J. Korean Wood Sci. Technol. 42(5): 579~589, 2014 http://dx.doi.org/DOI : 10.5658/WOOD.2014.42.5.579

Inhibitory Effects of *Camellia sinensis* Extract on the Development of Atopic Dermatitis-like Lesions in NC/Nga Mice[1]

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ABSTRACT

Atopic dermatitis (AD) syndrome is one of the most common and severe skin diseases in Korea; a large population has this disease. We examined the effects of the extract from the leaf and sprig of *Camellia sinensis* on the development of AD by using NC mice as a model of atopic dermatitis. Oral administration of the extract to NC/Nga mice treated with 2,4-dinitrochlorobenzene (DNCB) inhibited the development of AD-like skin lesions as shown by a significant decrease in the skin symptoms of the disease and a decrease in ear thickness and levels of immunoglobulin E (IgE) and thymus-and activation-regulated chemokine (TARC) level in the skin. Administration of the extract markedly suppressed the DNCB-induced mRNA expression of interleukin 4 (IL-4) and tumor necrosis factor α (TNF-α). The findings suggest that transdermal application of the extract may modulate in the skin of NC/Nga mice. The extract was effective for the prevention and treatment of AD.

Keywords : atopic dermatitis, NC/Nga mice, IgE, *Camellia sinensis*

1. INTRODUCTION

The incidence of AD has recently increased in industrialized countries, and although the onset of AD is usually during early infancy and childhood, it can also occur in adulthood (Cooper, 1994; Rudikoff and Lebwohl, 1998). Although topical steroids, emollients, and oral anti-histamines are used as the first-line therapy for AD, many patients are still concerned about the long-term use of these agents (Furue *et al.* 2003). Currently, treatment and prevention of AD through dietary intervention is receiving considerable attention. Recent studies indicate that intake of diet containing lactic acid bacteria and natural polyphenols such as astragalin and genistein suppresses AD-like skin lesions (Wakabayashi *et al.* 2008; Kotani *et al.* 2000). The prevalence of AD has increased progressively by 2 to 3 fold during the past three decades in industrialized countries with a lifetime prevalence of 10-20% in children and 1-3% in adults (Leung and Bieber, 2003; Schultz and Hanifin, 2002). Topical glucocorticoids are used for the treatment of AD as important and effective remedies. However, these agents can be used only for a short period of time and in limited skin regions, because long-term use of glucocorticoids caus-

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es a variety of potential side effects (Barnetson and White, 1992). Therefore, the use of complementary or alternative medicine (CAM), a main alternative resource being phytotherapy (use of natural extracts as therapeutic agents), has become increasingly popular for the treatment of AD, and CAM has been used to overcome the shortcomings of conventional therapy (Boneberger *et al.* 2010). Thus, because of an increase in the use of CAM in AD patients, further studies are required to establish their efficacy, safety, and therapeutic use (Boneberger *et al.* 2010).

C. sinensis, a tree from which a beverage commonly used in Asian countries is obtained, contains compounds having strong antioxidant capacities. It is a significant source of polyphenols, including (-)-epigallocatechin gallate (EGCG), (-)-epigallocatechin, (-)-epicatechin gallate, and (-)-epicatechin. These polyphenols have recently attracted attention as bioactive agents with anticancer, antidiabetic, antiviral, antimalarial, hepatoprotective, neuroprotective, and cardioprotective effects (Adhami *et al.* 2007; Noonan *et al.* 2007). However, little is known about the beneficial effects of *C. sinensis* extract on AD (Lee *et al.* 2006; Rowe *et al.* 2007).

Polyphenols obtained from *C. sinensis*, referred to type of catechins, are present in the leaves of the tea plant. Several in vitro (Trompezinski *et al.* 2003; Huang *et al.* 2005) and *in vivo* (Afaq *et al.* 2003; Hsu *et al.* 2007) studies have shown that a group of polyphenolic catechins, their major constituent being EGCG, exhibits multiple biological effects on the skin. Trompezinski *et al*. (Trompezinski *et al.* 2003) have shown that EGCG inhibits, in a dose-dependent manner, the upregulation of both vascular endothelial growth factor and IL-8 in TNF-α stimulated keratinocytes. Afaq *et al.* (Afaq *et al.* 2003) have shown that topical application of green tea polyphenol to SKH-1 hairless mice before ultraviolet-B exposure significantly decreased skin edema and infiltration of leukocytes via modulations in the mitogen-activated protein kinase and nuclear factor kappa B signaling pathways. These findings have suggested that EGCG may be a novel therapeutic agent for various inflammatory skin diseases such as AD. Kim *et al*. (Kim *et al.* 2012) showed that bath therapy with green tea extract is an effective, safe, and non-steroidal therapy for the treatment of patients with AD associated with *Malassezia sympodialis*.

