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RESEARCH ARTICLE

Effects of topical application of *Astragalus membranaceus* on allergic dermatitis

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Abstract

Astragalus membranaceus (AM) is one of the most popular health-promoting herbs in East Asia, and has been used in traditional medicine for more than 2000 years. This study was performed to examine whether AM suppresses atopic dermatitis (AD)-like skin lesions in BALB/c mice. Seven-week-old female BALB/c mice were sensitized with 1-chloro-2,4-dinitrobenzene (DNCB) to induce allergic dermatitis. Skin sections were stained with hematoxylin and eosin (H&E) to assess epidermal and dermal hyperplasia, which were determined by measuring the thicknesses of the epidermis and dermis, respectively. The serum immunoglobulin G (IgE) concentration was quantified by enzyme-linked immunosorbent assay (ELISA). In addition, the levels of interleukins (IL)-4, -5, -6, and -13 and tissue necrosis factor (TNF)- α were measured in mouse serum. Significance was determined by one-way analysis of variance (ANOVA). Topical AM markedly improved the AD skin lesions in DNCB-induced mice. The AD skin lesions were significantly thinner in the AM treatment group compared with untreated controls, and the hyperkeratosis disappeared. Topical treatment of AM also restored nuclear factor- κ B (NF- κ B) expression. In addition, the serum IgE level was reduced. AM suppressed the expression of Th2 cytokines (IL-4, -5, -6, and -13) and significantly decreased the TNF- α level. AM is effective for treating AD by regulating cytokines. AM may be an alternative or complementary therapeutic option for treating patients with AD. More in-depth studies are necessary to clarify the mechanisms of AM.

Keywords: *Astragalus membranaceus*, atopic dermatitis, cytokines, DNCB, IgE

Introduction

Atopic dermatitis (AD) is an immune disorder with a genetic and environmental basis, and is a chronic intractable disease.⁽¹⁾ Patients with AD usually have a personal or familial history of other atopic diseases.⁽²⁾ Population studies have suggested that AD affects 10–20% of all children in most countries at some time during childhood.⁽³⁾ Moreover, AD is a multifactorial disease, and its pathogenesis and etiology are not fully understood.⁽²⁾

AD is treated using steroids, antihistamines, immunosuppressive agents, and other medications. However, many studies have indicated that long-term use or even

abuse of these agents may cause various side effects. Consequently, recent studies have focused on complementary therapies based on alternative medicine.⁽⁴⁾

The root of *Astragalus membranaceus* (AM) is one of the most popular health-promoting herbs in East Asia, and has been used in traditional medicine for >2000 years.⁽⁵⁾ The main constituents of AM roots are polysaccharides, saponins, flavonoids, amino acids, and trace elements.⁽⁶⁾ Recently, AM was reported to enhance the levels of interleukin (IL)-4, but to reduce interferon (IFN)- γ secretion in T cell receptor (TCR)-stimulated T cells.⁽⁷⁾ In addition, AM in combination with other

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herbs (the Japanese herbal medicine *Hochu-ekki-to*) is used to treat allergic diseases.⁽⁸⁾ *Hochu-ekki-to* also suppresses dermatitis and serum immunoglobulin G (IgE) levels in NC/Nga mice.⁽⁹⁾ However, *Hochu-ekki-to* contains many different constituents, making it difficult to identify the active component(s).

This study was performed to investigate the protective effects of topical treatment of AM on AD-like skin lesions in BALB/c mice treated topically with 1-chloro-2,4-dinitrobenzene (DNCB) by histopathological examination and measurement of nuclear factor- κ B (NF- κ B) recruitment, serum IgE and cytokine levels.

Materials and Methods

Preparing the AM extract

AM was purchased from Jung-do Herb (Seoul, Korea). A 30-g sample of AM was extracted with 300 mL of 70% ethanol at room temperature for 24 h, and the suspension was filtered. The filtrate was concentrated under reduced pressure and lyophilized. The yield was 26% (dry weight 76.60 g), and the product was stored at 4°C. A voucher specimen (AM001) has been deposited with the Department of Prescriptionology at Kyung Hee University.

