CED
Clinical and Experimental Dermatology

In vivo assessment of the effect of taxifolin glycoside on atopic dermatitis-like skin lesions using biomedical tools in NC/Nga mice

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doi:10.1111/ced.12522

Summary

Background. Noninvasive methods of assessment are widely used in clinical trials. However, such methods have not been established in atopic dermatitis (AD), which is a chronic inflammatory skin disease.

Aim. To demonstrate, using biomedical tools, the benefits of a new substance, taxifolin glycoside (TAX), in an AD model, the NC/Nga mouse.

Methods. We evaluated the efficacy of topical TAX for AD by measuring clinical skin severity score, cytokine expression and serum IgE level, and by using biomedical measures (vapometry and corneometry). Topical TAX was applied to AD-induced NC/Nga mice for 3 weeks. The anti-inflammatory effects of this compound were demonstrated noninvasively using biomedical tools and immunological assays.

Results. Our method of AD assessment using biomedical tools is more objective and accurate than visual inspection. The results obtained using the biomedical tools were identical to those obtained using immunological assays.

Conclusions. *In vivo* biomedical tools are useful for diagnosing and monitoring treatment effects in AD.

Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease, characterized by intractable pruritus and relapsing eczematous skin lesions, and is caused by both genetic and environmental factors.[1] Although the pathological mechanism underlying AD is not fully understood, complex inflammatory immune abnormalities and skin barrier defects are believed to affect the onset and aggravation of AD.[2] AD is characterized by increases in eosinophil count and concentration of serum IgE, the representative indicator of AD. In adaptive immunological processes, T helper (Th) cells play a crucial role in induction of AD.[3] In the acute phase of AD, levels of Th2-related cytokines such as interleukin (IL)-4, IL-5 and IL-13 are increased, while those of Th1-related cytokines such as IL-2 and interferon (IFN)-γ show a reciprocal decrease.[4] In addition, impaired barrier function of the stratum corneum (SC) may play a key role in the development and aggravation of AD. Lower levels of ceramide, a major constituent of intercellular lipids in the SC, are thought to increase transepidermal water loss (TEWL) and decrease water capacitance in atopic dry skin.[2]

To date, topical corticosteroids with strong anti-inflammatory properties have been among the most commonly used therapies for treatment of AD. However, these therapeutic agents have limited value because of long-term side effects, rebound phenomenon and intermittent recurrences.[3] Topical calcineurin inhibitors are potential therapeutic alternatives to corticosteroids; however, local side effects, including

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Conflict of interest: the authors declare that they have no conflicts of interest.

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Accepted for publication 26 May 2014

erythema and burning sensation, are commonly noted.[5] Several studies evaluating therapies based on natural substances as potential agents have suggested that patients with AD may benefit from these raw materials.[3,6–8] One such agent, taxifolin glycoside (TAX), isolated from *Rhododendron mucronulatum*, is used in traditional Korean medicine for treatment of rheumatism, neuralgia and inflammation (Fig. 1).[9]

The NC/Nga mouse is a well-described animal model for AD because it has an increased concentration of serum IgE, chronic dryness and severe pruritus.[10,11] However, the low incidence of AD-like lesions, late onset of disease and poor reproducibility are disadvantages of this model.[12] To solve this problem, contact sensitizers such as 2,4,6-trinitro-1-chhlorobenzene (TNCB) or diphenylcyclopropenone have been adopted to induce AD-like lesions in NC/Nga mice. Repeated application to the same skin site results in an immediate-type response followed by a late reaction,[13] features that are representative of AD.

In this study, we evaluated the therapeutic effect of topical TAX on AD-like skin lesions in TNCB-treated NC/Nga mice. Efficacy was assessed by using biomedical tools, skin severity scores, cytokine expression in splenocytes and total serum levels of IgE.

Methods

The study was approved by the Institutional Animal Care and Use Committee of Korea University, and the procedures conformed to the Animal Care and Use Guideline of Korea University, Seoul, Korea.