To date, several animal models have been developed for studies on AD (Yang *et al.* 2013; Turner *et al.* 2013; Yoon *et al.* 2011). The AD model of NC/Nga mice is clinically and histologically very similar to human AD, when mice are raised under conventional conditions (Matsuoka *et al.* 2003).

NC/Nga mice originated from the Japanese fancy mice at Nagoya University (Japan) in 1957, and they were established as an inbred strain for creating an animal model of human AD. NC/Nga mice raised in air-uncontrolled conventional circumstances spontaneously develop AD-like skin lesions with a markedly elevated serum level of IgE, whereas NC/Nga mice maintained in specific pathogen-free (SPF) conditions do not show clinical signs of AD or IgE hyperproduction (Matsuda *et al.* 1997). However, a variety of antigens can be involved in the development of AD-like skin lesions in NC/Nga mice under conventional conditions, which makes it difficult to analyze the pathogenesis of AD-like skin lesions (Shiohara *et al.* 2004).

The objective of this study was to evaluate the effects of administration of *C. sinensis* extract on the progress of AD-like skin lesions in NC/Nga mice by continuous application of the DNCB. *C. sinensis* extract showed excellent anti-AD activity and thus could be possibly used to treat and prevent AD. This study may further provide insights on the potential of *C. sinensis* extract as an integral part of a novel therapeutic modality for AD in humans or animals.

2. MATERIALS and METHODS

2.1. Preparation of *C. sinensis* Extracts

For this study, the leaves and branches of *C. sinensis* were collected from the Hadong green tea institute (Hwagae-myeon, Hadong-gun, Gyeongnam-do, Korea, 35°N and 127°E) in Hadong, Korea. The leaves and branches were cut using a pair of pruning shears. The leaves and branches were spread out on a large flat surface to dry. To ensure heterogeneity among samples, all leaves were stored at room temperature until a constant fresh weight was obtained before use.

The leaves and branches were pretreated to perform a steaming treatment. Steaming of leaves and branches was performed in an autoclave set at a desired temperature of 121℃ for 30 min.

The extracts of pretreated leaves and branches were prepared under two conditions of hot water and ethanol treatment. The hot water extract of pretreated leaves and branches was exactly weighed (50 g) in an Erlenmeyer flask, 1000 mℓ distilled water was added to the flask, and the extract was autoclaved at the desired temperature of 121℃ for 30 min. The ethanol extract of pre-treated leaves and branches was exactly weighed (50 g) in an Erlenmeyer flask, 1000 mℓ of 80% ethanol solution was added into the flask, and the extract was placed in a shaking incubator at 25℃ for 48 h.

Then, the extracts were filtered through a glass filter (2G3). The hot water and ethanol extracts were concentrated in a rotary evaporator under reduced pressure at 40℃ and then freeze-dried. Both extracts were stored at 4℃ for subsequent analyses.

The yield from dried hot water and ethanol extracts was approximately 19.7% and 26.1% (w/w), respectively. The hot water extract and ethanol extract were mixed (1:1, w/w) for the experiment.

2.2. Experimental Animals and Diets Preparation

Specific pathogen-free female 5-week-old NC/Nga mice (25 ± 2 g) were purchased from SLC, Inc. (Shizuoka, Japan). Animals were maintained for 1 week before the start of the experiments. They were housed in an air-conditioned animal room with a temperature of 23 ± 2℃ and a humidity of 50 ± 10%. Mice were provided with solid feed (CRF-1, Oriental Yeast, Tokyo, Japan).

2.3. Chemicals and Treatment in NC/Nga Mice Test

2,4-dinitrochlorobenzene (DNCB) was purchased from Sigma-Aldrich (Milwaukee, WI, USA). The experimental schedule for the preparation of AD-like skin lesion in NC/Nga mice is summarized in Fig. 1.

