga mice during DPCP treatment. Application of 0.1% DPCP on the backs of the animals at 1-week intervals induced the AD-like skin lesions. The total clinical severity score was defined as the sum of scores graded as 0 (none), 1 (mild), 2 (moderate) and 3 (severe) for each of the seven aspects: erythema, haemorrhage, oedema, excoriation, erosion, scaling and dryness. The results are expressed as means ± S.D. (n = 5). (B) Changes in the total clinical severity score by topical application and intraperitoneal injection of DPCP.

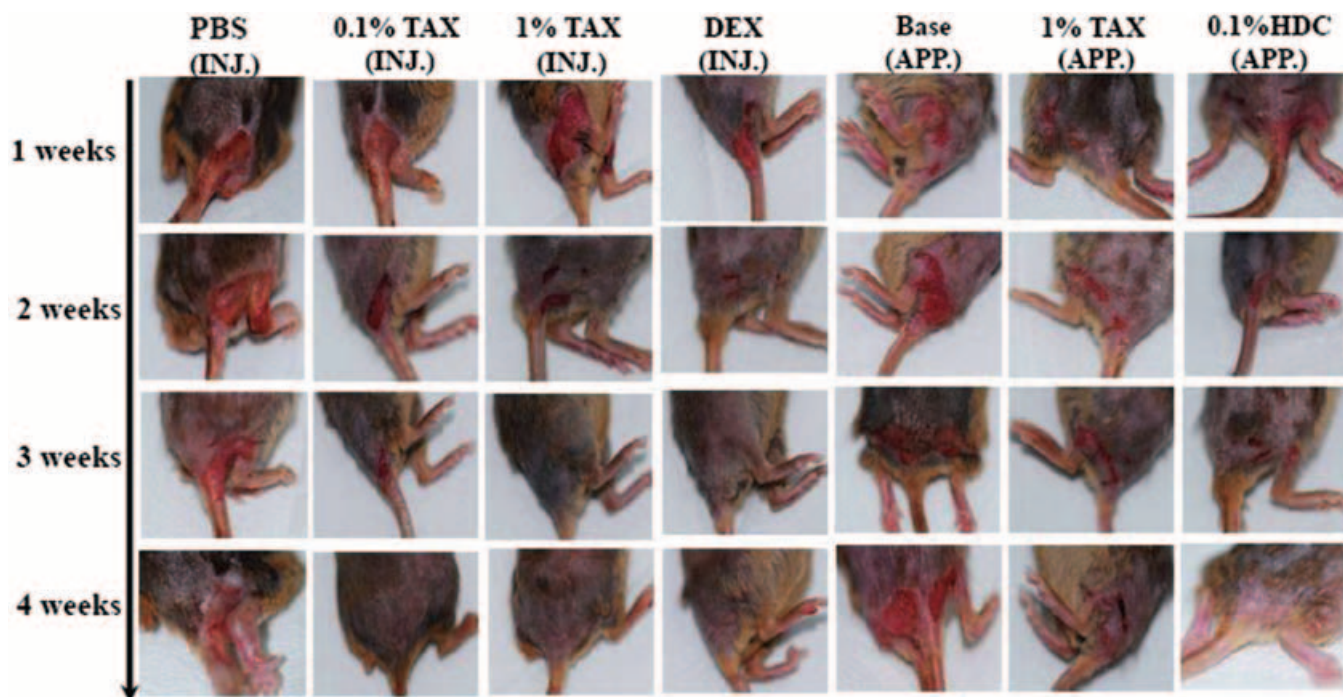


Figure 3. Clinical observation of AD-like skin lesions induced in NC/Nga mice. TAX: Taxifolin-glycoside, DEX: Dexamethasone, Base: Baseline, HDC: Hydrocortisone cream. INJ: injection, APP: application.

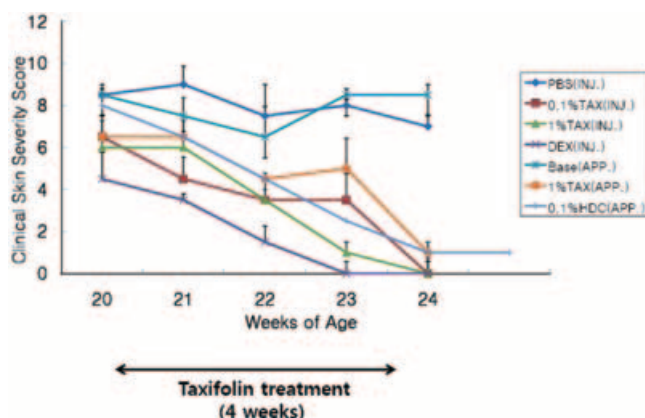


Figure 4. Changes in the total clinical severity score following topical application and intraperitoneal injection of TAX. The total clinical severity score was defined as the sum of scores graded as 0 (none), 1 (mild), 2 (moderate) and 3 (severe) for each of the seven aspects: erythema, haemorrhage, oedema, excoriation, erosion, scaling and dryness. The results are expressed as means $\pm$ S.D. ($n = 5$). TAX: Taxifolin-glycoside, DEX: Dexamethasone, Base: Baseline, HDC: Hydrocortisone cream. INJ: injection, APP: application.

