



RESEARCH ARTICLE

Effects of Anti-inflammatory and *Rehmanniae radix* Pharmacopuncture on Atopic Dermatitis in NC/Nga Mice

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Abstract

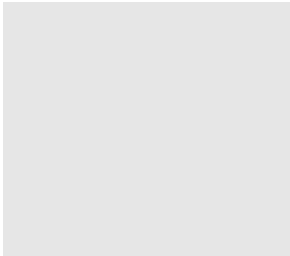
Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by pruritic and erythematous skin lesions. The purpose of this study was to investigate the suppressive effects of anti-inflammatory and *Rehmanniae radix* pharmacopuncture on the development of AD-like skin lesions in NC/Nga mice.

The AD was induced on the mice's back skin by using biostir AD. The experimental groups were divided into three groups, PPI (anti-inflammatory pharmacopuncture), PPII (*Rehmanniae radix* pharmacopuncture, hydrodistillation extraction) and PPIII (*Rehmanniae radix* pharmacopuncture, MeOH extraction). All mice were treated using a 1-mL syringe to inject 0.1 mL of pharmacopuncture at right and left acupoints (BL13) on alternate days. In the control group, normal saline was used instead of pharmacopuncture. The following factors were investigated: (1) optical observations made with a handscope and clinical skin scores were evaluated; (2) tissue (general/immune) mast cells and CCR3⁺ eosinophils, as well as vascular endothelial growth factor, fibroblast growth factor, and epidermal growth factor immunoreactive changes were evaluated; (3) CD4⁺ and CD8⁺ cells in the spleen were immunohistochemically examined; and, (4) the serum immunoglobulin (Ig)E level and lymphokines [interleukin (IL)-2, IL-4] were measured.

In the PPI and the PPIII groups, the clinical skin score, total number of mast cells, CCR3⁺ eosinophils immunoreaction, and total serum IgE, IL-2, and IL-4 levels were lower than the control group. The PPI and the PPIII groups also showed strong

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immunohistochemical reactions for vascular endothelial growth factor and fibroblast growth factor. The PPI group particularly showed a very strong immunohistochemical reaction for epidermal growth factor. All groups showed strong immune activity for CD8⁺. The PPIII group showed strong immunity for both CD4⁺ and CD8⁺.

From the above results, *Rehmanniae radix* pharmacopuncture (MeOH extraction) and anti-inflammatory pharmacopuncture exerted anti-allergic and anti-inflammatory effects, suggesting that they are promising agents for improving AD-related symptoms.

1. Introduction

Atopic dermatitis (AD) is chronic relapsing inflammation that manifests as severe pruritic and pathognomonic eczematous dermatitis. The cause of the disease is related to a variety of factors, of which genetic predisposition accompanied by assorted peculiar immune symptoms account for more than 50% of reported cases. Abnormal responses to medication, microbial infections, and substances in the environment are also known to be causes [1]. The disease is characterized by poorly defined erythema with edema, vesicles, and weeping in the acute stage and by skin thickening in the chronic stage [2].

The diagnosis of AD has been widely used during the 30 years since Hanifin and Rajka set up the diagnosis standard based on the patient's medical examination by interview and clinical tests [3]. The Korean atopy dermatitis society has been trying to establish a better diagnosis standard for Korea since the release of the AD diagnosis standard by Hanifin and Rajka [4].

Recently, the incidence of AD, along with that of allergic diseases, has steadily increased every year, and according to research, 47.2% of kindergarten students, 26% of elementary students, and 17.5% of middle school students have been diagnosed as having AD [5].

The field of Oriental medicine dealing with AD has defined the disease as abscesses induced by breast milk, fetal converged sores, fetal sores, fetal heat, breast milk sores and heat, dampness, dryness, qi deficiency, and blood deficiency [6]. The Dongeuihwangtang recommends prescribing Saengjihwangtang and Yangyubang as treatment for fetal heat [7].

In recent studies of these AD treatments, Na et al [8] used Jeseupwiryongtang-gagam and Park et al [9] used Yanghyulyoonbutang to treat NC/Nga mice. Yang et al [10] observed a change in cytokine levels after administering a variety of herbal medicines to a patient. Ahn et al reported that Gagam-palmultang was effective for changing transepidermal water loss [11].

With regard to external treatments, Yeo et al [12] and Song et al [13] reported that atopic cream and jawoongo ointment were effective for AD in NC/Nga mice. Kim et al [14] reportedly spread Hwangryunhaedoktang. With regard to pharmacopuncture treatment, Park et al reported the use of Ursi Fel pharmacopuncture [15], and Seo et al [16] reported the use of bee venom pharmacopuncture. However, studies or reports on the use of anti-inflammatory pharmacopuncture or *Rehmanniae radix* pharmacopuncture are hard to find.

In this study, the therapeutic effect of anti-inflammatory and *Rehmanniae radix* pharmacopuncture against biostir AD-induced AD was assessed in mice.

2. Materials and methods

2.1. Materials

The 30 experimental animals used in this study for the AD animal model were 7-week-old male NC/Nga mice (15–20 g) purchased from JAPAN SLC Inc. [17]. The animals had *ad-libitum* access to hard food (antibiotic deduction, Samyang Feed Co.) and were kept in a controlled environment at a temperature of 22 ± 2 °C and a humidity of $55 \pm 15\%$ with a 12-hour light-dark cycle. After the mice had become adjusted to the environment, the experiment was conducted. The experimental animals were distributed into five groups with six mice per group: normal group, control group, and three experimental (PPI, PPII, PPIII) groups. In the experimental groups, the PPI group received anti-inflammatory pharmacopuncture, the PPII group received *Rehmanniae radix* pharmacopuncture (hydrodistillation extraction) and the PPIII group received *Rehmanniae radix* pharmacopuncture (MeOH extraction). All institutional guidelines for the care and treatment of laboratory animal were followed. The *Rehmanniae radix* used in this experiment was purchased from Gwangmyeongdang Pharmaceuticals and was used in the Medical Herbs Laboratory at Woosuk University.

2.2. Methods

AD was induced by spreading an atopic-inducing reagent, biostir AD (Biostir Inc., Japan), which is a natural chemical found in mites, on the mice's skin for 3 weeks. The reagent was made from allergens of the *Dermatophagoides farinae*, the dust mite, and caused subclinical symptoms quite similar to those of atopy in humans. To induce AD, during the first phase we removed the NC/Nga mice's fur from the back to above the earflap by using a grainer with depilatory cream (Rasera™ depilatory gel, Reckitt Benckiser, France); excess depilatory cream was washed off. Next, biostir AD cream (100 mg) was spread on the backs and earflaps of all mice in all groups except the normal group by using a flat stick. During the second phase, to impede fat-forming element removal and damage the cuticle barrier, a 4% SDS (sodium dodecyl sulfate) aqueous solution (150 µL) was spread from the back to above the earflap and was allowed

to dry for 2 or 3 hours. Then measured amounts of biostir AD cream were spread as in the first phase. Application of biostir AD cream was conducted twice a week (Monday and Thursday) for 3 weeks for a total of six times.