Animals

Female 12-week-old NC/Nga mice (Central Laboratories, Animal Inc., Seoul, Korea were kept under specific pathogen-free conditions for 7 days before TNCB treatment to allow acclimatization and complete restoration of any damage to the skin barrier that might have occurred during transport.[14] Temperature was maintained at 23 ± 3 °C, humidity at 55% ± 15%, and light on a 12-h light/12-h dark cycle. Food and water were provided *ad libitum*.[15,16]

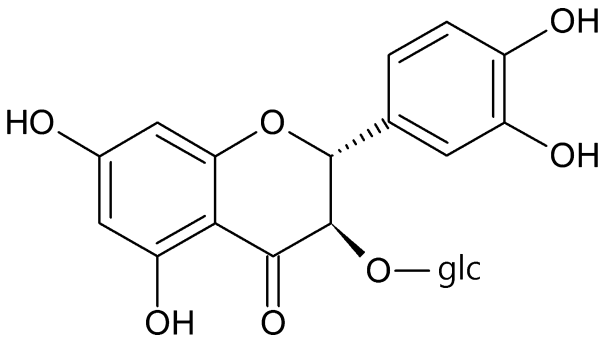

Figure 1 Chemical structure of taxifolin 3-*O*-β-D-glucopyranoside (Taxifolin glycoside).

Taxifolin glycoside

Taxifolin 3-*O*-β-D-glucopyranoside (3′,4′,5′,7′ tetrahydroxy flavonol glucoside) was isolated from the root of *R. mucronulatum*, and administered by topical application (Fig. 1).[13]

2,4,6-trinitro-1-chlorobenzene

TNCB was prepared and dissolved in a mixture of acetone and ethanol in a ratio of 1 : 4 as a 1% solution. The solution was used for induction of atopic-like skin lesions. The tape-stripping method was used to remove the dorsal hair of mice. After 7 days, 200 μL of 1% TNCB was applied topically to the dorsal skin 5 times per week for 2 weeks. In total, 15 mice were stimulated to induce an AD-like skin condition.

Taxifolin glycoside treatment

After induction of AD, 3 μL of cream was applied to the dorsal skin of the mice once per day. Three types of cream were used for treatment (provided by Professor CS Lee, College of Pharmacy, Chungang University, Korea).[17] Creams containing either 1% TAX (n = 5) or 1% TAX with liposome (TAX + L, n = 5) were applied to positive treatment groups, and a base cream with no TAX was applied to the negative treatment group (Base group, n = 5). The control group (n = 5) did not receive any treatment. Creams were applied after stabilization at a room temperature of 22 °C and relative humidity of 60% for 30 min, and stopped 12 h before the efficacy tests.[16]

Clinical assessment of atopic dermatitis

The clinical skin severity score was measured once a week by the SCORAD (SCORing Atopic Dermatitis) index once weekly during the 6-week experimental period. Areas of application were assessed on photographs. Assessment was performed by an investigator who was blinded to the treatment groups. Areas were scored macroscopically using the following diagnostic criteria, which are also used for evaluation of human

AD. Features such as development of erythema/haemorrhage, scarring/dryness, excoriation/erosion and oedema were scored as 0 (none), 1 (mild), 2 (moderate) or 3 (severe).[18] The total skin severity score was defined as the sum of individual scores. The maximum possible score was 12 (3 + 3 + 3 + 3).

Measurement of transepidermal water loss and skin hydration

TEWL and skin surface hydration (SSH) were measured to evaluate the functional state of the epidermal diffusion barrier. Several studies have suggested that reduced levels of ceramides in the SC are involved in the defective barrier function in AD, which is thought to accelerate TEWL and decrease skin hydration. TEWL and SSH measure the water evaporating index from the skin and the skin moisture by capacitance. In recent years, a study has focused on a significant correlation between corneometry, TEWL and AD severity.