of dermatitis was evaluated once a week. The development of (1) erythema/haemorrhage, (2) scarring/dryness, (3) oedema, and (4) excoriation/erosion was individually scored as 0 (none), 1 (mild), 2 (moderate) and 3 (severe). The sum of the individual scores was taken as the dermatitis score (Fig. 4) (Oystein *et al.*, 2009). Clinical signs and symptoms in conventional NC/Nga mice treated with DPCP began with itching, erythema and haemorrhage, followed by oedema, superficial erosion, deep excoriation, scaling and dryness of the skin, and retarded growth. Total severity scores increased with aging and reached a clinical score of more than 13 out of 15 points in 20-week-old females.

Eosinophil and IgE levels were decreased by TAX

Eosinophil and IgE levels were decreased after treatment with topical and intraperitoneal TAX administration (Fig. 5).

Th2-related cytokines were decreased by TAX

Cytokine (IL-4, IL-5 and IL-13) expressions were significantly inhibited in the TAX-treated group (Fig. 6).

Expression of iNOS and COX-2 in the skin lesion were decreased by TAX

The transcription and translation of COX-2 and iNOS in AD lesional skin were significantly suppressed by TAX treatment ($p < 0.05$) (Fig. 7).

DISCUSSION

In AD, Th1 cytokines such as tumour necrosis factor- α (TNF- α) and interleukin 1 (IL-1) from resident cells (keratinocyte, mast cell, dendritic cells) bind to receptors on the vascular endothelium, activating cellular signalling pathways and inducing expression of vascular endothelial cell adhesion molecules. These events initiate the process of inflammation.

In our previous study, TAX, which has been isolated from *Rhododendron mucronulatum* (RM), exhibited anti-inflammatory and inhibitory effects against dendritic cell-mediated immune responses (Kim *et al.*, 2008). Therefore, TAX may be an effective therapeutic agent for AD due to its inhibition of IL-1 β .

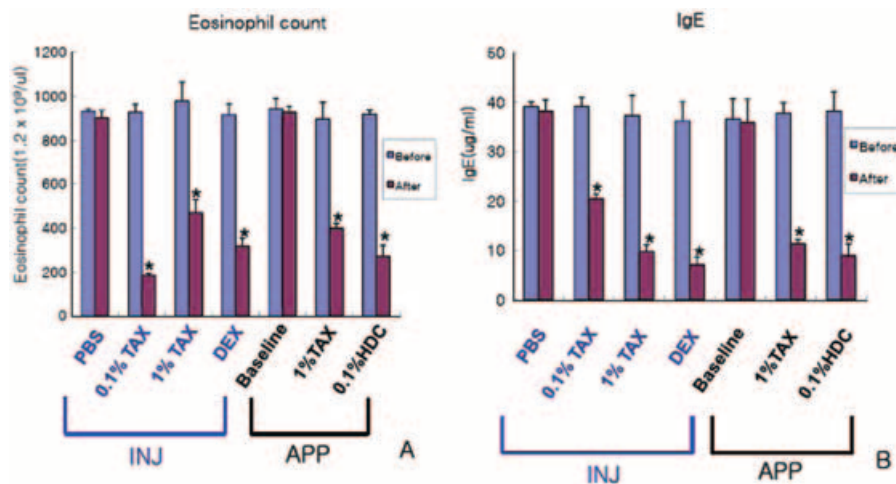


Figure 5. Effects of topical application and intraperitoneal injection of TAX on eosinophil count and IgE. (A) Eosinophil levels were decreased after treatment of the animals with TAX. TAX may thus improve eosinophil-related allergic diseases. (B) Both topical and intraperitoneal TAX treatment significantly down-regulated the IgE levels in NC/Nga mice. The results are expressed as means $\pm$ S.D. (* $P < 0.05$) ($n = 5$). TAX: Taxifolin-glycoside, DEX: Dexamethasone, Base: Baseline, HDC: Hydrocortisone cream. INJ: injection, APP: application.