The anti-inflammatory pharmacopuncture used for this experiment was provided by the Korean Pharmacopuncture Institute and its constituents are listed in Table 1.

The hydrodistillation extraction was kept in a pyrex bottle throughout the process. *Rehmanniae radix* (100 g) was placed in 1.5 L of water that had been distilled three times in a distillation extractor by using a reflux condenser at 170 °C for 3 hours. The hydrodistillation extract was kept refrigerated for 24 hours to allow inorganic salts to precipitate, which resulted in a clear supernatant liquid after the salinity had been adjusted to 0.98% and the pH to 7.25–7.35, so that the hydrodistillation extraction (1 L) could be obtained by filtering with a 0.1 µm filter (Advantec, Japan). The hydrodistillation extraction was placed into 20-mL vials that had been sterilized at high pressure for 30 minutes at 120 °C. The vials were capped with sterilized silicone stoppers and aluminium caps.

Rehmanniae radix (500 g) was extracted using an undiluted solution machine (Dongah, Korea). The liquid (117.54 g) was obtained from dried powder (33 g) by freeze drying. The reprocessed freeze-dried powder that had been added to MeOH (99.5%, Samchun Chemical, Korea) was extracted using an ultrasonic-waves machine (Hwashin Tech, Powersonic 420, Korea) for 2 hours. The end-product (10 g) was obtained using a decompression thickener (Eyela, Japan) after having been filtered through filter paper (Advantec No.2). Finally, 500 mg of the end product was diluted with 50 mL of distilled water.

Pharmacopuncture fluid injection was carried out using 26-gauge syringes. All experimental groups were treated daily with 0.1 mL of pharmacopuncture using syringes at the right and the left acupoints (BL13) on alternate days. For the control group, normal saline was used instead of pharmacopuncture.

To determine macroscopic changes on the skin surface, we used a handscope to make daily observations for 4 weeks. The subclinical macroscopic observations were made using the methods of Yamamoto et al [18]. The total clinical skin score for the AD-like lesions on the NC/Nga mice was defined as the sum of individual scores, graded as 0 (none), 1 (mild), 2 (moderate), or 3 (severe), for each of four signs and symptoms (erythema/hemorrhage, scarring/dryness, edema, excoriation/erosion) on the back skin. The total clinical skin score ranged from a minimum of 0 to

a maximum of 12 points. For accuracy, before the experiment, the animals were placed into groups so that the skin scores of the control group and experimental groups were 8 points on average.

To observe the change in mastocytes in skin tissue and to make general histologic observations, we sacrificed the mice at the 4th week after pharmacopuncture treatment. Twelve hours after flow-through fixing, we made an incision on the skin of the back parallel to or at a right angle to the spine line. The specimens were then embedded in paraffin through a dehydration process, and hematoxylin and eosin staining and toluidin blue dyeing was performed on 7-µm fragmentation sections.

To observe immunohistochemical changes in various cytokines and CCR3⁺ eosinophils in the skin tissue, we produced 7-µm thick paraffin sections. The paraffin sections were made by removing endogenous peroxidase from 0.3% H₂O₂ of 99.5% methanol, followed by processing normal serum (normal serum, 1:50) for 30 minutes to eliminate the nonspecific immune reaction. To observe the expected changes in the skin tissue, we used CCR3⁺ eosinophils (1:600), vascular endothelial growth factor (VEGF, 1:50), fibroblast growth factor (FGF, 1:100), and epidermal growth factor (EGF, 1:100) as the first antibodies. The proper dilution concentration of the first antibody was determined from the positive control test for each antibody because the concentration was found to depend on the kinds of antibody and the manufacturing company. Following the procedure of Hsu et al [19], the second antibody was washed with 0.1 M PBS (Phosphate Buffer Saline) for 5 minutes three times after the first antibody reaction that diluted biotinylated anti-immunoglobulin (Ig)G (Vector Laboratories, Inc.; 1:200). It was reacted with moisture at room temperature. Next, the second antibody was washed with 0.1 M PBS for 5 minutes three times again and was reacted with avidin-biotin-peroxidase complex for 30 minutes. Thereafter, it was washed with 0.1 M PBS for 15 minutes two times; then, it was reacted in a solution that had been prepared by melting 30 mg of 3-3' diaminobenzidine in 150 mL of 0.1 M PBS for 5 minutes, followed by the addition of 0.005% oxygenated water to induce a reaction that led to a brown color after about 15 minutes. After the tissue preparation, counter staining with Mayer hematoxylin was conducted for 5 seconds, and after pre-mounting, the specimens were observed through an optical microscope.

After pharmacopuncture treatment for 4 weeks, immunohistochemistry dyeing was conducted on 7-µm thick paraffin sections to observe the changes in CD4⁺ and CD8⁺ cells in the spleen and lymphoid organs. The first-order antibody used in this experiment was made from a dilution of rabbit polyclonal anti-CD4 (Sigma-Aldrich, USA) and mouse monoclonal CD8 (Santa Cruz Biotech, USA), each antibody being diluted at 1:50 and 1:300.

The measurement of IgE in the serum was conducted using an enzyme-linked immunosorbent assay (ELISA) kit (Gene Way Biotech, Inc.) and the ELISA method. Fifty-fold diluted serum sample and standard solution (100 µL) was placed into the wells of a 96-well microplate that had been coated with anti-mouse IgE antibody before being reacted with diluted buffer at room temperature for 30 minutes. Then, the sample was washed four times with washing

Table 1 Prescription for "anti-inflammatory" pharmacopuncture fluid.

Pharmacognostic name	Dose (g)
<i>Taraxaci herba</i>	39
<i>Lonicerae flos</i>	39
<i>Rehmanniae radix</i>	39
<i>Forsythiae fructus</i>	39
<i>Coptidis rhizome</i>	24
<i>Scutellariae radix</i>	24
<i>Phellodendri cortex</i>	24
<i>Gardeniae fructus</i>	24

buffer and was processed with HRP conjugated antibody (100 μ L), followed by cultivation for 30 minutes at room temperature with sunlight blocked. Finally, the sample was washed four times with washing buffer, then, 100 μ L of TMB (Tetramethylbenzidine) solution was put into each well, followed by exposure to light for 10 minutes. The reaction was terminated by adding 100 μ L of 0.3 M H_2SO_4 solution. After 10 minutes, the absorbance was measured at a 450 nm wavelength.

In order to observe lymphokines [interleukin (IL)-2 and IL-4] in the serum, we collected blood through the heart, and the collected blood was separated through centrifugation at 3000 rpm and 4 °C for 20 minutes. The collected blood was kept at -70 °C before being analyzed. The concentrations of IL-2 and IL-4 in the serum were measured using the sandwich ELISA method [20].

2.3. Statistical analysis

The data were expressed as means $\pm$ SD. The experimental data were analyzed by using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA). Statistical differences were further analyzed using the student *t* test to verify the results. A *p* value of <0.05 was considered statistically significant.