The mice were divided into four groups (n = 5 per group). To induce AD-like immunologic and skin lesions, DNCB was applied onto the dorsal skin and ears. After complete removal of hair on the dorsal surface of within an area of approximately 8 cm^2, 200 μℓ of 1% DNCB solution (dissolved in a 3:1 mixture of acetone and olive oil) was applied on their dorsal skin and ears for three consecutive days fors ensitization. Four days after sensitization, the dorsal skin and ears were challenged with 200 μℓ of 0.2% DNCB solution three times per week for eight weeks. After inducing AD, the extract was applied to the dorsal skin and ears of the mice six times per week for four weeks.

A lotion without the extract was applied to the dorsal skin and ears of the control and DNCB-treated mice. Animals were killed 64 days after the first application of DNCB. Blood was collected from the vena cava and the right ear was removed and used for histopathological examination.

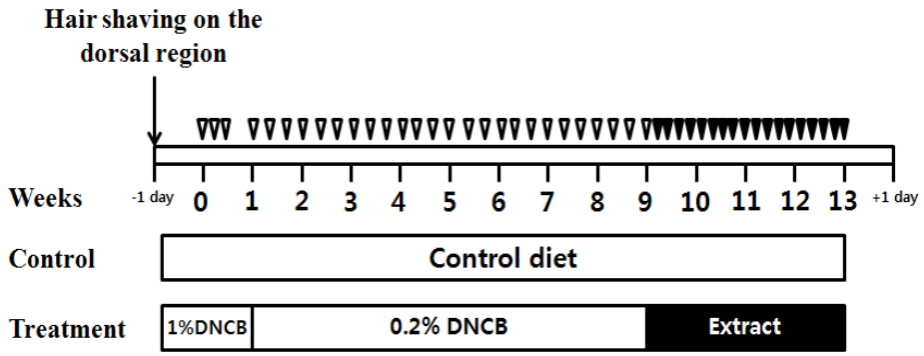

Fig. 1. Experimental schedule for the induction of AD-like lesion in NC/Nga mice. Deltas indicate the application of DNCB.

2.4. Body Weights and Ear Thickness of NC/Nga Mice

The body weights of the NC/Nga mice were monitored once a week during the experimental period. Ear thickness was measured with a micrometer (Mitutoyo, Kawasaki, Japan) on the day of sacrifice. The right ear was removed from each mouse and fixed in 10% formalin solution. It was subsequently embedded in paraffin, sectioned (4 μm), and stained with hematoxylin and eosin.

2.5. Quantitation of Total IgE, IL-10, and TARC (ELISA assay)

Enzyme-linked immunosorbent assay (ELISA) kits for IgE, IL-10, and TARC were obtained from R&D Systems (Minneapolis, MN, USA) and BD Biosciences (San Diego, CA, USA). Cytokines in serum level were measured by sandwich ELISA using the OptEIA Set mouse IgE kit from BD Biosciences, the DuoSet mouse IL-10, and the mouse CCL17/TARC kits from R&D Systems, according to the manufacturer's instructions.

Cytokine concentrations were calculated using a linear regression equation obtained from standard absorbance values.

2.6. RT-PCR Analysis

Total RNA from the spleen was isolated with Trizol (Molecular Research Center Inc., Cincinnati, USA) according to the manufacturer's instruction. cDNA was synthesized from total RNA (2 g) using oligo (dT) 18 primers (0.5 g) and the Prime RT Premix kit (Daejeon, Korea) according to the manufacturer's instruction. The primers used in this study were as follows: IL-4 (forward) 5'- AGT TGT CAT CCT GCT CTT CTT T -3', (reverse) 5'- GAC TGG GAC TCA TTC ATG GTG C -3'; TNF-α (forward) 5'- CAC CAC GCT CTT CTG TCT ACT GAA C -3', (reverse) 5'- CCG GAC TCC GTG ATG TCT AAG TAC T -3'; and GAPDH (forward) 5'-TGA ACG GGA AGC TCA CTG G-3' (reverse) 5'-TCC ACC ACC CTG TTG CTG TA-3'. The PCR reaction was performed using the Prime *Taq* Premix kit (GeNet Bio, Korea) and 0.1 mM GAPDH-specific primer pair. All samples were pre-denatured 94℃ for 5 minutes, followed by thirty-two thermal cycles (at 94℃ for 30 s, 55-62℃ for 30 s, and 72℃ for 1 minutes), and an additional extension at 72℃ for 7 minutes. Amplified cDNAs were subjected to elec-