Two humidity sensors inside the closed chamber of the measurement device (VapoMeter®; Delfin Technology Ltd, Kuopio, Finland) were used to monitor increases in the relative humidity difference and to measure the evaporation rate of water, which was not affected by ambient or body-induced airflow, and transepidermal water loss (TEWL) was calculated from the measured values.[19] We also measured skin surface hydration using another device (Corneometer CM825®; Courage & Khazaka, Köln, Germany) based on capacitance measurement of a dielectric medium.[20] Each measurement was obtained three times from the same area, and the average value was used. Measurements were obtained at 24% and 46% relative humidity once a week during the 6-week experimental period.[16]

Measurement of serum IgE

Elevation of serum IgE is one of the characteristics of patients with AD. Recently, many studies have suggested that IgE is the key factor in the pathogenesis of AD, and that an increase in its level may be used as a diagnostic and prognostic indicator for AD.[21–23]

The total amount of IgE in serum was measured using an ELISA kit (Bethyl Laboratories Inc. Montgomery, TX, USA), in accordance with the manufacturer's instructions.[24] In brief, each well was coated with captured antibodies, and plates were incubated overnight at 4 °C. The captured antibody solution was then aspirated from each well, and the wells were washed with washing solution (50 mmol/L Tris, 0.14 mol/L NaCl and 0.05% Tween 20) at pH 8.0, and 200 μL of blocking solution (50 mmol/L Tris, 0.14 mol/L NaCl and 1% bovine serum albumin) at pH 8.0 was added to each well. After 30 min of incubation, wells were washed. Serum samples and standards were diluted and plated in the wells, followed by incubation for 1 h. After washing each well, horseradish peroxidase-conjugated detection antibodies were diluted (1 : 20 000), transferred to each well, and then incubated for 1 h. After further washing, the enzyme reaction was initiated by addition of the substrate solution, and the plate was kept at room temperature for 30 min in the dark. The reaction was stopped by addition of 2 mol/L H_2SO_4, and absorbance was measured at 450 nm using an ELISA reader.

Splenocyte culture

Splenocytes were isolated from spleen tissue at the end of the experiment (when mice were around 18 weeks of age), and the mice were subsequently euthanized. Red blood cells (RBCs) were lysed in RBC lysis buffer (Sigma, St Louis, MO, USA), and the lysate was centrifuged at 9177 ***g*** for 15 min at 4 °C. After removal of the supernatant, cells were seeded at 1×10^6/well, and then cultured in 24-well plates. The supernatant containing cultured splenocytes was harvested, and cytokine levels were measured.[9]

Cytokine measurement

Recent studies have shown that an imbalance between Th1 and Th2 levels contributes to development of AD skin lesions.[25] In acute AD, Th2-related responses are prominent, and Th2-related cytokines such as IL-4, IL-5 and IL-13 must be suppressed for effective treatment of AD in the acute phase.[26]

Blood samples were collected from the mice at 18 weeks of age. Supernatants of cultured lymphocytes were analyzed for cytokine levels. A mouse cytokine enzyme immunoassay kit (Invitrogen, Carmarillo, CA, USA)[27] was used for measurement of IL-4 and IFN-γ.

Statistical analysis

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

Results

Clinical severity score

Topical application of 1% TNCB to the dorsal surface of the NC/Nga mice induced AD-like skin lesions, including erythema, erosion, scaling and oedema (Fig. 2). Skin severity scores showed a progressive increase after application of 1% TNCB, reaching a score of > 11 points at 2 weeks. TAX treatment resulted in a significant reduction in the severity of AD-like lesions (Fig. 3a). After 3 weeks of treatment, skin severity scores were significantly lower in the TAX and TAX + L groups than in the Base group ($P < 0.05$). Scores were reduced by 39.7%, 72.3% and 82.2% in the Base, TAX and TAX + L groups, respectively. No significant differences in skin severity scores were observed between the TAX and TAX + L groups (Fig. 3b).

