



Hataedock treatment has preventive therapeutic effects for atopic dermatitis through skin barrier protection in *Dermatophagoides farinae*-induced NC/Nga mice



Ho-Yeol Cha^{a,d,1}, Sang-hyun Ahn^{b,1}, Jin-Hong Cheon^{a,d}, Sun-Young Park^c, Kibong Kim^{a,d,*}

^a Department of Korean Pediatrics, Hospital of Korean Medicine, Pusan National University, Geumo-ro 20, Mulgeum-eup, Yangsan-si, Gyeongsangnam-do 50612, Republic of Korea

^b Department of Anatomy, College of Korean Medicine, Semyung University, Semyung-ro 65, Jecheon-si, Chungbuk 27136, Republic of Korea

^c Department of Physiology, College of Korean Medicine, Semyung University, Semyung-ro 65, Jecheon-si, Chungbuk 27136, Republic of Korea

^d Department of Korean Pediatrics, School of Korean Medicine, Pusan National University, Pusandaehak-ro 49, Mulgeum-eup, Yangsan-si, Gyeongsangnam-do 50612, Republic of Korea

ARTICLE INFO

Keywords:

Hataedock

Atopic dermatitis

Dermatophagoides farinae

Skin barrier protection

outside-in hypothesis

anti-inflammation

ABSTRACT

Ethnopharmacological relevance: Hataedock treatment is traditionally used for the purpose of preventing the future skin disease by feeding herbal extracts to the newborn in traditional Chinese and Korean medicine.

Aim of the study: This study investigated the preventive therapeutic effects of Hataedock (HTD) treatment for atopic dermatitis (AD) through skin barrier protection in *Dermatophagoides farinae*-induced NC/Nga mice.

Materials and methods: To the HTD treatment group, the extract of *Coptis japonica* Makino and *Glycyrrhiza uralensis* Fischer, which analyzed with High Performance Liquid Chromatography (HPLC)-fingerprint for quality consistency, was administered orally to the 3-week-old mice before inducing AD. After that, *Dermatophagoides farinae* was applied except the control group to induce AD-like skin lesions. We confirmed the effects of HTD on morphological changes, protection of skin barrier, regulation of Th2 differentiation, inflammation regulation and induction of apoptosis through histochemistry, immunohistochemistry, and Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay.

Results: HTD effectively reduced edema, angiogenesis and skin lesion. HTD also increased the levels of liver X receptor (LXR) and filaggrin but decreased the level of protein kinase C (PKC) ($p < 0.01$). The levels of interleukin-4 (IL-4), IL-13, signal transducer and activator of transcription-6 (STAT-6) and Cluster of differentiation 40 (CD40) were significantly reduced in the HTD treated group ($p < 0.01$). HTD also suppressed the mast cell degranulation and the level of the high-affinity IgE receptor (FcεRI), substance P, Matrix metalloproteinases-9 (MMP-9) and 5-hydroxytryptamine (5-HT) ($p < 0.01$). The levels of inflammatory factors such as nuclear factor-kappaB (NF-κB) p65, phosphorylated IκB (p-IκB) and inducible nitric oxide synthase (iNOS) were also decreased ($p < 0.01$). Apoptosis of inflammatory cells was also found to increase ($p < 0.01$).

Conclusion: Our results indicate that HTD effectively regulate the Th2 differentiation, mast cell activation and various inflammatory responses on AD-induced mice through protection of skin barrier. Therefore, HTD may have potential applications for alternative and preventive treatment in the management of AD.

Abbreviation: AD, Atopic dermatitis; HTD, Hataedock; DfE, *Dermatophagoides Farinae* Extract; HPLC, High Performance Liquid Chromatography; M/T, Masson trichrome method; LXR, liver X receptor; FLG, Filaggrin; PKC, protein kinase C; IL-4, interleukin-4; IL-13, interleukin-13; STAT-6, Signal transducer and activator of transcription-6; CD40, Cluster of differentiation 40; FcεRI, the high-affinity IgE receptor; MMP-9, Matrix metalloproteinases-9; 5-HT, 5-hydroxytryptamine; NF-κB, nuclear factor-kappaB; p-IκB, phosphorylated IκB; iNOS, inducible nitric oxide synthase; NMF, natural moisturizing factors; DAG, diacylglycerol; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling

* Corresponding author at: Department of Korean Pediatrics, Hospital of Korean Medicine, Pusan National University, Geumo-ro 20, Mulgeum-eup, Yangsan-si, Gyeongsangnam-do, 50612, Republic of Korea.

E-mail addresses: chahy0671@hanmail.net (H.-Y. Cha), dongana@hanmail.net (S.-h. Ahn), cheonjh@pusan.ac.kr (J.-H. Cheon), sue-7@hanmail.net (S.-Y. Park), kbb2630@gmail.com (K. Kim).

¹ These two authors contributed equally to this work.

<http://dx.doi.org/10.1016/j.jep.2017.06.001>

Available online 03 June 2017

0378-8741/ © 2017 Elsevier B.V. All rights reserved.

1. Introduction

Atopic dermatitis (AD) is a highly pruritic chronic inflammatory skin disease characterized by dryness, pruritus, and erythematous eczema and is often the initial step in the atopic march leading to the development of asthma and allergic rhinitis (Leung and Bieber, 2003; Leung et al., 2004). The cause of atopic dermatitis is not known, while the interactions of genetic, environmental and immunological factors appear to be important in determining disease expression (Oshio et al., 2009).

Traditionally, two competing hypotheses about the pathogenesis of AD are presented (Brandt and Sivaprasad, 2011). The main research direction of AD has been dominated on the inside-out hypothesis, which focused largely on primary abnormalities of the immune system that causes Th2-predominant inflammation and IgE-mediated sensitization (Williams et al., 2012). However, the outside-in hypothesis suggests that skin barrier abnormalities precede immune dysregulation (Elias et al., 2008). Recently, a growing number of studies, which support the outside-in hypothesis, have shown a highly significant association between disruption of the skin barrier and risk of developing early-onset, severe, persistent AD (Boguniewicz and Leung, 2010). Therefore, a dysfunctional and impaired skin barrier is now considered not just to be an epiphenomenon of AD, but a driver for inflammation and development of AD (Clausen et al., 2015).

Within traditional medicine, AD is believed to be associated with “fetal heat”, which are passed on to the baby from the mother in the womb (Im et al., 2002). This pathological heat can be caused by the lifestyle and diet of the mother during pregnancy. The heat can manifest as various diseases when the child is born and AD is the most common diseases expressed to the surface of the body. Therefore, after the child is born, they are given a drop of herbal extracts in the mouth to clear fetal heat from their bodies, which is called Hataedock (HTD) treatment. We have paid attention that HTD treatment is used to prevent the onset of symptoms before the manifestation of AD and other skin disease.

Traditionally, a simplified 2-herb formula consisting of *Coptis japonica* Makino and *Glycyrrhiza uralensis* Fischer in 1:1 ratio has been used for HTD treatment (Heo, 1613). Moreover, our previous studies have shown that HTD treatment effectively prevents AD through regulation of Th2 differentiation and inflammation under high-fat diet conditions (Cha et al., 2016). *Coptis japonica* Makino, a kind of classical heat-clearing and detoxifying herb, has been used for treating various diseases including intestinal infections, skin diseases, conjunctivitis, otitis, and hypertension (Muluye et al., 2014). *Glycyrrhiza uralensis* Fischer has been used in traditional medicine mainly for the treatment of peptic ulcer, hepatitis C, and pulmonary and skin diseases (Asl and Hosseinzadeh, 2008). Based on these things, it can be inferred that the combination of these effects play an important role in reducing skin inflammation in AD. However, information concerning how synergistic effects of two herbs affects AD symptoms prevention has yet to be explored.

In the present study, we aimed to evaluate the preventive therapeutic effects of HTD treatment and investigate its underlying pharmacological mechanisms using a *Dermatophagoides Farinae*

Extract (DfE)-induced NC/Nga AD model mice. In particular, based on the outside-in hypothesis, we investigated whether the protective effect of HTD treatment on the skin barrier affects the attenuation of AD symptoms.

2. Methods and materials

2.1. Preparation of HTD herb extract

Coptis japonica Makino and *Glycyrrhiza uralensis* Fischer were purchased from Omniherb (Yeongcheon, Korea). 100g of *Coptis japonica* Makino and 100g of *Glycyrrhiza uralensis* Fischer (1:1) were powdered and boiled for 3 h in distilled water. The resulting extract was filtrated, concentrated and freeze-dried. We obtained 31 g of the extract (yield: 15.5%) for use. The chemical consistency of HTD herb extract was validated by HPLC fingerprint analysis. The major content of two herbs used in this experiment is in accordance with Korean Pharmacopoeia (KFDA, 2016).