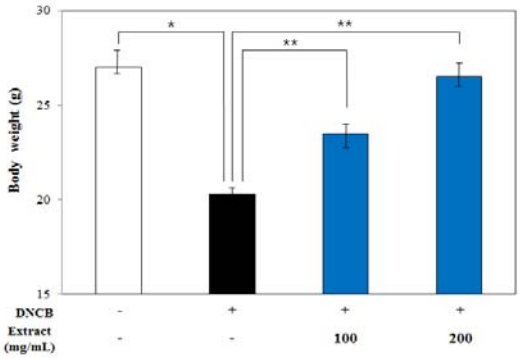

Fig. 2. Body weight of DNCB-induced NC/Nga mice by transdermal application of extract. Extract (100 mg and 200 mg/kg) was application to the NC/Nga mice sensitized and challenged with DNCB as described in materials and methods. Body weights in each group of NC/Nga mice were monitored once a week starting 1 week before sensitization. Values represent the means ± S.D. of 5 mice for each group. (*p < 0.1, compared to the control group; ** p < 0.01, compared to the DNCB-treated group)

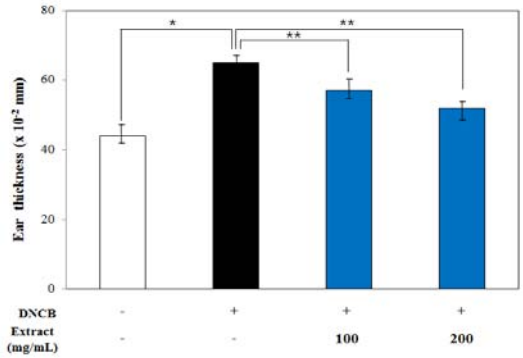

Fig. 3. Effects of *C. sinensis* extract on the ear thickness. Each value represents the mean ± SD of five mice. (*p < 0.1, compared to the control group; ** p < 0.01, compared to the DNCB-treated group)

trophoresis on 2% agarose gels and visualized by staining with ethidium bromide.

2.7. Histological Examination

Tissues from the right ear were removed and fixed in 10% paraformaldehyde solution for 24 hours. The tissues were embedded into the paraffin and blocks of 5 μm thickness were prepared. The sections were stained with haematoxylin and eosin (H&E) to differentiate inflammation and edema in epidermis, dermis, keratinocytes, neutrophils and eosinophils.

2.8. Statistical Analysis

All experiments were repeated at least three times. Results are reported as means ± standard error of the mean (SEM). Statistical significance was determined by a one-way analysis of variance (ANOVA), followed by the Tukey-Kramer multiple comparisons test. A significant value was defined as p < 0.05.

3. RESULT and DISCUSSION

3.1. Change Body Weight of NC/Nga Mice

The extract was applied to the dorsal skin and ears of the mice six times per week for four weeks. A lotion without the extract was applied to the dorsal skin and ears of control and DNCB-treated mice. The body weight of DNCB-treated mice was measured using a weighing balance. Each value represents the mean ± SD of five mice (Fig. 2). The body weight of the NC/Nga mice increased with an increase in the concentration of the extract. The mean body weights of the NC/Nga mice treated with the extract (200 mg/mℓ) were not significantly different from those of the normal NC/Nga mice. Our results are representative of five separate experiments with similar results. The body weights of mice treated with the extract were within normal limits.

3.2. Ear Thickness of NC/Nga Mice

Changes in the ear thickness of mice are shown in Fig. 3. The ear thickness was measured immediately before DNCB application and killing the mice. The ear thickness was 43.1 ± 1.4 ($\times 10^{-2}$ mm) in normal NC/Nga mice, 65.5 ± 2.5 ($\times 10^{-2}$ mm) in DNCB-treated NC/Nga mice, 56.4 ± 7.3 ($\times 10^{-2}$ mm) in NC/Nga mice treated with 100 mg/mℓ of the extract, and 49.1 ± 0.3 ($\times 10^{-2}$ mm) in NC/Nga mice treated with 200 mg/mℓ of the extract. This finding showed that the ear thickness of normal NC/Nga mice was significantly lower than that of NC/Nga treated with the extract. In DNCB-treated NC/Nga mice, the ear swelling subsided was certainly remission after application of the 200 mg/mℓ of the extract, and the ear thickness decreased to a value similar to that of the normal mice. These results indicated the protective effects of the extract on decreasing swelling of the ear.