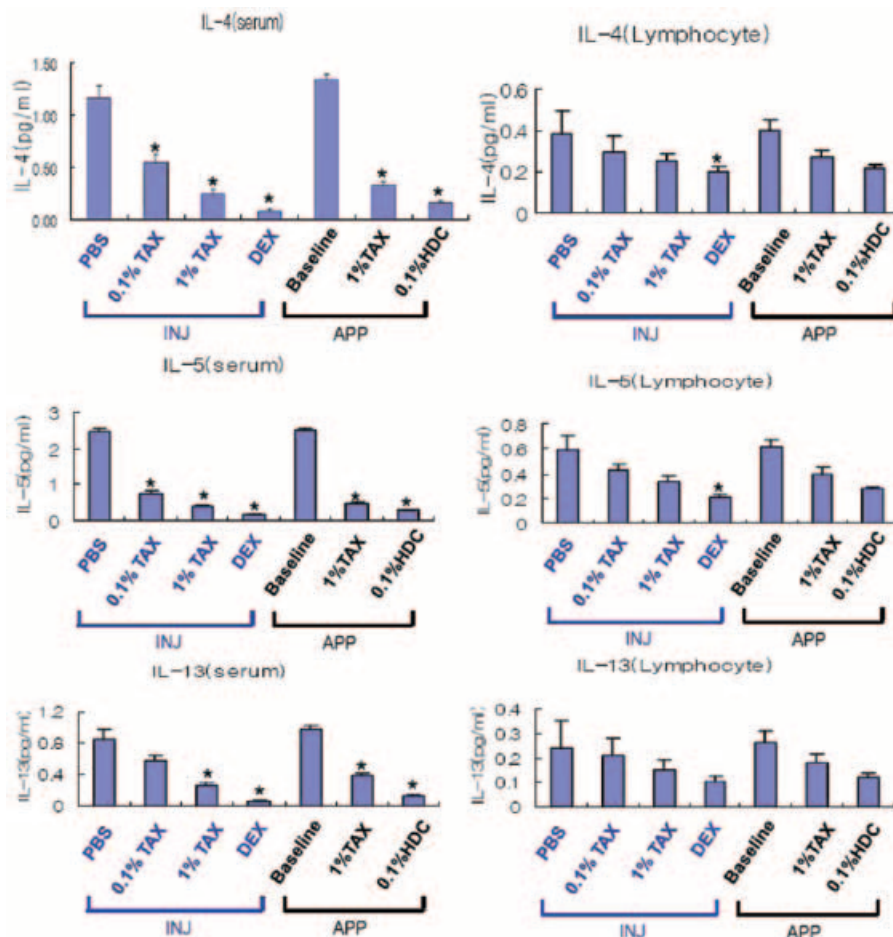


Figure 6. Effects of topical application and intraperitoneal injection of TAX on serum Th2 cytokines levels. The results are expressed as means $\pm$ S.D. (* $P < 0.05$) ($n = 5$). TAX: Taxifolin-glycoside, DEX: Dexamethasone, Base: Baseline, HDC: Hydrocortisone cream. INJ: injection, APP: application.

In an attempt to develop an appropriate treatment agent for AD from a natural substance, we performed *in vivo* experiments using NC/Nga mice to assess the anti-AD effect of TAX.

TAX was used in the current study in order to objectively evaluate its anti-AD efficacy. NC/Nga mice were used in a well-known experimental model of AD

(Vestergaard *et al.*, 1999; 2000; Hashimoto *et al.*, 2004). Immune hypersensitivity was provoked using the contact allergen, DPCP (Shahin, 2005). TAX was administered via topical skin application or intraperitoneal injection.

NC/Nga mice develop atopic dermatitis spontaneously, but in our study the NC/Nga mice did not develop