2.2. Animal and AD Induction

3-week-old male NC/Nga mice (13–15 g each) were purchased from Central Lab Animal Inc. (Seoul, Korea). These were divided into 3 groups (n = 10 per group) as follows: the normal group (Ctrl group), AD-induced with no treatment group (AE group), AD-induced with HTD treatment group (CGT group). For HTD treatment, 3-week-old mice in the CGT group were given the extract of *Coptis japonica* Makino and *Glycyrrhiza uralensis* Fischer on days 1, 2 and 3. After the complete removal of dorsal hairs from the mice, 1 mL of 5% sodium dodecyl sulfate (SDS) (Sigma-Aldrich, USA) was rubbed on the back of each mouse to remove the lipid lamella of the stratum corneum. On 4th, 5th, 6th, 8th, 9th, and 10th week, DfE (100 mg, Biostir Inc., Japan) was applied 2 times per week for weeks. On 11th week, the mice were deeply anesthetized and sacrificed. All animal experiments were approved by the Institutional Animal Care and Use Committee of Pusan National University (IACUC number: PNU-2015-0924). We followed the NIH Guide for the Care and Use of Laboratory Animals throughout this study. The experimental design is summarized in Fig. 1.

2.3. Fingerprinting analysis

To identify the constituents of the herb extract, High-performance liquid chromatography (HPLC)-based fingerprinting was performed with an Agilent 1200 Series HPLC System (Agilent Technologies, Santa Clara, USA). The column of Capcell PAKMGII C18 (3.0 × 150 mm, 3.0 μm; Shiseido, Tokyo, Japan) of 0.5% Aqueous formic acid/acetonitrile (A/B, v/v) with the gradient elution (5 min-2% B, 12 min-10% B, 20 min-25% B, 27 min-25% B, 35 min-80% B, 37 min-80% B, 40 min-30% B, 45 min-2% B) as the mobile phase, a flow rate of 0.6 mL/min at 35°C, UV detection at 260 nm, and the injection volume at 15 μL was used. A standard solution containing Palmatine, Berberine (ChemFaces, China), Liquiritin, and Liquiritigenin (Sigma-Aldrich, USA) was prepared by dissolving these compounds in distilled water (10 mg/100 mL). The solution was filtered through 0.45 μm syringe



Fig. 1. Experimental design. Before inducing AD, the extract of *Coptis japonica* Makino and *Glycyrrhiza uralensis* Fischer, which is traditionally used in HTD treatment, was administered orally to the CGT group on days 1, 2 and 3. Mice were challenged by DfE on 4th, 5th, 6th, 8th, 9th, and 10th week.

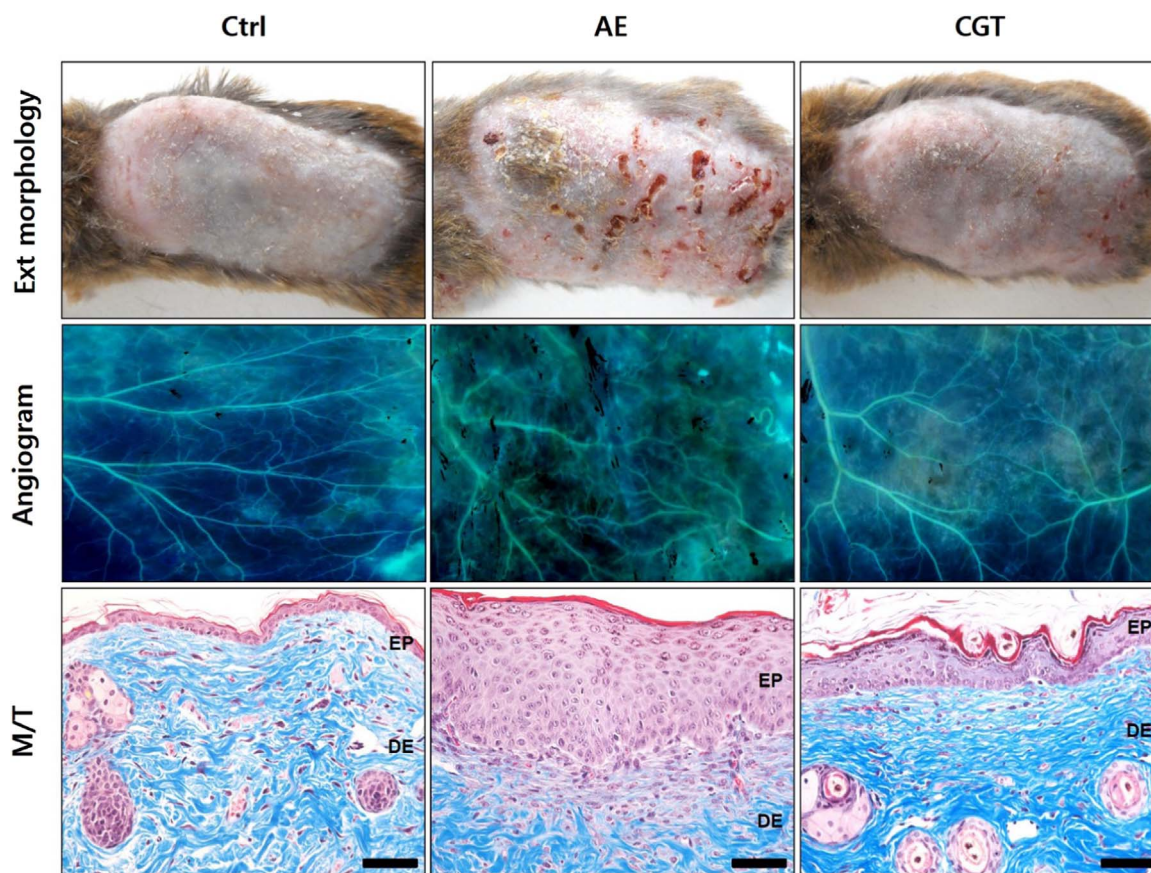


Fig. 2. The mitigative effect of HTD treatment for AD symptoms. Characteristic skin lesions in AD were alleviated in the CGT group compared with AE group. The distribution of capillary increased in AE group but decreased in CGT group ($\times 4$). In M/T result, spongiosis was decreased in CGT group, but increase in AE group (M/T histochemistry; bar size, 50 μ m). Ctrl: normal, AE: AD-induced with no treatment, CGT: AD-induced with HTD treatment, EP: epidermis, DE: dermis, and M/T: Masson trichrome method.

filter and subjected to HPLC analysis.

Compared with the standard compounds, four main chromatographic peaks were identified as follows: Palmatine (33.001 min), Berberine (33.775 min), Liquiritin (26.729 min), Liquiritigenin (34.672 min) (Cha et al., 2016). This fingerprint analysis revealed that HTD herb extract contains the known bioactive compounds of *Coptis japonica* Makino and *Glycyrrhiza uralensis* Fischer, including Palmatine, Berberine, Liquiritin, and Liquiritigenin. Therefore, we considered that the quality of HTD herb extract was controlled by HPLC fingerprint.

2.4. Tissue process & histochemistry

After the mice were sacrificed, dorsal skin biopsies were fixed in 10% neutral buffered formalin (NBF) at room temperature for 24 h and embedded in paraffin. After embedding, serial sectioning (5 μ m) were histochemically stained. To investigate histological changes such as epithelial hyperplasia, capillary distribution, and collagen fiber distribution, we performed Masson's trichrome staining. To investigate the distribution and morphological changes of the mast cells, we performed histochemical staining with Luna's stain.