3. Results

Macroscopic observations of AD were made on the back skins of NC/Nga mice after 3 weeks of biostir AD treatment had caused AD. When the clinical skin scores were measured using a handscope, the skin scores of the control

and the experimental groups showed few individual differences (Fig. 1). From weeks 1 to 2, a variety of symptoms, such as erythema/hemorrhage, scarring/dryness, edema, and excoriation/erosion were observed both in the control group and the experimental groups. After daily pharmacopuncture treatment for 4 weeks, in the NC/Nga mouse model for AD induced by biostir AD, the clinical skin score of the control group was 4.8 ± 0.4 , and that of the PPII group was 4.6 ± 0.5 , however the clinical skin score of the PPI group was 2.9 ± 0.2 and that of the PPIII group was 3.2 ± 0.5 . The score for the PPI group was significantly decreased ($p < 0.05$) compared with the control group (Table 2). Erythema/hemorrhage (mild, 1), scarring/dryness (moderate, 2), edema (mild, 1), and excoriation/erosion (mild, 1) were noted for the control and the PPII groups. Erythema/hemorrhage (mild, 1), scarring/dryness (mild, 1), edema (none, 0), and excoriation/erosion (mild, 1) were noted for the PPI and the PPIII groups (Fig. 2).

An observed microscopic feature was greater growth of the epidermis in the PPI group compared with the other groups. Degranulated mast cells were observed in the control and PPII groups (Fig. 3A and C). The changes in the numbers of mast cells in the skin tissue were 7.9 ± 2.3 for the normal group, 13.9 ± 4.5 for the control, 3.4 ± 2.4 for PPI, 16.2 ± 4.1 for PPII, and 3.0 ± 1.4 for PPIII group. The values were significantly decreased ($p < 0.05$) in the PPI and PPIII groups compared with control (Fig. 4).

The changes in levels of CCR3+ eosinophils in the skin tissue were 0.9 ± 0.8 for the normal group, 7.4 ± 2.0 for control, 1.3 ± 1.2 for PPI, 5.7 ± 1.8 for PPII, and 1.9 ± 1.2 for PPIII. The values for the PPI and PPIII groups were

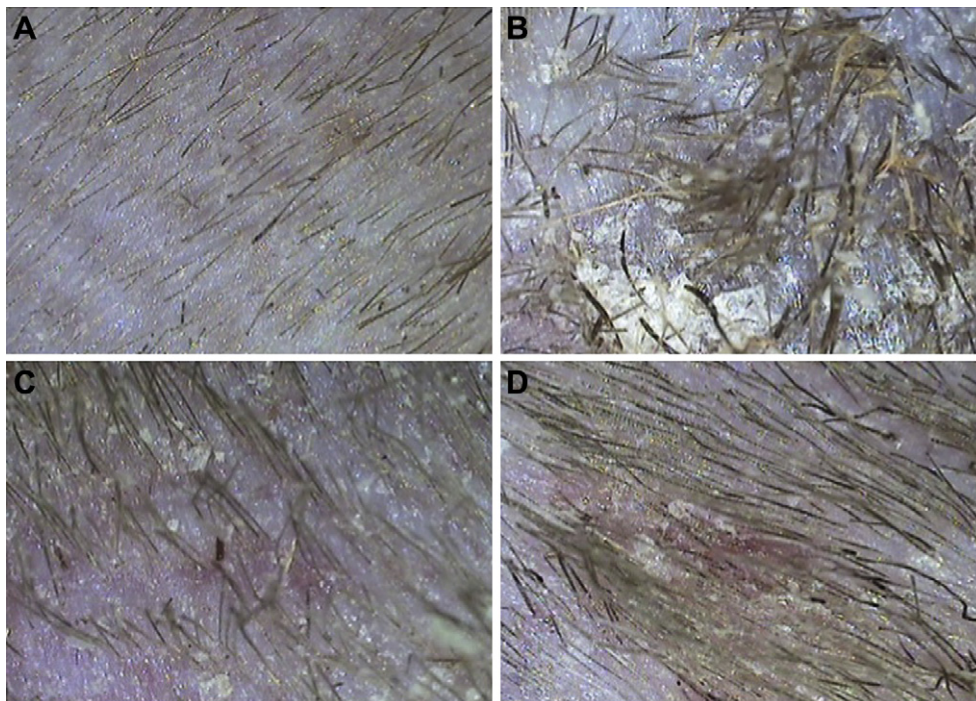


Figure 1 Handscopic observation of atopic dermatitis (AD) on the back skins of NC/Nga mice after a 3-week biostir AD application. (A) The backs of AD NC/Nga mice to which biostir was not applied (normal); and (B) scarring/dryness and edema, (C) scarring/dryness, edema, and erythema/hemorrhage, and (D) scarring/dryness, erythema/hemorrhage, edema, and excoriation/erosion following application of biostir AD for 3 weeks.

Table 2 Effects on clinical skin score.

Group	Clinical skin score, mean $\pm$ SD
Control	4.8 $\pm$ 0.4
PPI	2.9 $\pm$ 0.2*
PPII	4.6 $\pm$ 0.5
PPIII	3.2 $\pm$ 0.5

Control group, atopic dermatitis induced by biostir AD + saline pharmacopuncture; PPI group, atopic dermatitis induced by biostir AD + anti-inflammatory pharmacopuncture; PPII group, atopic dermatitis induced by biostir AD + *Rehmanniae radix* pharmacopuncture (hydrodistillation extraction); PPIII group, atopic dermatitis induced by biostir AD + *Rehmanniae radix* pharmacopuncture (methanol extraction).

* p value < 0.05 .

significantly decreased compared with the control group ($p < 0.05$) (Fig. 5).

The change in VEGF in the skin tissue, indicated a very small immunoreaction response in the control group, and a small immunoreaction response in the PPII group (Fig. 6C). VEGF immunoreaction expression was more intense in the epidermis of the PPI and PPIII groups than it was in the control and PPII groups (Fig. 6B and D).

A moderate immunohistochemical response of FGF was shown in the control, PPI, and PPIII groups (Fig. 7A, B, and D), but only a faint immunohistochemical reaction of FGF was found in the PPII group (Fig. 7C).

A faint EGF immunohistochemical response was shown in the control, PPII, and PPIII groups (Fig. 8A, C, and D), but a severe EGF immunohistochemical reaction was shown in the PPI group (Fig. 8B).

A moderate immunohistochemical reaction was observed for CD4⁺ immunohistochemical-reactive cells around the central artery and the periarterial lymphatic sheath. Among the experimental groups, a severe immunohistochemical reaction was noted in the PPIII group, and a faint immunohistochemical reaction was observed in the PPI group (Fig. 9).

Expression of the CD8⁺ immunoreaction was more intense around the central artery of the spleen and the periarterial lymphatic sheath. In the PPI, PPII, and PPIII groups, in order of increasing intensity, immunoreaction expression was more intense than it was in the control group (Fig. 10).