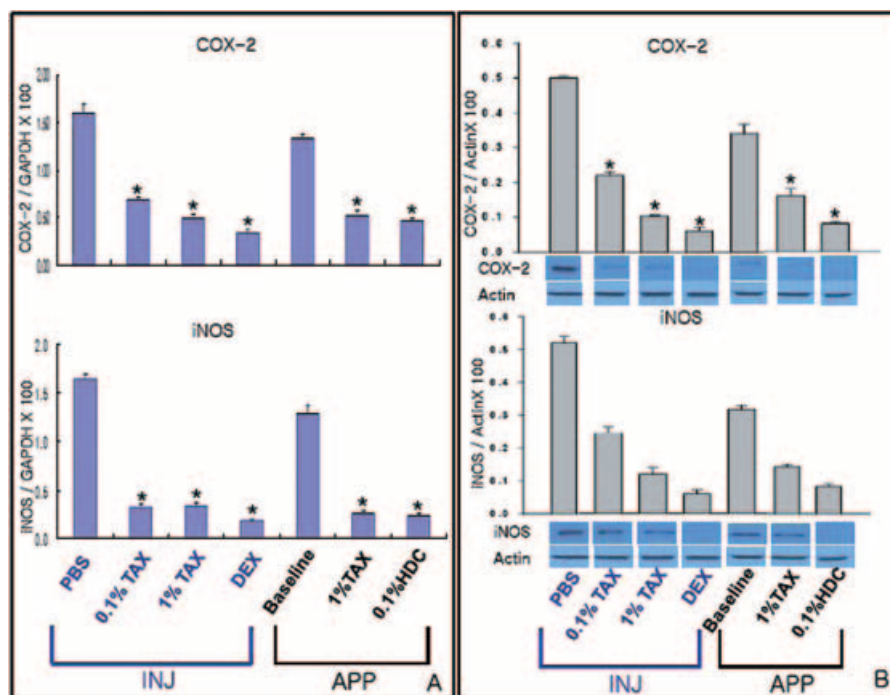


Figure 7. Effects of topical application and intraperitoneal injection of TAX on mRNA and protein expression of iNOS and COX-2. (A) The expression levels were determined by semi-quantitative RT-PCR. (B) The protein expression levels were determined by Western blotting. The results are expressed as means $\pm$ S.D. (* $P < 0.05$) ($n = 5$). TAX: Taxifolin-glycoside, DEX: Dexamethasone, Base: Baseline, HDC: Hydrocortisone cream. INJ: injection, APP: application.

atopic dermatitis skin lesions. We therefore used DPCP sensitization to evoke the allergic skin condition. There are some known differences between atopic dermatitis and allergic contact dermatitis, but, with time, the histological changes are similar. Because of this, patches are often used for the development of atopic dermatitis skin lesions in mice.

For objective assessment of AD, gross examination of skin lesions was performed using the SCORE method (Takano *et al.*, 2006). The clinical skin severity score and the indices of clinical findings were decreased after treatment with TAX.

Eosinophils, which play a crucial role in AD symptoms, infiltrate the dermis within 2–6 h of contact with allergens. Approximately 24–48 h later, eosinophils secrete eosinophil cationic protein (ECP) and thereby induce infiltration of other immune cells into the skin lesions (Bruijnzeel, 1993; Simon *et al.*, 2004), leading to the symptoms of AD. Eosinophil levels were decreased after treatment with TAX. Thus TAX might improve eosinophil-related allergic diseases (Fig. 5).

One of the characteristics often seen in the serum of patients with AD is an increased level of IgE. In recent years, this has been postulated as the key factor in the pathogenesis of AD and the possibility of using this as a prognostic indicator has also been proposed (Rousset *et al.*, 1991; Bos *et al.*, 1992). The increased level of IgE is also a diagnostic indicator of AD. Both topical and intraperitoneal TAX administration significantly down-regulated the IgE levels in NC/Nga mice (Fig. 5).

The aetiology of AD is based on cytokine abnormalities (Sun *et al.*, 1991; Jonathan *et al.*, 1999). An imbalance between Th1 and Th2 levels, i.e., a predominant Th2 response, contributes to the development of acute AD skin lesions, although the causes of the imbalance between Th1 and Th2 have not yet been elucidated. For

effective treatment of AD, Th2-related cytokines must be suppressed (Thepen *et al.*, 1996; Grewe *et al.*, 1998; Homey *et al.*, 2006). Therefore, the rate of suppression of Th2-related cytokines such as IL-4, IL-5 and IL-13 was measured in serum and supernatants of splenocytes. In the TAX-treated group, cytokine expression was significantly inhibited. These results suggest that TAX might exert an immunoregulatory function in the treatment of AD (Fig. 6).

One of the methods for examining the anti-inflammatory activity of a compound is to measure its effect on the rate of suppression of iNOS synthesis (Kharitonov *et al.*, 1994). There are two isoforms of COX: COX-1 and COX-2. COX-1 is constitutively expressed and plays a role in maintaining homeostasis within the body. In contrast, COX-2 is markedly expressed in inflammatory cells in response to stimulation with cytokines in inflammatory or other immune reactions. Thus, the rate of suppression of COX-2 synthesis is also a well-known and universal method for testing the anti-inflammatory effects of a compound (Luca *et al.*, 1999). The mRNA and protein expression levels of iNOS and COX-2 are inhibited in the TAX-treated group (Fig. 7). These results suggest that TAX might exert effective anti-inflammatory activity in NC/Nga mice and might be beneficial in the treatment of skin allergies such as AD.

Previous therapies have had limited value due to the side effects that develop during therapy and their recurrence after cessation of therapy. Thus, natural substance-based therapeutics such as TAX may be potential therapeutic agents for AD.

Acknowledgement

This work was supported by the Seoul R & BD program.

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