2.5. Immunohistochemistry

The proteolytic digestion of tissue sections was performed for 5 min with 20 μ g/ml of proteinase K and then incubated in blocking serum (10% normal goat serum) for 4 hours. The sections were incubated with goat anti-STAT6 (1:100, Santa Cruz Biotech, USA), goat anti-IL-4 (1:100, Santa Cruz Biotech, USA), goat anti-IL-13 (1:100, Santa Cruz Biotech, USA), goat anti-CD40 (1:100, Santa Cruz Biotech, USA), goat

anti-Fc ϵ receptor (1:100, Santa Cruz Biotech, USA), goat anti-Substance P (1:100, Santa Cruz Biotech, USA), goat anti-MMP-9 (1:200, Santa Cruz Biotech, USA), goat anti-5-HT (1:200, Santa Cruz Biotech, USA), goat anti-NF- κ B p65 (1:500, Santa Cruz Biotech, USA), goat anti-p-IkB (1:500, Santa Cruz Biotech, USA), goat anti-iNOS (1:200, Santa Cruz Biotech, USA), goat anti-LXR (1:200, Santa Cruz Biotech, USA), goat anti-filaggrin (1:200, Santa Cruz Biotech, USA), goat anti-PKC (1:100, Santa Cruz Biotech, USA), all of which are primary antibodies, for 72 hours in a 4°C humidified chamber. Next, the sections were exposed to secondary antibodies with biotinylated rabbit anti-goat IgG (1:100, Santa Cruz Biotech, USA) for 24 hours at room temperature. Sections were subsequently processed using an avidin/biotin complex kit (Vector Lab, USA) for 1 hour at room temperature. Finally, the slices were immersed in a 0.05 M Tris-HCl buffer (pH 7.4) containing 0.05% 3,3'-diaminobenzidine and 0.01% HCl, and then counter-stained with hematoxylin.

2.6. TUNEL assay

The Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed using reagent from the in situ apoptosis detection kit (Apoptag, Intergen, USA). The sections were digested with proteinase K for 5 minutes and then applied equilibration buffer for 5 seconds. The sections were subsequently incubated with working strength TdT enzyme (36 μ l TdT enzyme: 72 μ l reaction buffer). Sections were then incubated in a humidified chamber at 37 °C for 1 h, agitated for 10 min in strength stop/wash buffer, and treated with anti-digoxigenin peroxidase and DAB for 1 h. Then, we observed the sections counterstained with eosin using an optical microscope.

2.7. Image analysis and statistical analysis

Image analyses were carried out with the Image Pro Plus (Media cybernetics, USA). The results are shown as the mean $\pm$ standard error. Data analyses were performed using SPSS software packages (SPSS 23, SPSS Inc., USA) using a one-way ANOVA and Levene's (LSD) test with a significance level of $p < 0.01$.

3. Results

3.1. The mitigative effect of HTD treatment for AD symptoms

Macroscopically, the AD-like skin lesions were severely developed on the back skin by DfE sensitization in Nc/Nga mice. The obtained image of the AE group showed various pathological features, such as hemorrhage, erythema, edema, dryness and erosion. In the CGT group, clinical symptoms were more suppressed than in AE group (Fig. 2).

To investigate the regulatory effect of HTD treatment on angiogenesis associated with AD, we compared the angiogram of the AE group and the CGT group. Our angiogram showed that the CGT group inhibited angiogenesis more than the AE group (Fig. 2).

We investigated edema changes such as collagen fiber distribution and epithelial cell hyperplasia using Masson's Trichrome staining. In the AE group, the distribution of collagen fibers was reduced and epithelial hyperplasia was observed. These results represent the basic histological pattern of inflammatory skin damage caused by persistent application of DfE in the AE group. In contrast, the CGT group showed less skin damage in most regions (Fig. 2).

3.2. The maintenance of epidermal barrier

To estimate the protective effects of skin barrier by HTD treatment, we analyzed the levels of liver X receptor (LXR), Filaggrin, and protein kinase C(PKC)-positive reactions. The levels of LXR-positive reaction that were seen widely in the cytoplasm of cells in the stratum corneum and the stratum granulosum were decreased in the AE group, but the levels of the CGT group were increased by 36% ($p < 0.01$) as compared with the AE group. The CGT group also showed a 264% ($p < 0.01$) increase in filaggrin as compared with the AE group (Fig. 3).

We used immunohistochemical staining to identify the PKC-positive reactions in damaged keratinocytes and intercellular space. An increase in the levels of PKC was observed in the AE group compared with the Ctrl group. In contrast, the CGT group showed a 81% ($p < 0.01$) decrease in PKC as compared with the AE group (Fig. 3).

3.3. The regulation of Th2 differentiation

The regulation of Th2 differentiation was estimated by measuring the interleukin (IL)-4, IL-13, Signal transducer and activator of transcription (STAT)-6 and Cluster of differentiation (CD)40-positive reaction in the cytoplasm of dermal papilla cells. Compared with the AE group, HTD treatment significantly decreased the levels of IL-4, IL-13, STAT-6 and CD40-positive reactions. The levels of IL-4 in the CGT group were shown to be decreased by 77% ($p < 0.01$) as compared with the AE group. The CGT group showed a 62% ($p < 0.01$) decrease in IL-13 as compared with the AE group. The CGT group showed a 26% ($p < 0.01$) decrease in STAT-6 as compared with the AE group. In addition, CD40-positive reaction levels were decreased by 89% ($p < 0.01$) in the CGT group (Fig. 4).

3.4. The regulation of mast cells activation

The results using Luna's staining indicated that many granulated mast cells from the dermal papilla to the area around the subcutaneous layer were appeared in the AE group. In contrast, relatively few granulated mast cells were found in the AE group (Fig. 5).

The regulation of mast cells activation was estimated by measuring the high-affinity IgE receptor (FcεRI), Substance P, Matrix metalloproteinases (MMP)-9 and 5-hydroxytryptamine (HT)-positive reaction in dermal papilla. Compared with the AE group, HTD treatment significantly decreased the levels of FcεRI, Substance P, MMP-9 and 5-HT-positive reactions. The levels of FcεRI in the CGT group were shown to be decreased by 35% ($p < 0.01$) as compared with the AE group. The levels of Substance P in the CGT group were also shown to be decreased by 59% ($p < 0.01$) as compared with the AE group (Fig. 5). The levels of MMP-9 in the CGT group were shown to be decreased by 71% ($p < 0.01$) as compared with the AE group. The levels of 5-HT in the CGT group were shown to be decreased by 53% ($p < 0.01$) as compared with the AE group (Fig. 5).

3.5. Down-regulation of inflammation

To determine whether HTD treatment regulate the inflammatory response, we measured the levels of nuclear factor-kappaB (NF-κB) p65, phosphorylated IκB (p-IκB) and inducible nitric oxide synthase (iNOS)-positive reactions in stratum basale and dermal papilla. The results of the immunohistochemical staining showed the appearance of NF-κB p65, p-IκB and iNOS-positive reactions in the cytoplasm. Marked decreases of NF-κB p65, p-IκB and iNOS-positive reactions were observed in the CGT group. The CGT group showed a 39% ($p < 0.01$) decrease in NF-κB p65 as compared with the AE group. The CGT group also showed a 56% ($p < 0.01$) decrease in p-IκB as compared with the HDE group. In addition, the levels of iNOS in the CGT group were shown to be decreased by 63% ($p < 0.01$) as compared with the HDE group (Fig. 6).

The results of TUNEL assay were used to estimate the effect of HTD treatment on inflammatory regulation through apoptosis in dermal papilla cell. Unlike the indicators described above, the apoptotic cells in the CGT group were remarkably increased by 257% ($p < 0.01$) as compared to the AE group (Fig. 6).

4. Discussion

In the present study, we evaluated the preventive therapeutic effects of HTD treatment for AD using a DfE-induced NC/Nga AD model mice. In the AE group, repeated application of DfE caused increased eczematous injury, as well as angiogenesis and spongiosis in inflamed tissues (Fig. 2). In addition, LXR and filaggrin-positive reaction was remarkably decreased (Fig. 3). However, the level of PKC, IL-4, IL-13, STAT-6, CD40, FcεRI, substance P, MMP-9, 5-HT, NF-κB p65, p-IκB, and iNOS were elevated by DfE in inflamed tissues (Figs. 4,5,6). In contrast, in the CGT group, HTD treatment effectively relieved skin barrier injury, angiogenesis and spongiosis (Fig. 2). After HTD treatment, LXR, filaggrin-positive reaction was also increased (Fig. 3). PKC, IL-4, IL-13, STAT-6, CD40, FcεRI, substance P, MMP-9, 5-HT, NF-κB p65, p-IκB, and iNOS-positive reaction were decreased in the CGT group compared to the AE group (Figs. 4,5,6). These results imply that HTD treatment can effectively maintain the skin barrier function and prevent disruption of skin barrier, leading to regulating the Th2 cytokine cascade, suppressing mast cell activation and attenuating inflammation.