The serum IgE level was about 172.4 $\pm$ 0.3 ng/mL in the control group, compared with 126.6 $\pm$ 0.9 ng/mL in the normal group. The serum IgE levels in the experimental groups were 127.9 $\pm$ 4.1 ng/mL for the PPI group, and 141.8 $\pm$ 3.6 ng/mL for the PPIII group, both of which were significantly lower than the control group ($p < 0.05$). However, the value for the PPII group, 203.1 $\pm$ 6.9 ng/mL, was higher than that for the control group (Fig. 11).

The serum IL-2 level was about 0.100 $\pm$ 0.010 pg/mL in the control group, compared with 0.202 $\pm$ 0.021 pg/mL in the normal group. Among the experimental groups, the IL-2 levels for the PPI, PPII, and PPIII groups were 0.146 $\pm$ 0.012 pg/mL, 0.202 $\pm$ 0.019 pg/mL, and 0.185 $\pm$ 0.012 pg/mL, respectively. All groups showed levels that were significantly higher than the control group ($p < 0.05$) (Fig. 12).

The serum IL-4 level was 48.5 $\pm$ 0.6 pg/mL for the control group, which was similar to the level for the normal group (48.6 $\pm$ 0.4 pg/mL). Among the experimental groups, the serum IL-4 levels for the PPI, PPII, and PPIII groups were

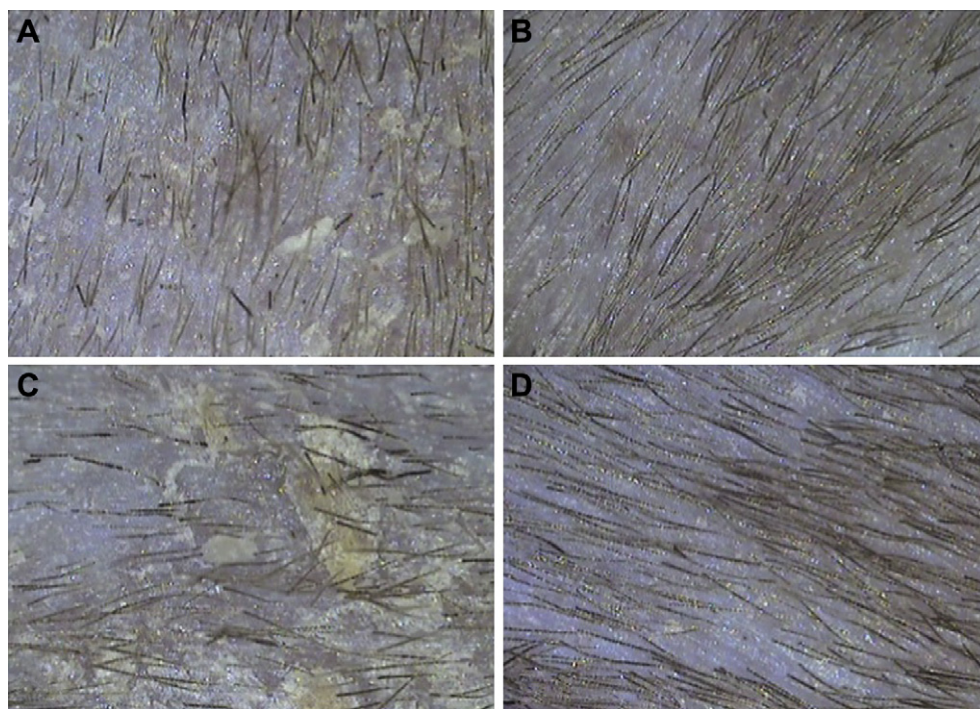


Figure 2 Handscopic observations of the skin: (A) control group and (B–D) experimental groups. Handscopic features of the control (A) and the PPII (C) groups showed more serious skin lesions (scarring/dryness) than the PPI (B) and the PPIII (D) groups.

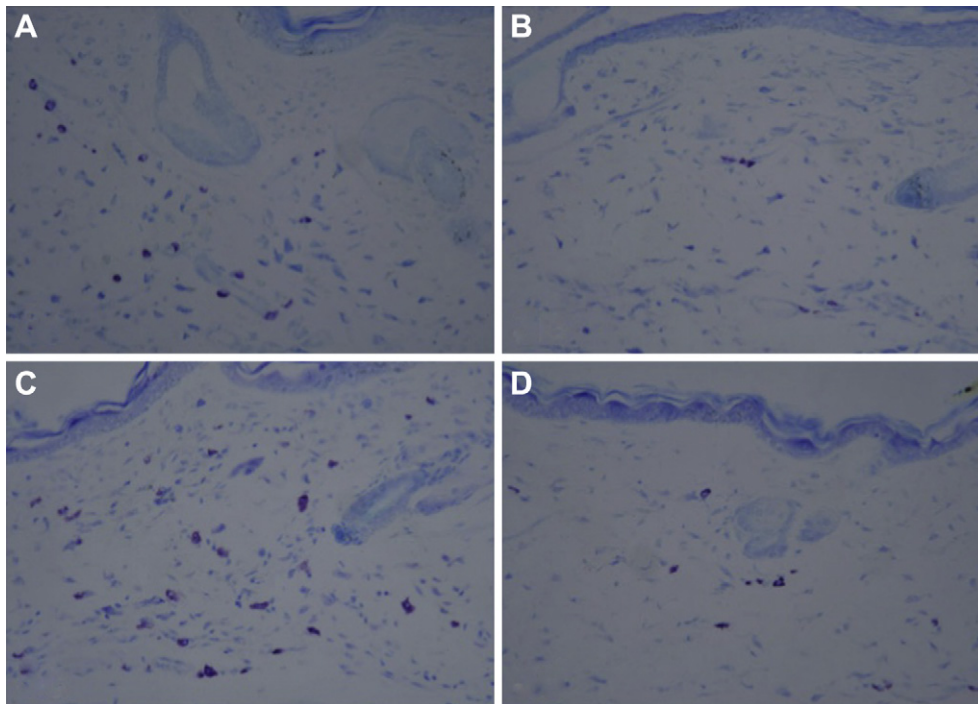


Figure 3 Histological section of the skin (toluidin blue, $\times 200$): (A) control group and (B–D) experimental groups. The distribution of mast cells in the skin was decreased more in the PPI (B) and the PPIII (D) groups than control (A) and the PPII group (C). Degranulated mast cells were increased more in the control (A) and PPII (C) groups than the PPI (B) and PPIII (D) groups.

47.5 ± 0.5 pg/mL, 47.2 ± 0.4 pg/mL, and 47.1 ± 0.4 pg/mL, respectively. All experimental groups showed levels lower than the control group (Fig. 13).

4. Discussion

AD is a disease that was initially proposed by Wise & Sulzberger in the 1930's [21]. According to the diagnostic criteria based on medical examination interviews and clinical aspects proposed by Hanifin & Rajka in 1980 [3], if a disease meets three conditions out of pruritus, distinguishing features and part of the body, chronic

dermatitis, and a family history of the AD, in addition to three accompanying symptoms (for example, xerosis, immediate skin-test reactivity, raised serum IgE, nipple eczema, cheilitis, etc.) a diagnosis for AD can be made. However, so far, no fundamental medical treatment methods for AD exist [22].