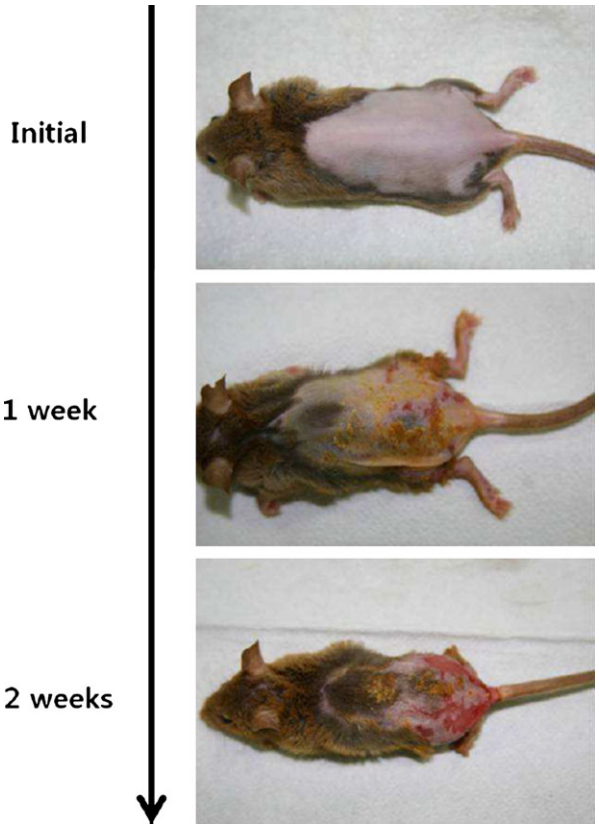

Figure 2 Clinical observation of atopic dermatitis (AD)-like skin lesions induced by 1% 2,4,6-trinitro-1-chlorobenzene (TNCB) in NC/Nga mice. Induction by 1% TNCB resulted in a significant increase in the skin severity score.

Skin barrier function

After 2 weeks of application of 1% TNCB, TEWL increased significantly in the Base, TAX and TAX + L groups compared with the control group ($P < 0.05$). After 3 weeks of treatment, TEWL decreased significantly in the taxifolin-treated groups (TAX and TAX + L) compared with the Base group (Fig. 3c).

Values of skin hydration using the Corneometer corresponded to the results of TEWL obtained using the VapoMeter®. Skin hydration was significantly reduced after application of 1% TNCB for 2 weeks. After 3 weeks of treatment, skin hydration progressively increased in the TAX + L group, reaching the level of the control group ($P < 0.05$; Fig. 3d). The TAX + L group had a better therapeutic effect than the TAX group (Fig. 3c,d). There was a significant negative correlation between corneometry and TEWL for TAX ($r = -0.57$, $P < 0.001$) and TAX + L ($r = -0.61$, $P < 0.001$) using the Pearson correlation coefficient (r).[28]

Total serum IgE

After 3 weeks of treatment, total serum IgE levels were downregulated in the TAX and TAX + L groups compared with the Base group (Fig. 4a), and IgE levels in the TAX + L group were reduced more than those of the TAX group.

Cytokines

In this study, expression of IL-4 was reduced significantly in the TAX and TAX + L groups compared with the Base group after 3 weeks of treatment ($P < 0.05$; Fig. 4b). However, expression of IFN-γ, a Th1-related cytokine, increased in the TAX and TAX + L groups, and showed a negligible difference in the Base group compared with the control group after 3 weeks of treatment (Fig. 4c). These results suggest that TAX performs an immunoregulatory function in acute AD.

Discussion

AD is a chronic inflammatory skin disease that occurs in response to various genetic and environmental factors. AD occurs mainly in infants and children; however, there has been a rapid increase in its incidence in adult patients. Several studies have been conducted for more precise determination of the pathogenesis and aggravating factors of AD. However, recent efforts have concentrated on finding new therapeutic agents for the control of skin moisture and inflammation with