We have noted that many existing therapeutic approaches to AD focus on the direction of relieving symptoms after damage has occurred. However, we came to think that if we understand the pathophysiological mechanism of AD and prevent it from manifesting, it might be a more effective treatment for AD. So we paid attention to the HTD treatment in traditional medicine. As described above, HTD treatment is a way to use herbal medicine to the newborn as a method of removing "fetal heat" beforehand as a cause of the child's dermatitis in traditional medicine. We wanted to explain the mechanism, from a modern medical point of view, what effects of HTD treatment would prevent dermatitis. Therefore, we have investigated whether HTD

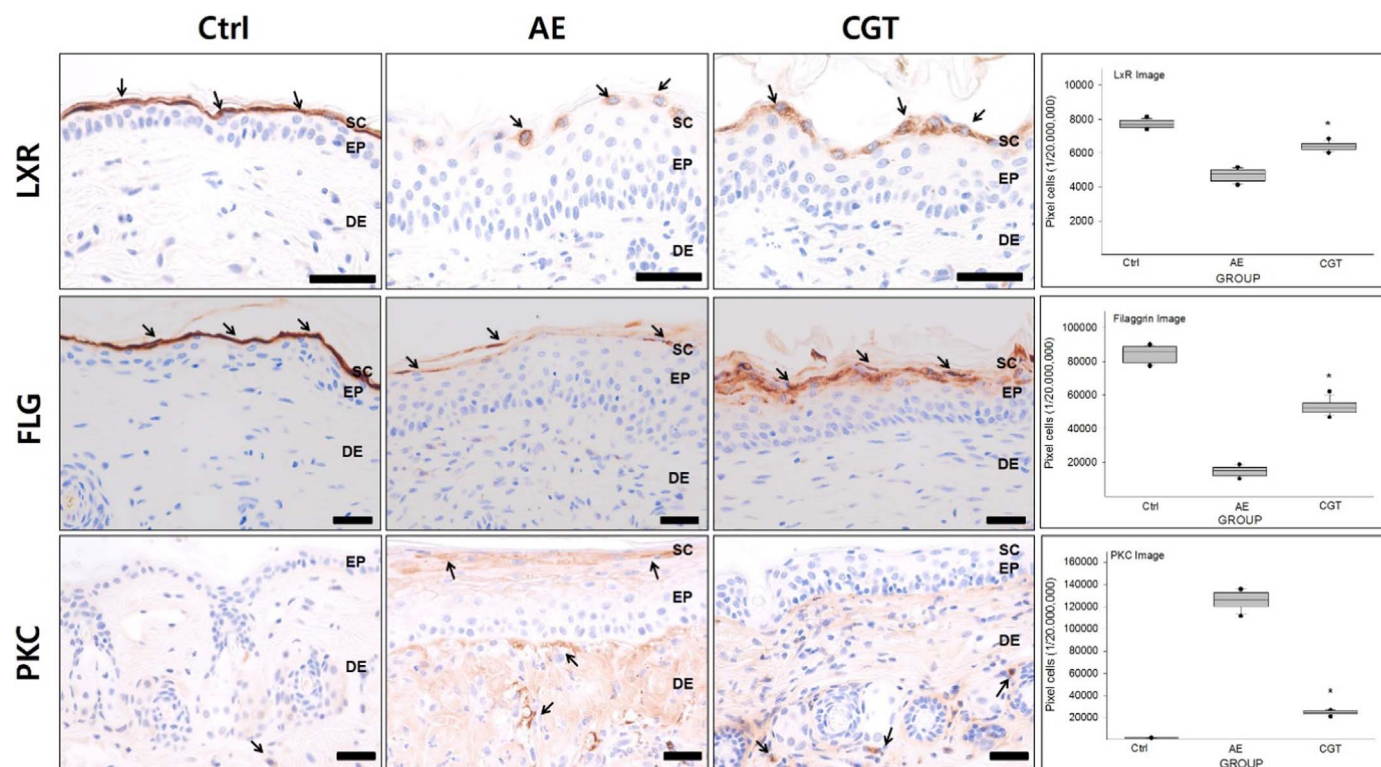


Fig. 3. The maintenance of epidermal barrier. The LXR-positive reaction (arrow indicates dark brown) in AE was remarkably decreased but increased in CGT (LXR immunohistochemistry; bar size, 25 μ m). The filaggrin-positive reaction (arrow indicates dark brown) in AE was also decreased but increased in CGT (filaggrin immunohistochemistry; bar size, 50 μ m). In contrast, the PKC-positive reactions (arrow indicates dark brown) in CGT remarkably decreased compared with AE. (PKC immunohistochemistry; bar size, 50 μ m). Data of LXR, filaggrin and PKC image analysis was also showing the same result ($p < 0.01$). Ctrl: normal, AE: AD-induced with no treatment, CGT: AD-induced with HTD treatment, EP: epidermis, DE: dermis, SC: stratum corneum, FLG: filaggrin. * $p < 0.01$, compared with the AE group.

treatment is effective in protecting the skin barrier in terms of the outside-in hypothesis that skin barrier defects can be the cause of AD. In addition, we also investigated whether HTD treatment affects immunologic regulation and attenuation of inflammation. In summary, previous studies have focused on assessing the effectiveness of therapeutic interventions in AD after symptom onset. However, this study differs from previous studies in that it evaluates the preventive effect by taking the herbal extracts before the onset of AD symptoms.

4.1. The mitigative effect of HTD treatment for AD symptoms

Repetitive application of DfE induced AD symptoms in NC/Nga mice (Matsuoka et al., 2003). We investigated whether HTD treatment can relieve DfE-induced AD symptoms and responses in NC/Nga mice. In our results, continual repetitive application of DfE without HTD treatment (the AE group) induced eczematous injury involving severe clinical manifestations including erythema, excoriation, crust and lichenification in NC/Nga mice. Moreover, increased angiogenesis and spongiosis were observed in the AE group. On the other hand, it was found that the skin lesions of CGT group as well as angiogenesis and spongiosis were significantly suppressed by HTD treatment. Therefore, these results suggest that HTD treatment was effective in preventing barrier disruption and maintaining skin integrity, thereby reducing susceptibility to allergic sensitization.

4.2. The maintenance of epidermal barrier

We analyzed the levels of LXR, filaggrin, and PKC-positive reactions in order to evaluate the protective effects of epidermal barrier by HTD treatment. LXRs are ligand-dependent transcriptional factors that regulate the expression of genes involved in the cellular efflux of excess cholesterol and decrease the expression of inflammatory mediators

(Edwards et al., 2002; Fowler et al., 2003). In the skin, activation of LXRs stimulates keratinocyte differentiation and improves epidermal barrier functions (Schmuth et al., 2008).

Filaggrin, key proteins for terminal differentiation of the epidermal keratinocytes, has a crucial structural and functional role in the epidermis with significant impact on the homeostasis of the skin (Cabanillas and Novak, 2016). Filaggrin plays a vital role in maintaining skin hydration by preserving stratum corneum integrity and the production of natural moisturizing factors (NMF) (Levin et al., 2013). Thus, it has been described that inherited or acquired filaggrin deficiency essentially contributes to the pathogenesis of AD (Cabanillas and Novak, 2016).

Protein kinase C (PKC) is a well-known family of homologous serine/threonine kinases that play central roles in the regulation of various cellular functions in numerous cell types (Griner and Kazanietz, 2007). Conventional isoforms of PKC are activated by signals such as increases in the concentration of diacylglycerol (DAG) or calcium ions (Ca^{2+}) (Huang, 1989). Because ceramides are important determinant of water-retaining property and barrier function of the stratum corneum, the deficiency of ceramides may provide an pathological basis for the dry and barrier defect skin of AD patients (Hara et al., 2000). Intracellularly, ceramide can compete with DAG at the DAG binding site on distinct PKC isozymes, and interfere with PKC functions (Jones and Murray, 1995). Thus, a defect in ceramide generation could increase PKC activation, leading to overproduction of other pro-inflammatory cytokines by keratinocytes and barrier disruption in AD (Pastore et al., 2006).