In terms of Oriental medicine, the causes of AD are considered to be mothers infected by seasonal qi and pathogen toxins, external contraction of wind, dampness and heat, over-consumption of spicy food, and abuse of warm drugs. The symptoms of AD are considered to be high fever, reddened complexion, closed and flushed eyes, red

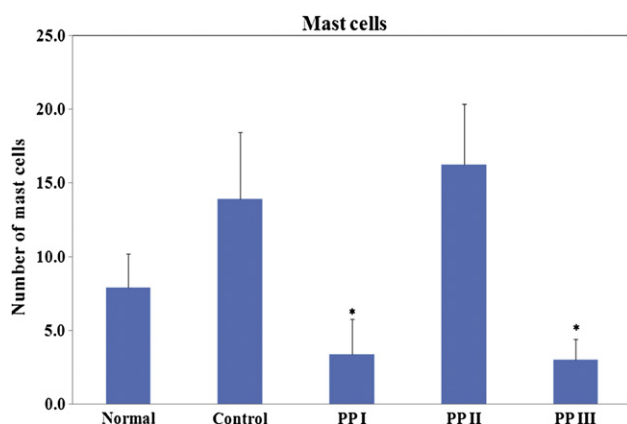


Figure 4 Changes in the number of mast cells. The number of mast cells in the skin was significantly lower in the PPI and PPIII groups than in the control and PPII groups. Each datum represents the mean $\pm$ SD for six mice. * $p < 0.05$.

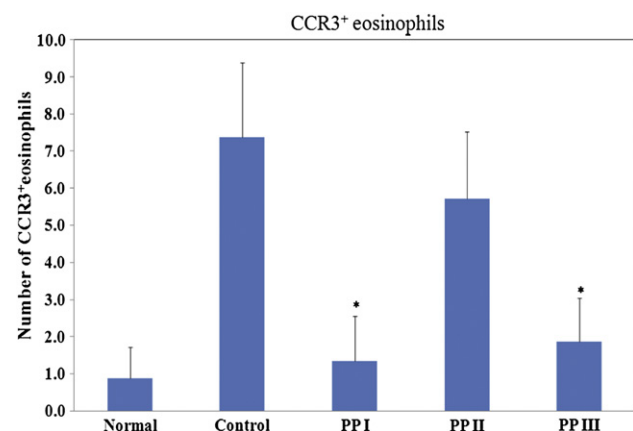


Figure 5 Changes in CCR3⁺ eosinophils. The number of CCR3⁺ eosinophils in the skin was much lower in the PPI and PPIII groups than in the control and PPII groups. Each datum represents the mean $\pm$ SD for six mice. * $p < 0.05$.

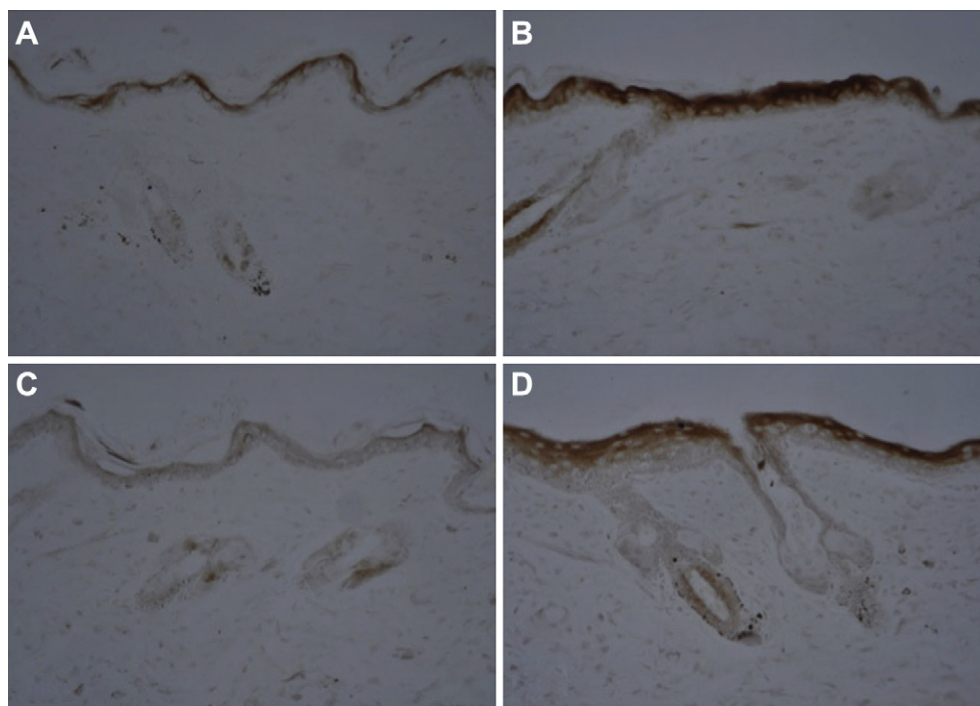


Figure 6 VEGF immunohistochemical staining ($\times 200$). (A) Control group and (B–D) experimental groups. Expression of the VEGF immunoreaction was more intense in the epidermis of the PPI and PPIII groups (B and D, respectively) than the control and PPII groups (A and C, respectively). VEGF = vascular endothelial growth factor.

and yellow urine, constipation, and itching. Clearing the heart and detoxification are basic methods of treatment, and they are divided into two categories: those based on dampness and those on thin body type [23].

Currently, AD is usually treated using humectants, steroids, antihistamines, immunomodulators, etc. [24]. In the case of long-term use of steroids, however, there are problems such as abuse and various side effects [25].