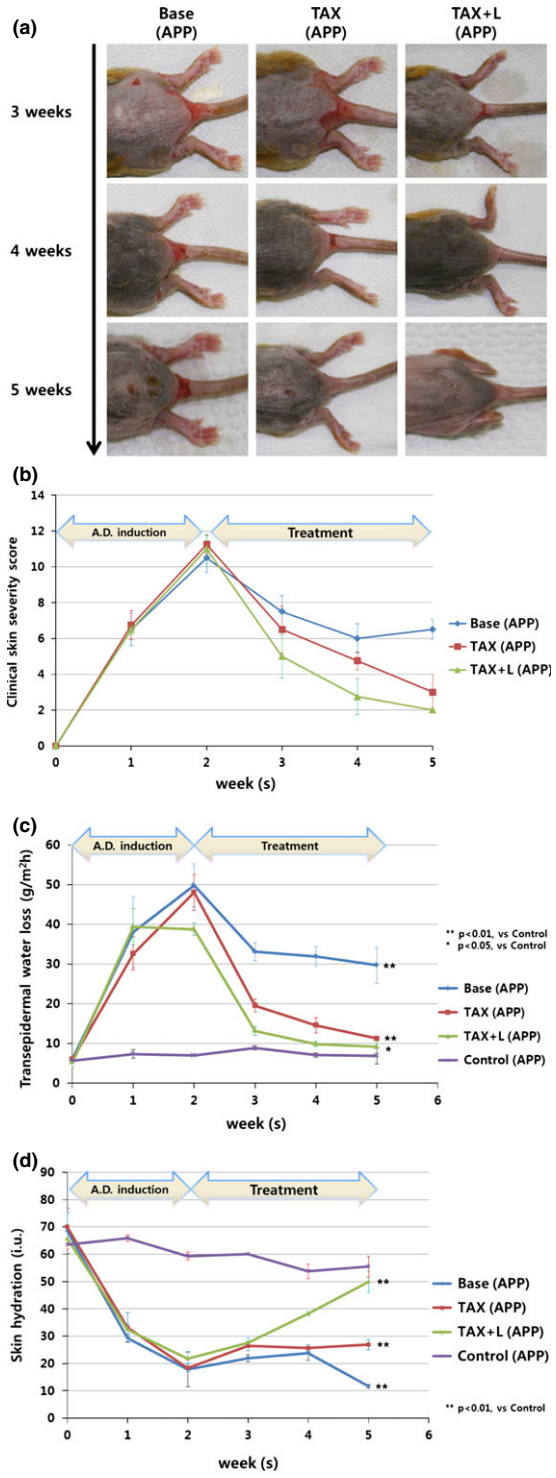

Figure 3 All the statistical differences between the groups were determined by an unpaired *t*-test. (a) Clinical observation of atopic dermatitis (AD)-like skin lesions in NC/Nga mice. Taxifolin glycoside (TAX) treatment resulted in reduced skin severity scores of AD-like skin lesions induced in NC/Nga mice. (b) Changes in the total clinical skin severity score with topical application of 1% TNCB followed by treatment with TAX. (c) Changes in transepidermal water loss measured using the VapoMeter®. (d) Changes in skin hydration measured using the Corneometer CM825®. Skin hydration showed a significant reduction in the AD induction groups after 2 weeks of treatment, and showed a progressive increase in the TAX + liposome (TAX + L) group after 3 weeks of treatment. Base, negative control; i.u., instrumental units; TA, topical application.

long-term safety, which can be used as an alternative to corticosteroids.

The flavonoid TAX is a natural substance isolated from various plants such as *R. mucronulatum, Rhizoma smilacis glabrae* and *Silybum marianum*.[29–31] The anti-inflammatory and anticancer effects of this compound have been demonstrated. Taxifolin also prevents hydrogen peroxide-induced cell death in human keratinocytes and mouse fibroblasts,[32] and lipopolysaccharide-induced production of tumour necrosis factor by murine macrophages.[33] In addition, it has an antioxidant effect,[34] and inhibits cell growth and cell death in prostate, breast, and colorectal cancer cell lines.[35–38] The anti-inflammatory effects of taxifolin glycoside on dendritic cell-mediated immune responses have also been demonstrated in recent studies. Taxifolin was found to inhibit cytokine production and formation of reactive oxygen species and nitric oxide, and induced changes in intracellular Ca^{2+} levels during dendritic cell responses stimulated by exposure to microbial products or IL-1β *in vitro*. These results suggest that TAX is an effective therapeutic agent for treatment of AD through inhibition of IL-1β.[9,17]