In the present study, we show that the level of LXR-positive reaction was remarkably decreased in the AE group but that the levels of the CGT group were increased. Moreover, the levels of filaggrin-positive reaction were also increased in the CGT group. On the other hand, the levels of PKC positive reaction were remarkably increased in

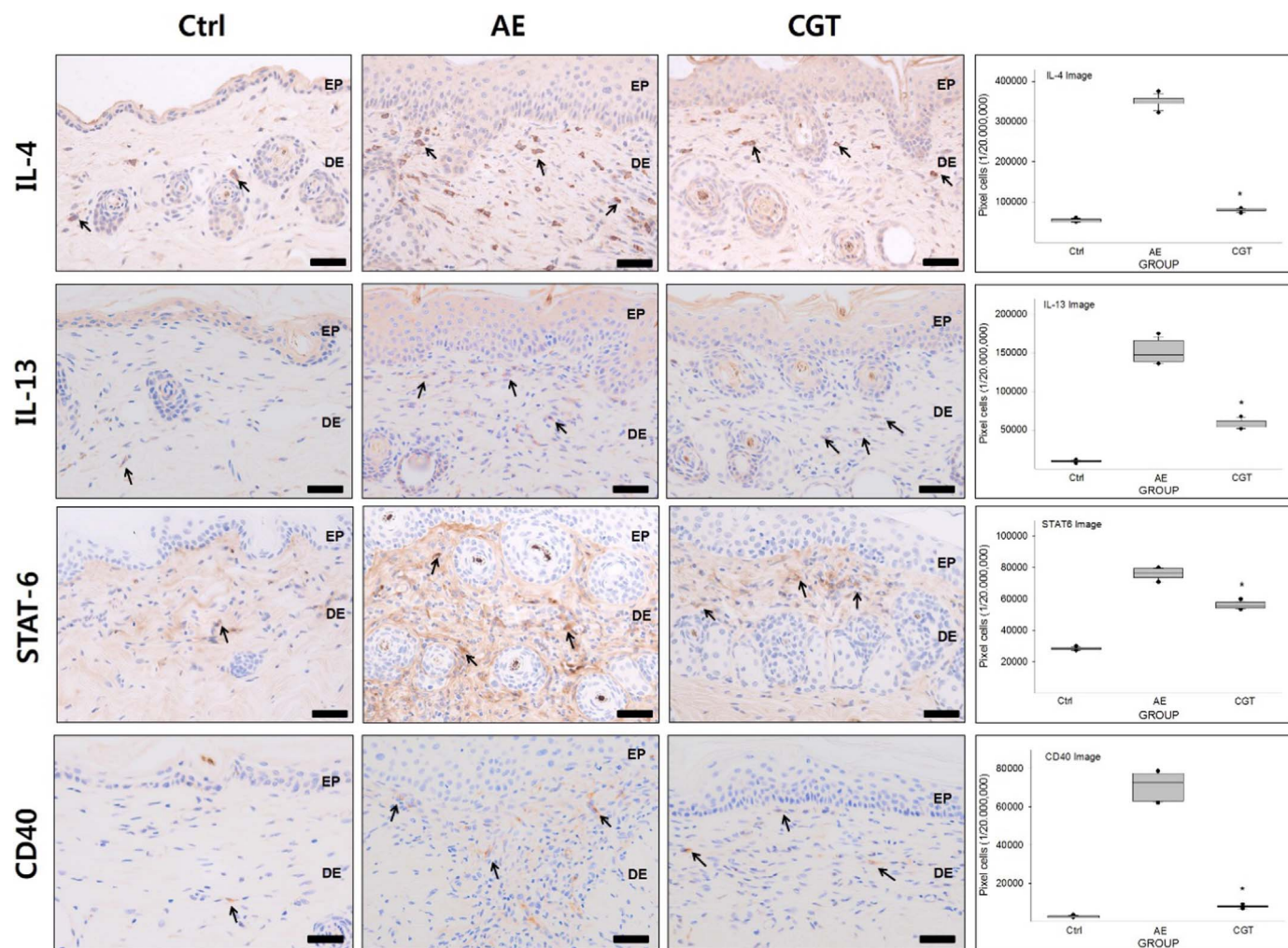


Fig. 4. The regulation of Th2 differentiation. The IL-4, IL-13, STAT-6 and CD40-positive reactions (arrow indicates dark brown) were decreased in the CGT group compared with the AE group (Immunohistochemistry; bar size, 50 μ m). Data of IL-4, IL-13, STAT-6 and CD40 image analysis were also showing the same results ($p < 0.01$). Ctrl: normal, AE: AD-induced with no treatment, CGT: AD-induced with HTD treatment, EP: epidermis, DE: dermis. * $p < 0.01$, compared with the AE group.

the AE group but that the levels of the CGT group were decreased (Fig. 3).

These results indicate that HTD treatment increases the expression of LXR and filaggrin and inhibits the PKC activity. Therefore, HTD treatment might contribute to maintaining and strengthening the barrier function of the skin, leading to reducing the passage of antigens through the epidermis, and thereby suppress allergic sensitization.

4.3. The regulation of Th2 differentiation

It has been known that Th2 cells play an important role in the pathophysiology of allergic reactions. AD is characterized by a dysregulated Th2 predominant immune response and markedly elevated IgE synthesis (Boguniewicz and Leung, 2011; Jirapongsananuruk et al., 1998). Th2 cells mediate these responses by producing various cytokines. Interleukin (IL)-4 and IL-13, expressed by Th2 cells, are key cytokines that promote acute inflammatory processes and develop AD (Brandt and Sivaprasad, 2011). Both IL-4 and IL-13, which share a common receptor subunit, activate signal transducer and activator of transcription factor-6 (STAT-6) (Hershey, 2003). STAT-6 is tightly connected to IL-4 and IL-13 signaling, and has been noted as a hub molecule in Th2 polarization of the immune system (Hebenstreit et al., 2006).

An increase in numbers of CD40 receptors on B cells has been previously reported in patients with AD (Renz et al., 1994). Cross-

linking CD40 has been identified as a significant costimulatory signal required for proliferation of B cells and switch recombination to IgE synthesis in the presence of IL-4 and IL-13 (Gauchat et al., 1993; Jirapongsananuruk et al., 1998; Karras et al., 1997).

To investigate whether HTD treatment regulates the Th2 differentiation, we performed a comparison of the IL-4, IL-13, STAT-6, CD40 levels of between the AE group and CGT group. Th2 cytokines including IL-4 and IL-13 produced by activated T lymphocytes were increased by DfE application in AE group. The level of STAT-6 and CD40-positive reaction also increased in AE group. Whereas CGT group treated with HTD displayed significantly reduced production of IL-4, IL-13 and STAT-6, CD40 levels. This finding suggests that HTD treatment regulate the Th2 cytokine cascade, which leads to IgE synthesis and allergic sensitization.

4.4. The regulation of mast cells activation

It is generally accepted that mast cells contribute to allergic reactions, including the development of skin lesions in AD. Mast cells are activated by Fc ϵ RI (Kawakami et al., 2009). When mast cells are triggered by an allergen that binds to serum IgE attached to their Fc ϵ RI, they release cytokines and their secretory granules (Amin, 2012).

Among various neuropeptides, substance P has been assumed to play an important role for mediating itch-scratch and stress-scratch