3.3. Serum Total IgE, IL-10 and TARC Levels of NC/Nga Mice

The serum IgE levels are shown in Fig. 4A. Repeated application of DNCB increased the serum IgE levels in sensitized NC/Nga mice. Mast cells are key effectors in IgE-mediated allergic disorders. Upon activation, mast cells undergo degranulation and release a variety of biologically active substances such as IL-4, IL-5, and IL-13, which play an important role in the development of AD (Amin, 2012; Wesolowski and Paumet, 2011). An increase in serum IgE levels is an important characteristic of AD. To examine whether the extract decreases the increase in IgE induced by DNCB in NC/Nga mice, we measured the serum IgE levels by using enzyme-linked immunoassay (ELISA) analysis.

We examined the serum levels of total IgE in NC/Nga mice for four weeks. The total IgE levels in NC/Nga mice treated with the extract were significantly lower than those in the DNCB-treated NC/Nga mice. The serum IgE levels in the DNCB-treated NC/Nga mice (38.26 ± 5.51 ng/mℓ) were higher than those in the normal NC/Nga mice ($p < 0.5$), whereas the serum IgE levels in the NC/Nga mice treated were markedly decreased to 18.34 ± 6.31 ng/mℓ (extract 200 mg/mℓ) ($p < 0.05$).

IL-10 is an important mediator that prevents allergic inflammation, and development of AD-like skin lesions might be associated with suppression of regulatory cytokines. IL-10 induces allergen-specific T-cell proliferation and suppresses the secretion of Th1- and Th2-type cytokines. Compared to normal controls, patients with severe AD show a decrease in IL-10 production (Pickard *et al.* 2007).

We examined IL-10 production from axillary lymph node cells obtained from normal mice and mice treated with the extract for four weeks. Compared to the lymph node cells from DNCB-treated NC/Nga mice, those from the mice treated with the extract showed a marked increase in the IL-10 levels compared DNCB-induced NC/Nga mice (Fig. 4B).

The serum TARC levels in NC/Nga mice treated with the extract significantly decreased during the four weeks ($p < 0.05$). Development of skin lesions in DNCB-treated NC/Nga mice induced a marked decrease in the expression of TARC (Fig. 4C). The NC/Nga mice treated with the extract (100 and 200 mg/mℓ) had lower TARC levels than the control mice. Overexpression of TARC by the basal keratinocytes as the lesions develop in the skin of the NC/Nga mice suggests that TARC plays a key role in the pathogenesis of the lesions. When mice keratinocytes were cultured in the presence of several inflammatory cytokines, TNF-α was the most potent inducer of TARC. In the present study, transdermal

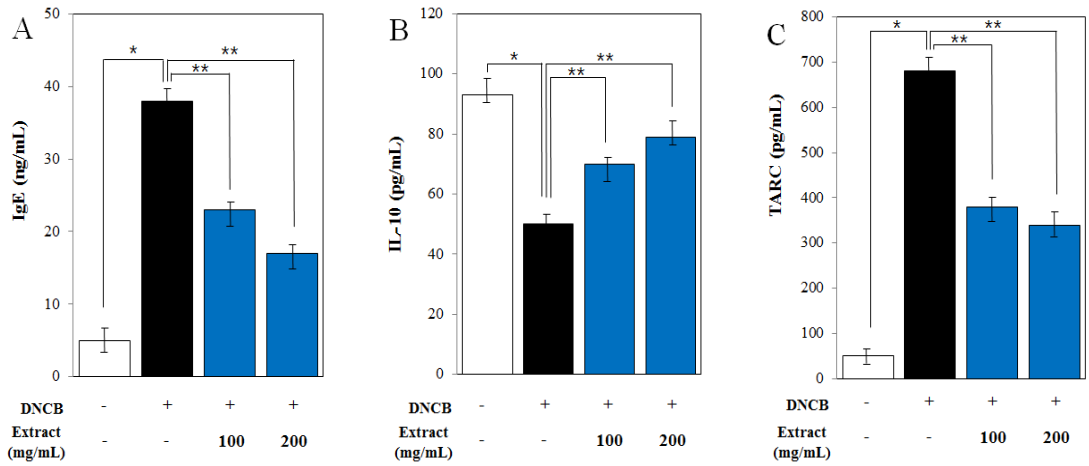

Fig. 4. Change in serum of IgE, IL-10 and TARC by *C. sinensis* application of DNCB in NC/Nga mice. The serum level of IgE, IL-10 and TARC was determined using mouse IgE specific ELISA kit. Each value represents the mean ± SD of five mice. (A: IgE, B: IL-10, C: TARC) (*p < 0.5, compared to the control group; ** p < 0.05, compared to the DNCB-treated group.)