AM was characterized based on the content of formononetin (molecular weight 268.26), and determined using reverse-phase high-performance liquid chromatography (HPLC). The HPLC apparatus was a Gilson System equipped with a 234 Autosampler, a UV/VIS-155 detector, and a 321 HPLC Pump (Gilson, Seoul, Korea). A Luna 4.60 $\times$ 250 mm C₁₈ reversed-phase column with 5 μ m particles (Phenomenex, Torrance, CA, USA) was used. The mobile phase consisted of 0.1% acetic acid and acetonitrile (HPLC grade; J.T. Baker Co. Ltd., Phillipsburg, NJ, USA) in a ratio specified by the following binary gradient with linear interpolation: 0 min 30%, 30 min 50%, 50 min 55%. The column eluent was monitored at UV 250 nm, following which all solvents were degassed with a micromembrane filter (PTFE; Advantec, Tokyo, Japan). Chromatography was performed at room temperature at a flow rate of 0.5 mL/min and 10 μ L was analyzed for 50 min.

Animal treatment

BALB/c mice (female, 7 weeks old) were purchased from Dae-Han Bio (Seoul, Korea). The animals were given water and food *ad libitum* throughout the experiment. They were kept at a constant temperature (23°C) and humidity (55%) with a 12-h light/dark cycle in pathogen-free conditions. Animal care and experimental procedures were performed in accordance with the "Guide for the Care and Use of Laboratory Animals" (Department of Health, Education and Welfare, NIH publication #78-23, 1996).

After a 10-day adaptation period, the hair on the upper back was shaved. AD induction was started on the following day (designated as day 0). Mice were

assigned to one of three groups of five mice each: Vehicle (control group); DNCB (negative control group), mice sensitized with DNCB; and AM, mice sensitized with DNCB and treated with AM. The barrier function of the shaved dorsal skin was disrupted by applying 100 μ L of 4% sodium dodecyl sulfate (SDS). Three hours later, the shaved dorsal skin was treated topically with 100 μ L of 1% DNCB in acetone/olive oil 4:1 (vol/vol). These procedures were repeated for 3 days (days 0–2). After 3 days of treatment, the mice were not treated for 5 days (day 3–7). Then, the treatment was repeated with 0.5% DNCB (days 8–16). After the SDS and DNCB treatments, AM group was treated with 1% AM in phosphate-buffered saline (PBS), whereas vehicle and DNCB groups were treated with PBS. The mice were sacrificed on day 17.

Histology

Skin sections were stained with hematoxylin and eosin (H&E) as described previously.⁽¹⁰⁾ to assess the epidermal and dermal hyperplasia by measuring the thicknesses of the epidermis and dermis in all three groups. Briefly, skin specimens of 1 $\times$ 0.5 cm were fixed in 10% neutralized formalin for 24 h, embedded in paraffin, and cut into sections 4- μ m thick. Digital images were obtained at a magnification of $\times$ 200 and the thicknesses were measured using Leica Application Suite (LAS; Leica Microsystems, Buffalo Grove, IL, USA).

Western blot analysis for NF- κ B

Nuclear proteins (40 μ g) were mixed with 2 $\times$ SDS sample buffer, heated at 95°C for 5 min, and separated by SDS-polyacrylamide (10%) gel electrophoresis. The proteins were then electrotransferred to a nitrocellulose membrane. The membrane was blocked with 1% skimmed milk in TBS containing 0.5% Tween 20 (TBST) for 1 h at room temperature (RT). After the membrane was lightly washed with TBST, it was incubated overnight at 4°C with anti-NF- κ B antibody (Ab) (1:1000 dilution in TBST) and then washed three times with TBST. Anti-rabbit IgG, the secondary Ab (1:2000 dilution in the blocking solution), was added, followed by incubation for 1 h at RT, then proteins were visualized using an enhanced chemiluminescence detection reagent (Amersham Pharmacia, Piscataway, NJ, USA).

Determination of serum IgE

Mouse total serum samples were prepared from blood collected by cardiac puncture in heparinized tubes. The blood was centrifuged at 14,000 rpm for 30 min, after which the supernatant and serum were collected. The mouse serum IgE concentrations were quantified using a Mouse IgE ELISA kit (Cat. No. KT-401; Kamiya Biomedical, Seattle, WA, USA), according to the manufacturer's protocol. Serum IgE level was measured by determining the absorbance at 450 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Molecular Devices, Downingtown, PA, USA).