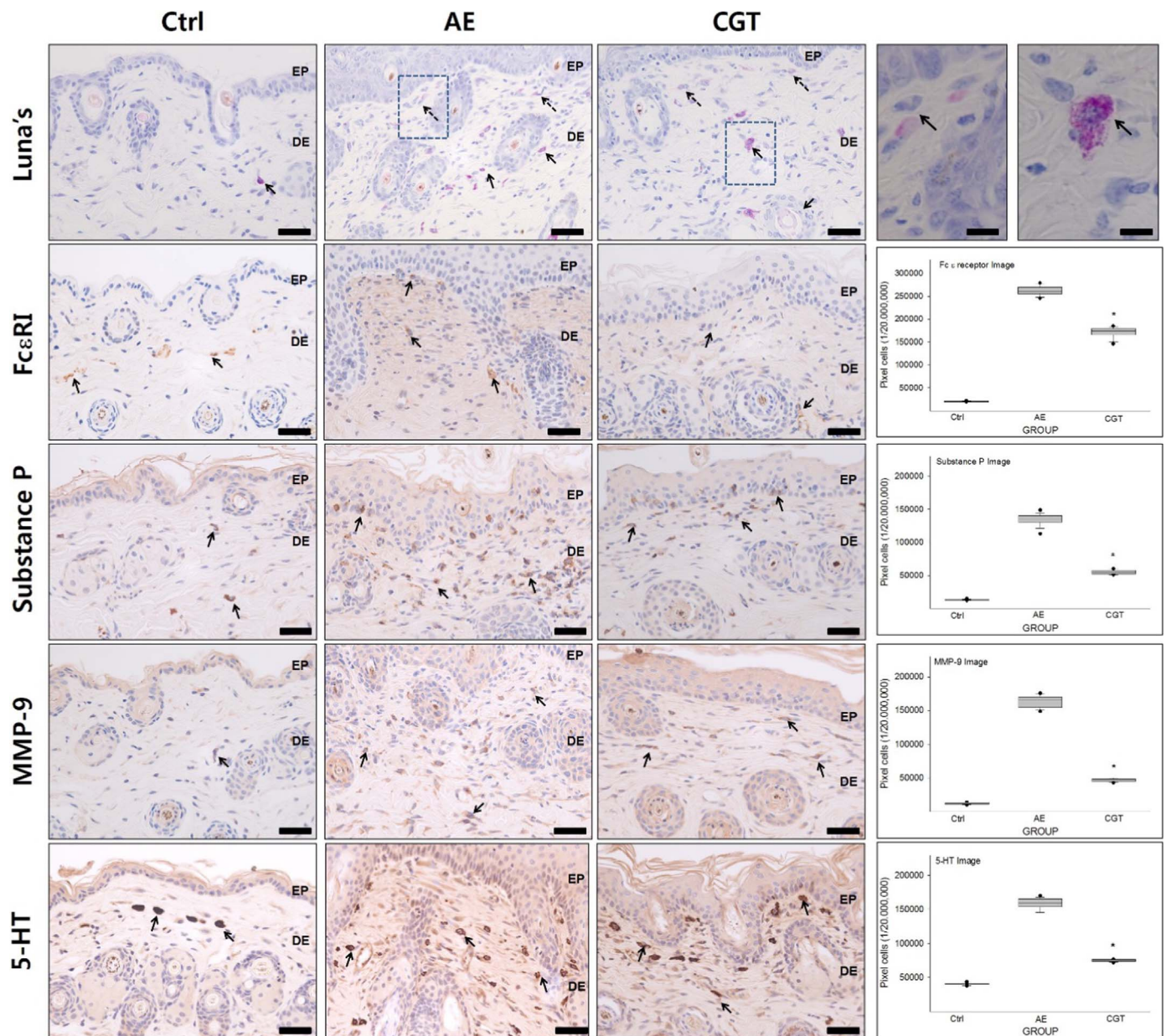


Fig. 5. The regulation of mast cells activation. In Luna's staining, the distribution of granulated mast cell (arrows indicate purple) was increased in the CGT group but decreased in the AE group (Luna's method; bar size, 50 μ m; square box, enlarged granulated mast cell; bar size, 10 μ m). The Fc ϵ RI, Substance P, MMP-9 and 5-HT- positive reactions (arrow indicates dark brown) were decreased in the CGT group compared with the AE group (Immunohistochemistry; bar size, 50 μ m). Data of Fc ϵ RI, Substance P, MMP-9 and 5-HT image analysis were also showing the same results ($p < 0.01$). Ctrl: normal, AE: AD-induced with no treatment, CGT: AD-induced with HTD treatment, EP: epidermis, DE: dermis. * $p < 0.01$, compared with the AE group.

cycles (Hosokawa et al., 2009). Numerous studies have shown that substance P evokes histamine release from mast cells (Suzuki et al., 1995). It is also known that both the number of substance P-containing mast cells and the amount of substance P within mast cells are increased in the lesional skin of AD patients (Toyoda et al., 2000). Taken together, these suggest that substance P may play an important role in the mechanisms underlying AD pathogenesis (Salomon and Baran, 2008).

The MMPs have long been associated with inflammation and tissue remodeling is now well recognized (Kahari and Saarialho-Kere, 1997; Parks et al., 2004). Human mast cell activated by T cells leads to secretion of MMP-9 (Baram et al., 2001). Recently, it was reported that skin wash samples from atopic dermatitis lesions showed the elevated levels of MMP9 and MMP activity (Harper et al., 2010). These reports raise the possibility of a functional role for MMP-9 in the pathogenesis of AD.

Serotonin, also known as 5-HT, is an important mediator of bidirectional contact between the skin and the neuroendocrine system (Nordlind et al., 2008). 5-HT may contribute to inflammation and pruritus in the skin (Palmqvist et al., 2016). It have been reported that patients with AD have an increased levels of 5-HT in plasma compared with patients with psoriasis and healthy controls (Soga et al., 2007).

To estimate whether HTD treatment regulates the mast cells activity, we measured the level of Fc ϵ RI, substance P, MMP-9 and 5-HT as indicators or mediators of mast cell activation. As shown in Fig. 5, DfE application increased the levels of Fc ϵ RI, substance P, MMP-9 and 5-HT-positive reactions in AE group. In contrast, the results showed decreased Fc ϵ RI, substance P, MMP-9 and 5-HT levels in CGT group. Therefore, it could be understood that HTD treatment could suppress mast cell activation, thereby alleviating the development of skin lesions in AD.

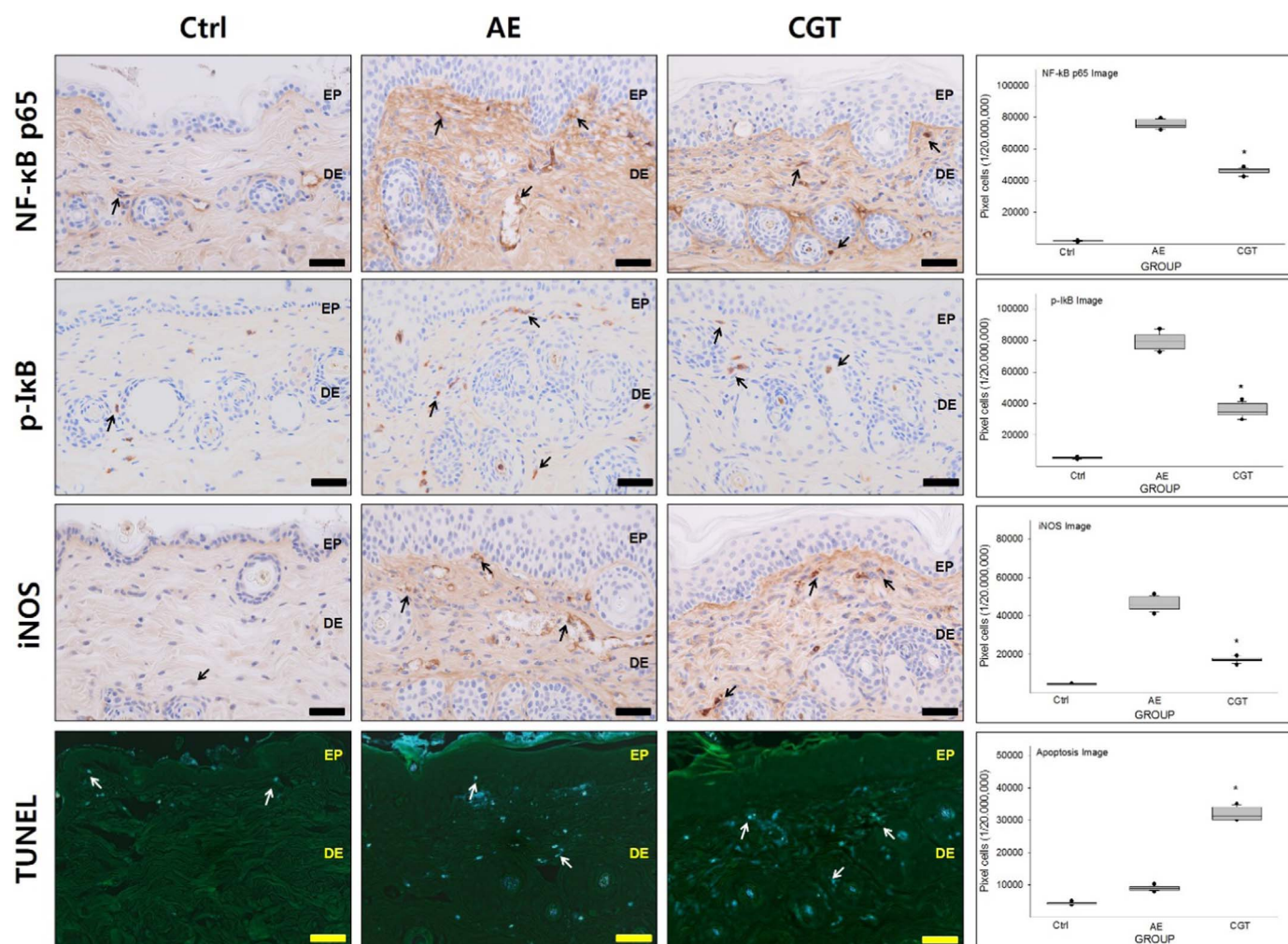


Fig. 6. Down-regulation of inflammation. The NF- κ B p65, p-I κ B and iNOS-positive reactions (arrow indicates dark brown) were decreased in the CGT group compared with the AE group (Immunohistochemistry; bar size, 50 μ m). Data of NF- κ B p65, p-I κ B and iNOS image analysis were also showing the same results ($p < 0.01$). In TUNEL assay, the apoptotic body (arrow indicates yellow-green fluorescence) in the CGT group was remarkably increased compared to the AE group (TUNEL assay; bar size, 50 μ m). Ctrl: normal, AE: AD-induced with no treatment, CGT: AD-induced with HTD treatment, EP: epidermis, DE: dermis. * $p < 0.01$, compared with the AE group.