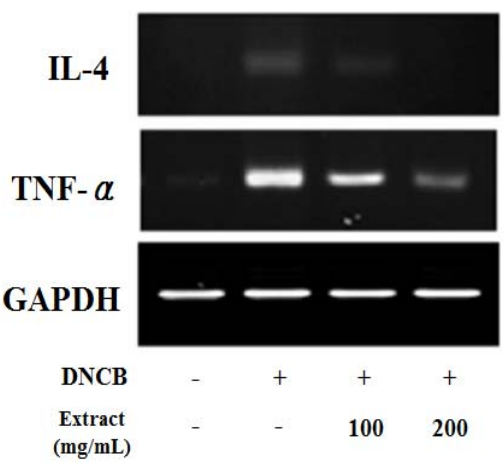

Fig. 5. Expression of IL-4, TNF-α and GAPDH mRNA in mice treated repeatedly with DNCB and *C. sinensis* extract. Total RNA was prepared and analyzed for the expression of IL-4, TNF-α and GAPDH mRNA by RT-PCR. Results are shown representatives of five observations.

application of the extract modulated the serum IgE, IL-10, and TARC levels (IgE, 16.2 ng/mℓ in mice treated with 200 mg/mℓ of the extract; IL-10, 78.8 pg/mℓ in mice treated with for 200 mg/mℓ of the extract; and TARC, 337.9 pg/mℓ in mice treated with 200 mg/mℓ of the extract).

3.4. IL-4 and TNF-α Induce Stabilization of Eotaxin mRNA

The mRNA expression levels of IL-4, TNF-α, and GAPDH examined using real-time polymerase chain reaction (RT-PCR) are shown in Fig. 5. An imbalance between Th1 and Th2 responses is thought to under immune system diseases such as AD, which are characterized by Th2-dominated allergic inflammation (Spergel *et al.* 1999; Vestergaard *et al.* 1999). The expression of TNF-α is upregulated in mast cells in the skin of AD lesions (Ackermann and Harvima, 1998) and induces expression of adhesion molecules in the endothelium in lesional AD skin (Vries *et al.* 1998).

Therefore, we examined changes in the mRNA expression of Th1 and Th2 type cytokines in the spleen. Compared to DNCB-treated NC/Nga mice,