The atopic disease reaction represents one of the most widely studied immune responses, and it follows application to the skin of chemically reactive haptene.[15] The TNCB hapten-induced murine model shows that many of the immunological alterations associated with the development of skin lesions, such as Th2-dominated immune responses, increased eosinophil counts and greatly increased serum IgE levels, are possibly a secondary process, rather than being the cause of the diseases. Some studies have indicated that Th2-derived cytokines play an important role in the improvement of atopic diseases. Specific atopic diseases correlate with specific allergic disorders, and these inflammatory reactions are dependent on Th2 cytokines.[29] In previous studies, it was shown that AD, asthma and allergic rhinitis are correlated.[39] AD induces immune response defects, including increased eosinophil counts and IgE levels, and an imbalance in the spectrum of Th1/Th2 responses. For effective treatment of atopic diseases, Th2-related cytokines must be suppressed.[4,30] Previous reports have shown that taxifolin significantly inhibits cytokine expression, and the taxifolin

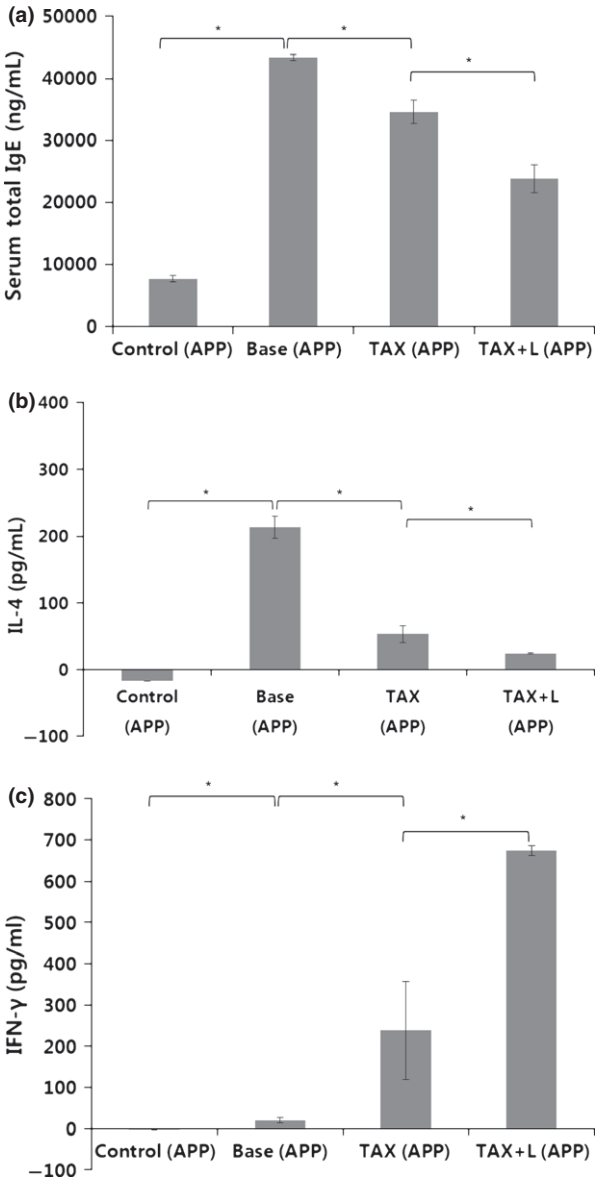

Figure 4 (a) Serum levels of IgE; (b) expression of interleukin (IL)-4; and (c) expression of interferon (IFN)-γ after 3 weeks of treatment. All data are presented as mean ± SEM. The statistical differences between the groups were determined by an unpaired *t*-test (*$P < 0.05$, $n = 4$). Base, negative control; TAX, taxifolin glycoside; TAX + L, taxifolin glycoside with liposome; APP, topical application.

contained in an antioxidant supplement (Pycnogenol®; Horphag Research Ltd, London, UK) has valuable anti-allergic, antiviral and anti-inflammatory properties.[39,40] These results suggest that taxifolin might perform an immune regulatory function in the treatment of autoimmune Th2 diseases.