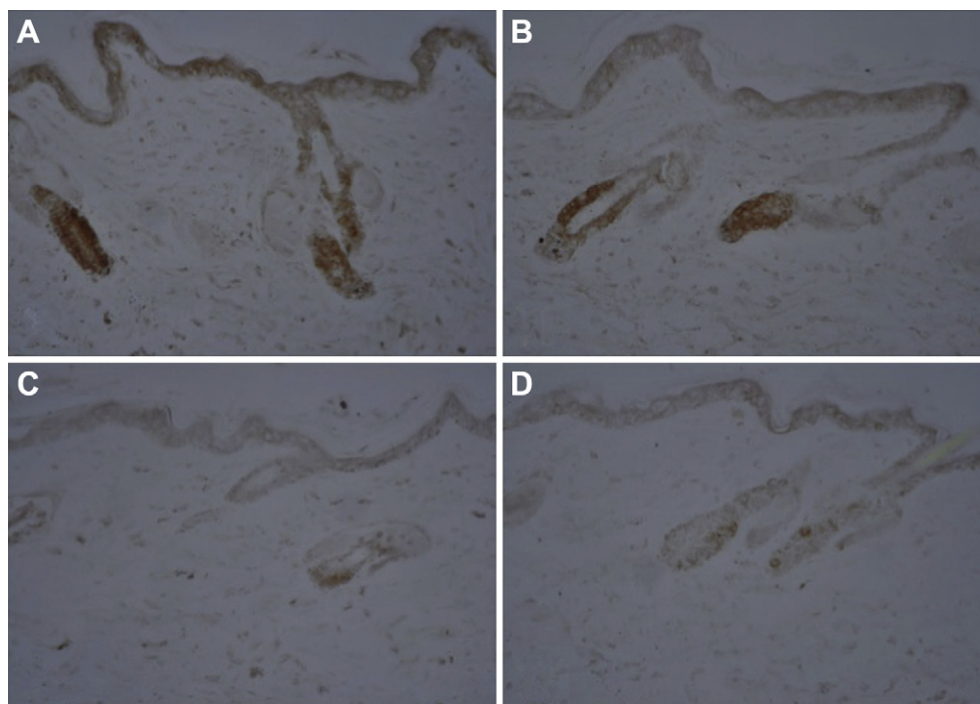


Figure 7 FGF immunohistochemical staining ($\times 200$). (A) Control group and (B–D) experimental groups. Expression of the FGF immunoreaction was more intense in the epidermis of the control (A), the PPI (B) and the PPIII groups (D) compared with the PPII group (C). FGF = fibroblast growth factor.

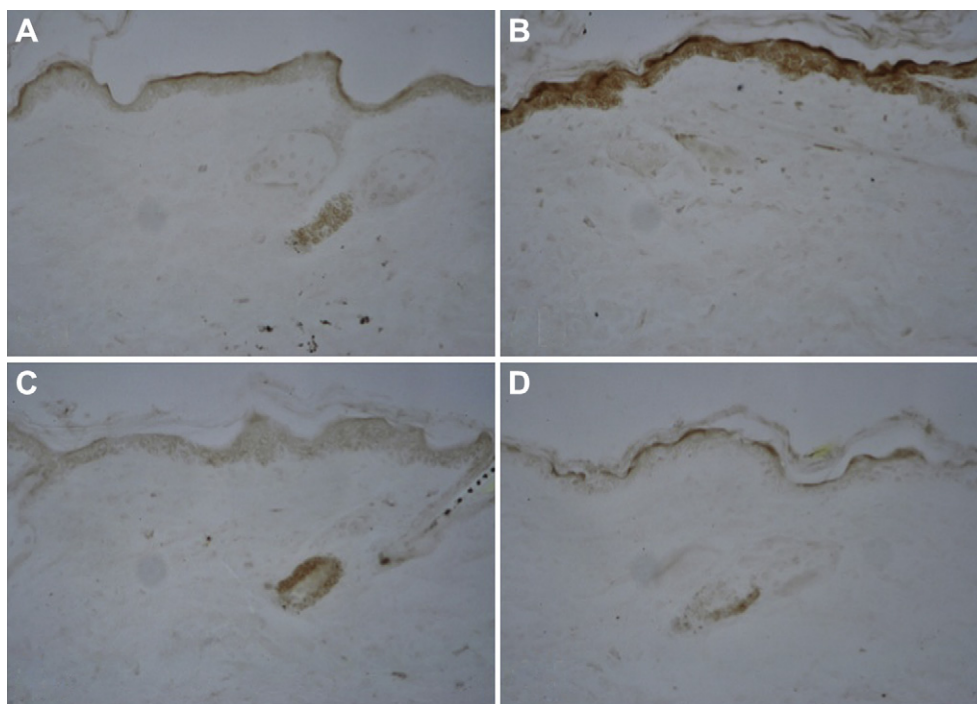


Figure 8 EGF immunohistochemical staining ($\times 200$). (A) Control group and (B–D) experimental groups. Expression of the EGF immunoreaction was more intense in the epidermis of the PPI group (B) than the other groups (A, C and D). EGF = epidermal growth factor.

Therefore, safer and more effective drugs need to be developed. Accordingly, the effectiveness of treating immune skin disease using anti-inflammatory and *Rehmanniae radix* pharmacopuncture needs to be studied.

Rehmanniae radix is the fresh root of *Rehmannia glutinosa* and *R. gluticosa* LIBOSCH. f. *hueichingensis*, a perennial plant belonging to Scrophulariaceae. It is cold and has a sweet and bitter taste. Also, it selectively affects some

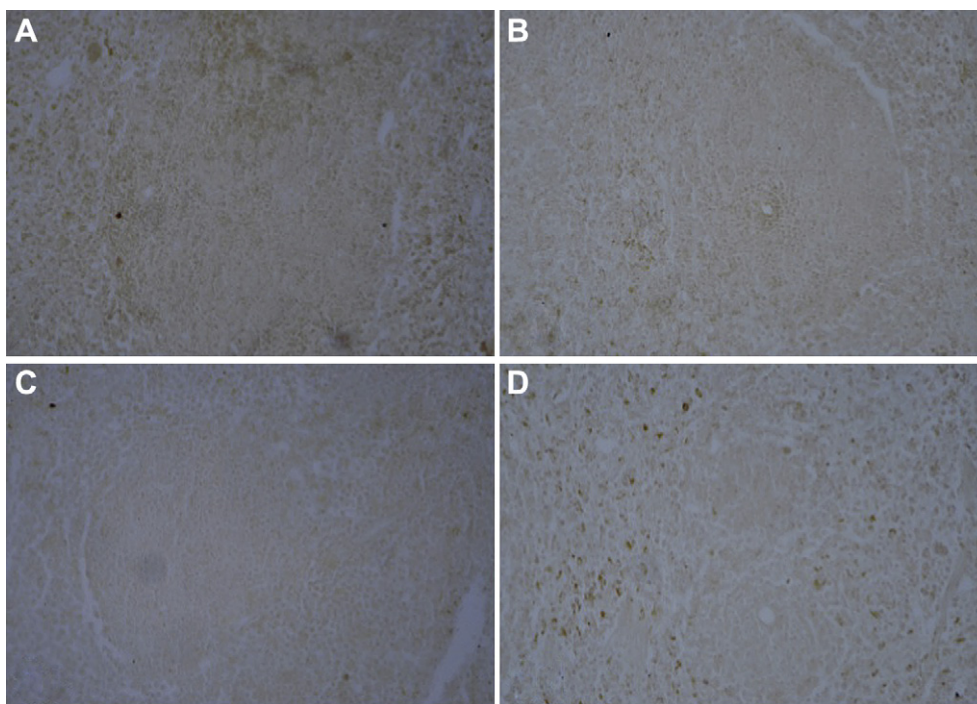


Figure 9 $CD4^+$ immunohistochemical staining of the spleen ($\times 200$). (A) Control group and (B–D) experimental groups. Expression of the $CD4^+$ immunoreaction was more intense in the periarterial lymphatic sheath for the PPIII group (D) than the control (A), the PPI (B) and the PPIII groups (C).