4.5. Down-regulation of inflammation

NF- κ B plays a crucial role in the allergic inflammation by enhancing the production of inflammatory cytokines and chemokines involved in the pathogenesis of AD (Dajee et al., 2006). In resting cells, NF- κ B is maintained in an inactive state through cytoplasmic retention by inhibitor κ B (I κ B) proteins that mask the nuclear localization sequence of NF- κ B (Bourke et al., 2000). p-I κ B is a central step in NF- κ B activation, leading to nuclear translocation of NF- κ B subunits (Hayden and Ghosh, 2008). It is now well-documented that various proinflammatory cytokines bind to their cognate receptors and induce the expression of iNOS via NF- κ B activation (Pahan et al., 2001). Excess nitric oxide (NO) arising from iNOS activity plays important roles in the pathogenesis of vasodilation and erythema in AD skin (Taniuchi et al., 2001). Therefore, the regulation of iNOS via the NF- κ B pathway is an important mechanism in inflammatory processes (Aktan, 2004).

Apoptosis and the clearance of apoptotic cells are crucial to the resolution process to chronic inflammation (Lawrence and Gilroy, 2007). Recent studies have reported that dysregulated apoptosis in skin-homing T cells, eosinophils and keratinocytes contributes to the initiation and persistence of AD (Trautmann et al., 2000). In particular, the anti-apoptotic activity of NF- κ B can maintain the inflammatory response through persistent leukocyte activation (Lawrence et al.,

2001).

We analyzed both the levels of NF- κ B p65, p-I κ B, iNOS-positive reactions on the NF- κ B pathway and the percentage of apoptotic cells in order to investigate the effects of HTD treatment on the inflammatory response in AD. Marked decreases of NF- κ B p65, p-I κ B, iNOS were observed in the CGT group but increased in the AE group. In contrast, the percentage of apoptotic cells, measured by TUNEL assay, increased significantly in CGT group, whereas no significant change was observed in AE group (Fig. 6). These findings suggest that HTD treatment, which interferes with NF- κ B activity leading to inhibition of iNOS expression and inducing apoptosis, may suppress the persistence and aggravation of inflammation in AD.

We have designed this experiment to find out the effect of Hataedock treatment, which is traditionally known to prevent the skin dermatitis of children. The preliminary treatment of newborn baby who did not develop the disease was considered to be limited to practical clinical applications if its safety and efficacy were not secured. For this reason, we sought to confirm whether Hataedock treatment was effective in AD through this animal experiment. As a result of this study, it is thought that the Hataedock treatment has a preventive effect against AD due to the combination of factors such as immunologic regulation and protection of skin barrier. In the future, our researchers plan to extend this experiment using substances other than the extract

of *Coptis japonica* Makino and *Glycyrrhiza uralensis* Fischer. Although it has traditionally been used in children, our goal is to obtain evidence for Hataedock treatment to be applicable to clinical care within the institutional healthcare system.

5. Conclusions

In this study, we demonstrated that HTD treatment effectively suppressed the development of symptoms of AD using a DfE-induced NC/Nga AD model mice. These results imply that HTD treatment may alleviate skin inflammation, regulate the activation of mast cells and the Th2 cytokine cascade by maintaining skin barrier function and preventing skin barrier damage. In conclusion, HTD treatment can be used as a preventive treatment for AD.

Author contributions

HY Cha, SH Ahn, KB Kim designed the study; SH Ahn conducted the experiments; SY Park performed HPLC analysis; HY Cha, SH Ahn and JH Cheon analyzed the data; KB Kim oversaw the study.

Conflict of interest

The authors assert no conflict of interest associated with this project.

Acknowledgments

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (No. NRF-2016R1D1A1B03930474).