In this study, we performed *in vivo* experiments using NC/Nga mice for the evaluation of the anti-AD effects of topically applied TAX. The NC/Nga mouse is a well-known natural model, with symptoms of AD that are similar to those observed in humans. TNCB is a valuable sensitizer for the rapid induction of AD symptoms, and these appeared after 2 weeks of application of 1% TNCB in our study.

For objective assessment, we attempted to evaluate the therapeutic effect using a multidisciplinary approach. First, visual analysis was performed macroscopically using the ScorAD index, the clinical skin severity score for AD.[6,41] Second, we performed laboratory analyses using ELISA to measure the level of serum IgE and the expression of Th1- and Th2-related cytokines (IFN-γ and IL-4, respectively), which are key factors in the pathogenesis of AD.[21,42]

Th2 cytokines play an important role in the pathogenesis of AD in NC/Nga mice, similar to their role in human AD. IFN-γ is a Th1 (proinflammatory) cytokine, which inhibits the differentiation of naive T cells to Th2 cells as well as the production of Th2 cytokines. IFN-γ can correct the Th1/Th2 imbalance, and is effective in the treatment of AD.[43,44] Systemic elevation of plasma levels of total IgE and cytokines correlates with AD-like skin lesions in NC/Nga mice, with considerable infiltration of CD4+ T cells, producing IL-4 and IL-5, and degranulation of mast cells and eosinophils.[45,46] Kitagaki reported that Th2-type cytokines, especially IL-4, are predominantly produced by T cells and mast cells in the chronic contact hypersensitivity model.[47] In the current study, repeated exposure of mouse skin to TNCB caused an increase in mRNA levels of IL-4 and IFN-γ, while topical application of TAX and TAX + L was found to cause changes in IL-4, but not IFN-γ mRNA expression.[48] Finally, we evaluated skin barrier function. Damage to the skin barrier is one of the main features in human AD, and NC/Nga mice also develop similar symptoms, including an increase in TEWL and abnormal skin conductance.[16] In the current study, noninvasive bioengineering devices, the VapoMeter® and Corneometer CM825®, were used to measure TEWL and the moisture status, respectively, of the SC. There was a better result in the TAX + L than in the TAX group. As liposomes can improve permeability of the drug into the epidermis and dermis,[21,25] the therapeutic effect might be maximized if taxifolin is administered with liposome. The results suggest that addition of liposome, which has been widely used as a nontoxic nanocarrier to promote skin permeation of a drug,[49] is superior to cream alone for the treatment of AD, because of greater flux values.

The NC/Nga mouse is the most commonly used disease model of AD.[50] Pathophysiological observations in

NC/Nga mice closely resemble those in human AD. Therefore, this is a useful model for analyzing pathological mechanisms of human AD.[44] The NC/Nga mouse model may provide significant evidence for understanding the pathophysiology of AD, and may also aid in drug discovery for potential treatment of AD.[51]

Conclusion

In this study, we demonstrated the therapeutic efficacy of topical taxifolin in NC/Nga mice *in vivo*. Visual assessment identified improvement of the clinical features of AD after treatment. Laboratory analysis using ELISA showed that topical taxifolin inhibited Th2-related cytokines and serum IgE, which are key pathogenic factors of AD. There was also improvement in skin barrier function after treatment as measured by noninvasive bioengineering devices. These results suggest that there is a potential application of TAX as an alternative therapeutic agent for treatment of AD. In addition, more objective and noninvasive methods may be valuable for evaluating and monitoring treatment efficacy in allergic skin diseases such as AD.

What's already known about this topic?

- Noninvasive methods have been widely used for clinical trials.
- However, such methods have not been established in AD, which is a chronic inflammatory skin disease.

What does this study add?

- This study aimed to demonstrate, using biomedical tools, the benefits of a new substance, TAX, in an AD model, the NC/Nga mouse.
- TAX produced improvement in clinical features of AD and in skin barrier function.
- TAX inhibited Th2-related cytokines and serum IgE.
- TAX seemed to work more efficiently when combined with liposomes.

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