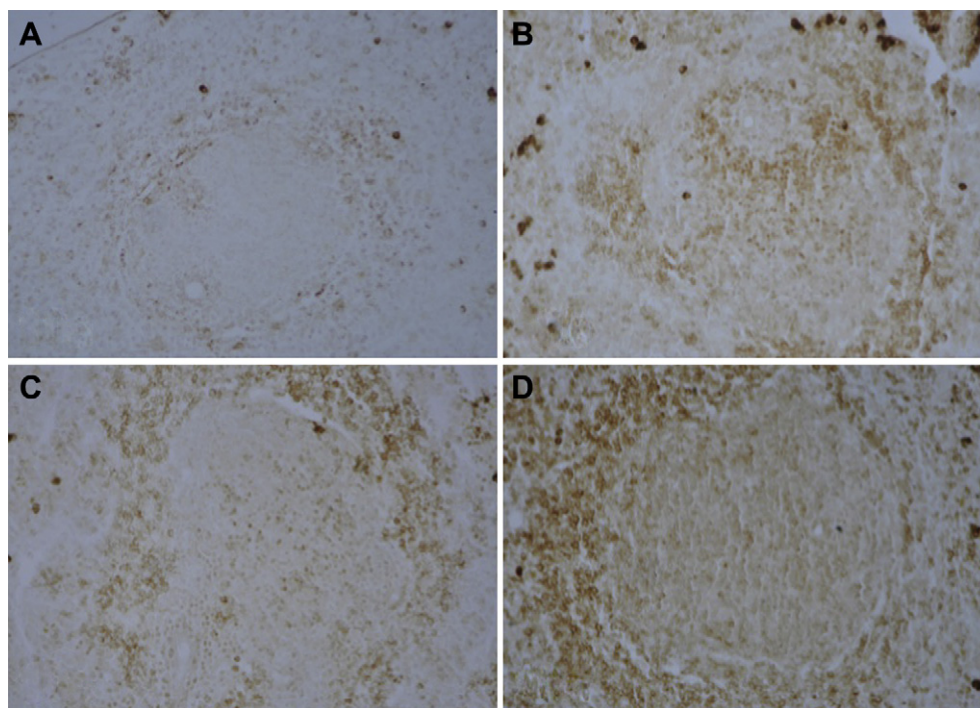


Figure 10 CD8⁺ immunohistochemical staining of the spleen (×200). (A) Control group and (B–D) experimental groups. Expression of the CD8⁺ immunoreaction was more intense in the periarterial lymphatic sheath for the PPI (B), PPII (C) and PPIII groups (D) compared with the control group (A).

parts of the body such as the heart, liver, and kidneys. Known drug actions are clearing the heart, and engendering fluid, tonifying blood, and stopping the flow of blood. It has been used as a medicine to treat heat disease and yin damage, tongue atrophy and anadypsis, outbreaks of macula, hematemesis, epistaxis, and swollen and painful throats [26].

Rehmanniae radix has also been reported to be effective for treating hypertension [27] and diabetes [28], and for the regeneration of hepatic tissue [29]. Shin et al [30] reported that in clinical trials, *Rehmanniae radix* pharmacopuncture was effective in controlling the autonomic nervous system.

Kim et al reported that *Rehmannia glutinosa* was effective for protein expression related to angiogenesis, cell survival, and inflammation [31]. Shin et al reported that *Rehmanniae radix* was effective for treating allergic contact dermatitis [32]. However, few studies and reports on using *Rehmanniae radix* pharmacopuncture to treat AD exist.

Anti-inflammatory pharmacopuncture involves a prescription that adds drugs such as *Taraxaci herba*, *Lonicerae flos*, *Rehmanniae radix*, and *Forsythiae fructus* to a Hwangryunhaedoktang [33]. Hwangryunhaedoktang, which was mentioned in Zhouhoufang and Waitaimiyao, is

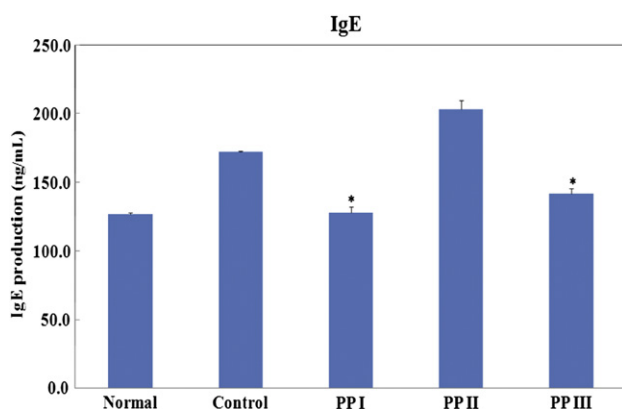


Figure 11 Effects on the serum IgE level. The levels of serum IgE were significantly lower in the PPI and PPIII groups than in the control and PPII groups. Each datum represents the mean $\pm$ SD for six mice. * $p < 0.05$. Ig = immunoglobulin.

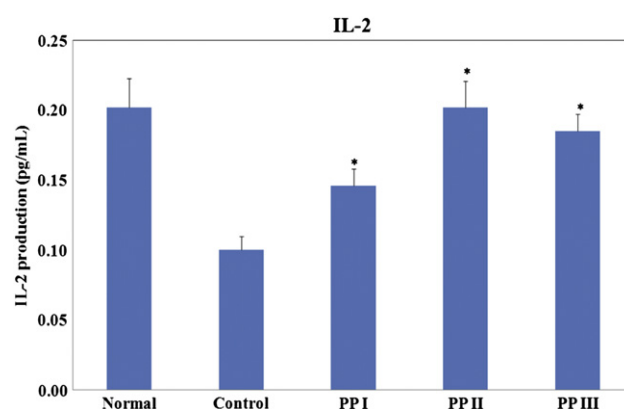


Figure 12 Effects on the serum IL-2 level. The levels of IL-2 were significantly larger in the PPI, PPII, and PPIII groups than in the control group. Each datum represents the mean $\pm$ SD for six mice. * $p < 0.05$. IL = interleukin.

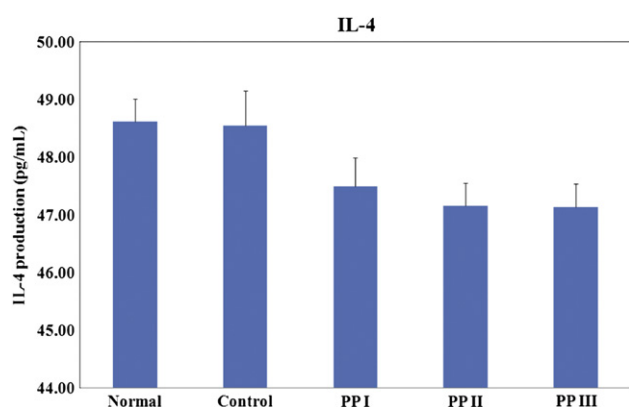


Figure 13 Effects on the serum IL-4 level. The levels of IL-4 were lower in the PPI, PPII, and PPIII groups than in the control group. Each datum represents the mean $\pm$ SD for six mice. IL = interleukin.

a prescription to treat excess heat, and it has been used to treat various skin diseases such as measles, smallpox, dermatolosis, pruritus cutaneus, urticaria, etc. [34].