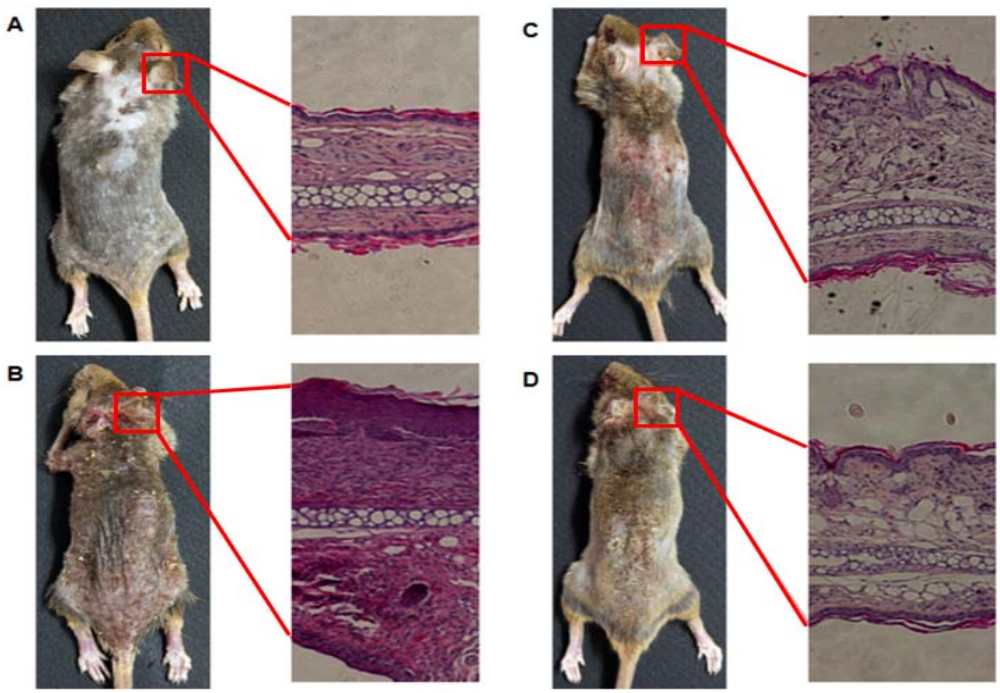

Fig. 6. Comparison of the histopathology of ear lesions in DNCB-induced NC/Nga mice after repeated application of extract. The ears of control (A), DNCB (B), DNCB plus extract (100 and 200 mg/mℓ C, D)-treated NC/Nga mice were excised, fixed with 10% formaldehyde, embedded in paraffin and thin sections were made. The skin sections were stained with hematoxylin and eosin.

the mice treated extract showed a marked decrease in the mRNA expression of IL-4 and TNF-α (Fig. 5). These findings suggest that transdermal application of the extract may modulate the immune response of DNCB-treated NC/Nga mice. In addition, these results suggest that the expression of IL-4 and TNF-α was inhibited in the NC/Nga mice treated with the extract.

3.5. Histological Evaluation of The Lesional Skin

We performed histopathological observations of the ear lesions in DNCB-treated NC/Nga mice (Fig. 6).

Epidermal thickening of the ear skin, infiltration of inflammatory cells, and increased elevated mast cell degranulation are histopathological features in AD-induced NC/Nga mice. Compared to the normal mice, those treated with the DNCB showed significant hyperkeratosis of the ear lesions, thickening of the dermis and epidermis, and accumulation of inflammatory cells. These effects were inhibited by application of lotion containing the extract. These results indicate that the extract effectively decreased the symptoms of AD in NC/Nga mice. In the ear treated with 200 mg/mℓ of the extract (Fig. 6D.), the result was certainly remission as compared between DNCB-induced NC/Nga mice and 100 mg/mℓ extract treated NC/Nga mice ear (Fig. 6B, C). In addition, no significant difference was observed in body weights of the two groups of NC/Nga mice during the experimental period (Fig. 2). These results

suggest that treatment of the NC/Nga mice with the extract decreased the development of symptoms of dermatitis without changes in body weight.

4. CONCLUSION

In this study, we showed that application of *C. sinensis* extract significantly reduces ear thickness, clinical signs, and serum IgE and TARC levels in a DNCB-treated NC/Nga mice model. The NC/Nga mice model has been used for the estimation of drug candidates developed for the treatment of AD. Here, we showed that typical symptoms of AD could be observed in NC/Nga mice by repeated topical application of DNCB. Moreover, treatment with *C. sinensis* extract decreased infiltration of inflammatory cells into the DNCB-induced AD-like skin lesions. These results highlighted protective effects of *C. sinensis* extract on development of dermatitis in DNCB-treated NC/Nga mice. Topical application of DNCB on the back and ear of NC/Nga mice was effective for inducing severe and controlled dermatitis within 4 weeks. Ear swelling and skin lesions were observed and IL-4 and IgE levels markedly increased. Further, histopathological analysis showed that infiltration of leukocytes, especially mast cells, into the ear was observed in DNCB-treated NC/Nga mice. The production of proinflammatory cytokines such as TNF-α by epidermal cells is one of the main events mediating the initiation of AD. TNF-α and other proinflammatory cytokines produced at the initiation stage of AD induce the expression of a variety of chemokines and adhesion molecules, which direct the recruitment, proliferation, and survival of leukocytes within the skin.

Treatment of DNCB-treated NC/Nga mice with *C. sinensis* extract is directed at symptom relief and reduction in cutaneous inflammation. Our results showed that application of *C. sinensis* extract decreased the severity of AD and prevented the development in AD-induced mice with no apparent side effects. Further studies are required to evaluate the clinical efficacy and the preventive effects of the *C. sinensis* extract on patients with AD; we believe that these agents may constitute alternative or complementary therapy for AD.

ACKNOWLEDGEMENT

This study was carried out with the support of “Forest Science & Technology Projects (project No. S211313L010140)” provide by Korea Forest Service.

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