References

- Aktan, F., 2004. iNOS-mediated nitric oxide production and its regulation. *Life Sci.* 75 (6), 639–653.
- Amin, K., 2012. The role of mast cells in allergic inflammation. *Respiratory Med.* 106 (1), 9–14.
- Asl, M.N., Hosseinzadeh, H., 2008. Review of Pharmacological Effects of Glycyrrhiza sp. and its Bioactive Compounds. *Phytother. Res.* 22 (6), 709–724.
- Baram, D., Vaday, G.G., Salamon, P., Drucker, I., Hershkovich, R., Mekori, Y.A., 2001. Human mast cells release metalloproteinase-9 on contact with activated T cells: juxtacrine regulation by TNF- α . *J. Immunol.* 167 (7), 4008–4016.
- Boguniewicz, M., Leung, D.Y.M., 2010. Recent insights into atopic dermatitis and implications for management of infectious complications. *J. Allergy Clin. Immunol.* 125 (1), 4.
- Boguniewicz, M., Leung, D.Y.M., 2011. Atopic dermatitis: a disease of altered skin barrier and immune dysregulation. *Immunol. Rev.* 242 (1), 233–246.
- Bourke, E., Kennedy, E.J., Moynagh, P.N., 2000. Loss of Ikappa B-beta is associated with prolonged NF-kappa B activity in human glial cells. *J. Biol. Chem.* 275 (51), 39996–40002.
- Brandt, E.B., Sivaprasad, U., 2011. Th2 cytokines and atopic dermatitis. *J. Clin. Cellular Immunol.* 2 (3), 110.
- Cabanillas, B., Novak, N., 2016. Atopic dermatitis and filaggrin. *Current Opinion Immunol.* 42, 1–8.
- Cha, H.Y., Ahn, S.H., Cheon, J.H., Park, I.S., Kim, J.T., Kim, K., 2016. Hataedock treatment has preventive therapeutic effects in atopic dermatitis-induced NC/Nga mice under high-fat diet conditions. *Evidence-based Complement. Altern. Med.* 1739760.
- Clausen, M.-L., Agner, T., Thomsen, S.F., 2015. Skin barrier dysfunction and the atopic march. *Current Treat. Options Allergy* 2 (3), 218–227.
- Dajee, M., Muchamuel, T., Schryver, B., Oo, A., Alleman-Sposeto, J., De Vry, C.G., Prasad, S., Ruhmundt, D., Shyamsundar, R., Mutnick, D., Mai, K., Le, T., Parham, C., Zhang, J., Komuves, L., Colby, T., Hudak, S., McEvoy, L.M., Ehrhardt, R.O., 2006. Blockade of experimental atopic dermatitis via topical NF-kB decoy oligonucleotide. *J. Invest. Dermatol.* 126 (8), 1792–1803.
- Edwards, P.A., Kennedy, M.A., Mak, P.A., 2002. LXRs: oxysterol-activated nuclear receptors that regulate genes controlling lipid homeostasis. *Vascular Pharmacol.* 38 (4), 249–256.
- Elias, P.M., Hatano, Y., Williams, M.L., 2008. Basis for the barrier abnormality in atopic dermatitis: outside-inside-outside pathogenic mechanisms. *J. Allergy Clinical Immunol.* 121 (6), 1337–1343.
- Fowler, A.J., Sheu, M.Y., Schmuth, M., Kao, J., Fluhr, J.W., Rhein, L., Collins, J.L., Willson, T.M., Mangelsdorf, D.J., Elias, P.M., Feingold, K.R., 2003. Liver X receptor activators display anti-inflammatory activity in irritant and allergic contact dermatitis models: liver-X-receptor-specific inhibition of inflammation and primary cytokine production. *J. Invest. Dermatol.* 120 (2), 246–255.
- Gauchat, J.F., Henchoz, S., Mazzei, G., Aubry, J.P., Brunner, T., Blasey, H., Life, P., Talabot, D., Flores-Romo, L., Thompson, J., et al., 1993. Induction of human IgE synthesis in B cells by mast cells and basophils. *Nature* 365 (6444), 340–343.
- Griner, E.M., Kazanietz, M.G., 2007. Protein kinase C and other diacylglycerol effectors in cancer. *Nat. Rev. Cancer* 7 (4), 281–294.
- Hara, J., Higuchi, K., Okamoto, R., Kawashima, M., Imokawa, G., 2000. High-expression of sphingomyelin deacylase is an important determinant of ceramide deficiency leading to barrier disruption in atopic dermatitis. *J. Invest. Dermatol.* 115 (3), 406–413.
- Harper, J.L., Godwin, H., Green, A., Wilkes, L.E., Holden, N.J., Moffatt, M., Cookson, W.O., Layton, G., Chandler, S., 2010. A study of matrix metalloproteinase expression and activity in atopic dermatitis using a novel skin wash sampling assay for functional biomarker analysis. *Br. J. Dermatol.* 162 (2), 397–403.
- Hayden, M.S., Ghosh, S., 2008. Shared principles in NF-kB signaling. *Cell* 132 (3), 344–362.
- Hebenstreit, D., Wirsberger, G., Horejs-Hoeck, J., Duschl, A., 2006. Signaling mechanisms, interaction partners, and target genes of STAT6. *Cytokine Growth Factor Rev.* 17 (3), 173–188.
- Heo, J., 1613. Dongui BogamRoyal Clinic. Joseon Dynasty.
- Hershey, G.K.K., 2003. IL-13 receptors and signaling pathways: an evolving web. *J. Allergy Clin. Immunol.* 111 (4), 677–690.
- Hosokawa, C., Takeuchi, S., Furue, M., 2009. Severity scores, itch scores and plasma substance P levels in atopic dermatitis treated with standard topical therapy with oral olopatadine hydrochloride. *J. Dermatology* 36 (4), 185–190.
- Huang, K.-P., 1989. The mechanism of protein kinase C activation. *Trends Neurosci.* 12 (11), 425–432.
- Im, G., Jeong, H.W., Kim, H.S., Jeong, W.Y., 2002. Oriental medical approach on the allergic disease. *J. Kor Oriental Physiol. Pathol.* 16 (5), 831–839.
- Jirapongsananurak, O., Hofer, M.F., Trumble, A.E., Norris, D.A., Leung, D.Y., 1998. Enhanced expression of B7.2 (CD86) in patients with atopic dermatitis: a potential role in the modulation of IgE synthesis. *J. Immunol.* 160 (9), 4622–4627.
- Jones, M.J., Murray, A.W., 1995. Evidence that ceramide selectively inhibits protein kinase C- α translocation and modulates bradykinin activation of phospholipase D. *J. Biol. Chem.* 270 (10), 5007–5013.
- Kahari, V.M., Saarialho-Kere, U., 1997. Matrix metalloproteinases in skin. *Exp. Dermatol.* 6 (5), 199–213.
- Karras, J.G., Wang, Z., Huo, L., Frank, D.A., Rothstein, T.L., 1997. Induction of STAT protein signaling through the CD40 receptor in B lymphocytes: distinct STAT activation following surface Ig and CD40 receptor engagement. *J. Immunol.* 159 (9), 4350–4355.
- Kawakami, T., Ando, T., Kimura, M., Wilson, B.S., Kawakami, Y., 2009. Mast cells in atopic dermatitis. *Current Opinion Immunol.* 21 (6), 666–678.
- KFDA, 2016. Korean Pharmacopoeia.
- Lawrence, T., Gilroy, D.W., 2007. Chronic inflammation: a failure of resolution? *Int. J. Exp. Pathol.* 88 (2), 85–94.
- Lawrence, T., Gilroy, D.W., Colville-Nash, P.R., Willoughby, D.A., 2001. Possible new role for NF-kappaB in the resolution of inflammation. *Nat. Med.* 7 (12), 1291–1297.
- Leung, D.Y.M., Bieber, T., 2003. Atopic dermatitis. *Lancet* 361 (9352), 151–160.
- Leung, D.Y.M., Boguniewicz, M., Howell, M.D., Nomura, I., Hamid, Q.A., 2004. New insights into atopic dermatitis. *J. Clin. Invest.* 113 (5), 651–657.
- Levin, J., Friedlander, S.F., Del Rosso, J.Q., 2013. Atopic dermatitis and the stratum corneum: Part 1: the role of filaggrin in the stratum corneum barrier and atopic skin. *J. Clin. Aesthetic Dermatol.* 6 (10), 16–22.
- Matsuoka, H., Maki, N., Yoshida, S., Arai, M., Wang, J., Oikawa, Y., Ikeda, T., Hirota, N., Nakagawa, H., Ishii, A., 2003. A mouse model of the atopic eczema/dermatitis syndrome by repeated application of a crude extract of house-dust mite *Dermatophagoides farinae*. *Allergy* 58 (2), 139–145.
- Mulvey, R.A., Bian, Y., Alemu, P.N., 2014. Anti-inflammatory and antimicrobial effects of heat-clearing chinese herbs: a current review. *J. Trad. Complementary Med.* 4 (2), 93–98.
- Nordlind, S., Azmitia, E.C., Slominski, A., 2008. The skin as a mirror of the soul: exploring the possible roles of serotonin. *Exp. Dermatol.* 17 (4), 301–311.
- Oshio, T., Sasaki, Y., Funakoshi-Tago, M., Aizu-Yokota, E., Sonoda, Y., Matsuoka, H., Kasahara, T., 2009. Dermatophagoides farinae extract induces severe atopic dermatitis in NC/Nga mice, which is effectively suppressed by the administration of tacrolimus ointment. *Int. Immunopharmacol.* 9 (4), 403–411.
- Pahan, K., Sheikh, F.G., Liu, X., Hilger, S., McKinney, M., Petro, T.M., 2001. Induction of nitric-oxide synthase and activation of NF-kappaB by interleukin-12 p40 in microglial cells. *J. Biol. Chem.* 276 (11), 7899–7905.
- Palmqvist, N., Siller, M., Klint, C., Sjodin, A., 2016. A human and animal model-based approach to investigating the anti-inflammatory profile and potential of the 5-HT2B receptor antagonist AM1030. *J. Inflammation (London, England)* 13, 20.
- Parks, W.C., Wilson, C.L., Lopez-Boado, Y.S., 2004. Matrix metalloproteinases as modulators of inflammation and innate immunity. *Nature reviews. Immunology* 4 (8), 617–629.
- Pastore, S., Mascia, F., Girolomoni, G., 2006. The contribution of keratinocytes to the pathogenesis of atopic dermatitis. *Eur. J. Dermatol. : EJD* 16 (2), 125–131.
- Renz, H., Brodie, C., Bradley, K., Leung, D.Y., Gelfand, E.W., 1994. Enhancement of IgE production by anti-CD40 antibody in atopic dermatitis. *J. Allergy Clin. Immunol.* 93 (3), 658–668.
- Salomon, J., Baran, E., 2008. The role of selected neuropeptides in pathogenesis of atopic dermatitis. *J. Eur. Acad. Dermatol. Venereol.* 22 (2), 223–228.
- Schmuth, M., Jiang, Y.J., Dubrac, S., Elias, P.M., Feingold, K.R., 2008. Thematic review

- series: skin lipids. Peroxisome proliferator-activated receptors and liver X receptors in epidermal biology. *J. Lipid Res.* 49 (3), 499–509.
- Soga, F., Katoh, N., Inoue, T., Kishimoto, S., 2007. Serotonin activates human monocytes and prevents apoptosis. *J. Invest. Dermatol.* 127 (8), 1947–1955.
- Suzuki, H., Miura, S., Liu, Y.Y., Tsuchiya, M., Ishii, H., 1995. Substance P induces degranulation of mast cells and leukocyte adhesion to venular endothelium. *Peptides* 16 (8), 1447–1452.
- Taniuchi, S., Kojima, T., Hara Mt, K., Yamamoto, A., Sasai, M., Takahashi, H., Kobayashi, Y., 2001. Increased serum nitrate levels in infants with atopic dermatitis. *Allergy* 56 (7), 693–695.
- Toyoda, M., Makino, T., Kagoura, M., Morohashi, M., 2000. Immunolocalization of substance P in human skin mast cells. *Arch. Dermatol. Res.* 292 (8), 418–421.
- Trautmann, A., Akdis, M., Blaser, K., Akdis, C.A., 2000. Role of dysregulated apoptosis in atopic dermatitis. *Apoptosis : Int. J. Programm. Cell Death* 5 (5), 425–429.
- Williams, H.C., Chalmers, J.R., Simpson, E.L., 2012. Prevention of atopic dermatitis. *F1000 Medicine Reports* 4, 24.