In the experimental studies, anti-inflammatory pharmacopuncture was effective in suppressing colon-inflammation-induced c-Fos [33] and lipopolysaccharide-induced inflammation-related cytokines [35]. In clinical studies, anti-inflammatory pharmacopuncture was effective in treating inflammation and pain [36], as well as in treating cancer patients with accompanying pain [37] and patients with supraspinatus tendinosis and subdeltoid bursitis [38]. However, few studies and reports on the use of anti-inflammatory pharmacopuncture to treat AD exist. Accordingly, this experimental study investigated the effects on AD-like skin lesions of anti-inflammatory and *Rehmanniae radix* pharmacopuncture at acupoints (BL13). The *Rehmanniae radix* pharmacopuncture was prepared by using either hydrodistillation extraction or methanol extraction.

Pharmacopuncture is a new kind of acupuncture treatment based on two major Oriental medical theories, meridian and herbal medicine. Many Oriental herbal medicines are injected at acupoints or at disease-related points to treat various symptoms [39].

For the injection of pharmacopuncture fluid, Fei shu (BL13) is an acupoint that belongs to the bladder meridian. Its characteristics are controlling lung qi, relieving fatigue, cooling deficiency heat, and harmonizing blood nourishment, and it is effective for treating pneumonia, pulmonary tuberculosis, coughing, asthma, bronchitis, inferior heat, night sweats, chest pressure, dyspnea, gastropathy, pruritus cutaneus, diarrhea, vomiting, hiccups, deafness, diabetes, shortness of breath, and flushing [39]. In this study, the pharmacopuncture was injected at BL13 for the treatment of AD.

After daily pharmacopuncture treatment for a total of 4 weeks, the clinical skin score of skin lesions were lower in the PPI and the PPIII groups than in the control and PPII groups.

According to observations of the general histological changes in skin tissue, a more active proliferation of epithelial cells was observed in the PPI group than in the control, PPII, and PPIII groups.

Increases in the concentration of mast cells in tissue are known to be caused by type I hypersensitivity [40]. Therefore, the significant decreases in the numbers of mast cells in the PPI and PPIII groups show that the anti-inflammatory and *Rehmanniae radix* pharmacopuncture (methanol extraction) is effective for treating AD. In addition, decreases in the CCR3⁺ eosinophils in the PPI and PPIII groups demonstrate that anti-inflammatory and *Rehmanniae radix* pharmacopuncture (methanol extraction) is effective for AD [41].

VEGF, FGF, and EGF directly affect vascular endothelial cells and work to stimulate angiogenesis. If cell proliferation is stimulated by cell injury, cell death, or physically modified tissue, these work as growth factors [40]. The VEGF results indicate that the healing process was active in the PPI and PPIII groups. From the FGF data, a moderate immunostaining response could be observed in the control, PPI, and PPIII groups, and from the EGF data, a satisfactory healing process for AD could be observed in the PPI group.

The cellular immune reaction is continued by T cells (T lymphocytes) that come from the thymus. T cells also regulate the immune response. Each T cell has an antigen-specific T cell receptor (TcR) on the cell surface and recognizes specific antigens via the TcR [40]. T cells are generally divided into two cells by the types of proteins that exist in the cell membrane. One is the CD4⁺ T cell, a helper T cell, and the other is the CD8⁺ T cell, a killer and suppressor T cell. CD4⁺ T cells are also divided into two cell types. One is the Th1 cell that mostly secretes IL-2 and IFN- γ . The other is the Th2 cell, which mostly secretes IL-4, IL-5, and IL-6. The Th1 cell works to help activate macrophages and killer T cells. The Th2 cell works to help antibody production. In contrast to the CD4⁺ T cells, the CD8⁺ T cell makes cells such as cytotoxic cells [42].

In this study, the strongest immunostaining response induced by *Rehmanniae radix* pharmacopuncture (methanol extraction) in the case of the CD4⁺ T cell was observed in the PPIII group. In the case of the CD8⁺ T cell, strong immunostaining responses were observed in all the experimental groups. Therefore, all the pharmacopunctures are thought to affect the hypersensitivity that causes AD.

IgE is a skin sensitization antibody, which has a characteristic of combining with mast cells. If an interaction occurs between the IgE on the mast cell's surface and an antigen, some substances that cause allergic reactions, such as histamines, may be secreted [42]. An increase in the IgE level and an outbreak of eosinophilia in the blood are initial symptoms of an allergic disease [43]. Therefore, the significant decreases in the IgE levels in the PPI and PPIII groups show that the anti-inflammatory and *Rehmanniae radix* (methanol extraction) pharmacopuncture is effective for treating AD.

IL-2 causes the activation and proliferation of T cells [42], and IL-4 is involved in IgE production, secretion of mucus, and eosinocyte production. IL-2 and IL-4 are representative cytokines related to the Th2 cell response, along with IL-5, IL-6, IL-13, etc. [44]. In this study, the IL-2 level was significantly increased in all experimental groups compared with the control group and the IL-4 level was significantly decreased in all experimental groups compared with the control group. This is consistent with other studies [32,35,45].

Catalpol has been reported to be the main medicinal component among the main ingredients of the *Rehmannia*

glutinosa rhizome [26], and it is significantly associated with the other ingredients [46]. Zhu et al reported that the extraction ratios of catalpol and 5-HMF are different depending on whether supercritical carbon dioxide extraction or hydrodistillation extraction is used [47]. According to Heo's study, four kinds of ingredients were detected by using a gas chromatography/mass spectrometry ingredient analysis of freeze-dried extracted *Rehmanniae radix* pharmacopuncture [48]. According to Chi et al, during a methanol extraction, fructose, glucose, sucrose as a saccharide, and β -sitosterol, campesterol, and stigmasterol as sterols were isolated and identified [49]. However, when the ingredients were extracted, some differences in the detected ingredients were noted for different solvents, extraction methods, and inspection methods fused for the extracts. Therefore, studies to analyze the active ingredients in the *Rehmanniae radix* pharmacopuncture for treating AD are needed in the future.

According to Yoon et al, the anti-inflammatory pharmacopuncture also contained berberine and coptisine as main ingredients of the coptidis rhizome, as determined by using high-performance thin-layer chromatography and some putative ingredients, such as epiberberine, palmatine, etc., were detected [35]. In this study, the anti-inflammatory pharmacopuncture had an effect on AD that could be related with the study by Kim et al on the inhibitory properties of Hwangryunhaedoktang for the development of AD in NC/Nga mice [14].

As a result of this study, injecting the anti-inflammatory and *Rehmanniae radix* (methanol extraction) pharmacopuncture at an acupoint (BL13) is expected to be an effective treatment for AD. In addition, analysis of the components of *Rehmanniae radix* and clinical studies based on various sampling methods should be continually pursued in the future.

Conflict of interest

The authors declare that there are no conflicts of interest.

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