Cytokine determination (IL-4, -5, -6, and -13, and TNF- α)

Cytokine levels in the serum samples were determined using a Milliplex® Map Mouse Cytokine/Chemokine kit (Millipore, Billerica, MA, USA), according to the manufacturer's protocol. Bio-Plex™ 200 (Bio-Rad, Hercules, CA, USA) was used to determine the cytokine levels (IL-4, -5, -6, and -13 and tissue necrosis factor (TNF)- α) in the serum samples. The multiplex cytokine assay was carried out in triplicate, and the data were analyzed using Bio-Plex Manager ver. 4.1.1 (Bio-Rad).

Statistical analysis

Significance was determined by one-way analysis of variance (ANOVA) and nonparametric tests. In all analyses, $P < 0.05$ was taken to indicate statistical significance.

Results

Characterization of AM using HPLC systems

Formonetin was selected as standard materials by AM constituent research, and analyzed with HPLC qualitatively.

The retention times of formonetin itself and formonetin in AM extract were 31.7 min (Figure 1).

Effects of AM on skin lesions and histological changes

None of the animals died during the study and no noticeable adverse effects were observed in any animal. Histological analysis was performed to investigate the effects of treatment on skin thickness. Photographs of typical specimens stained with H&E are shown in Figure 2. The skin thickness of the DNCB-induced mice was increased significantly

compared with the control group. In comparison with the positive control group, the group treated with topical AM had significantly less thickening and hyperplasia of both the epidermis and dermis.

Effects of AM on NF- κ B activation

NF- κ B is an important transcription factor that mediates the transcription of many proinflammatory cytokine genes. In order to investigate the inhibitory effect of AM on NF- κ B activation, NF- κ B activation was analyzed in nuclear protein from dorsal skin tissues. AM treatment significantly suppressed the expression of the NF- κ B ~49% compared to DNCB treatment group (Figure 3A).

Effects of AM on serum IgE level

The total serum IgE level was measured to assess the induction of AD at the end of the experiment. On completion of the second 0.5% DNCB challenge, mice had significantly higher levels of serum IgE (~4.3-fold increase) compared to the vehicle group (Figure 3), implying the successful induction of AD-like immune alteration. The topical application of AM on the DNCB-induced skin showed a tendency to inhibit the increase in total serum IgE, but not significantly (Figure 3B).

Effects of AM on cytokine expression

The serum levels of the cytokines IL-4, -5, -6, and -13, and TNF- α were measured to clarify the inhibitory mechanism of action of AM on the development of dermatitis. DNCB-induced mice showed significantly increased cytokine expression levels compared to the control group. The cytokine levels in the AM-treated mice were not as high, and decreased toward the level of the control

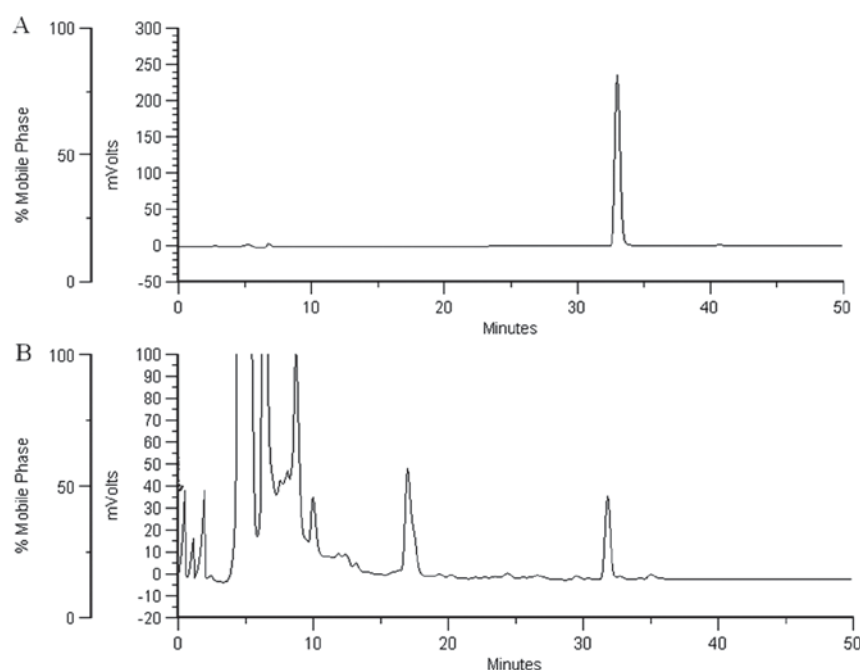


Figure 1. Standardization of *Astragalus membranaceus* (AM) using high-performance liquid chromatography (HPLC) systems. HPLC chromatograms of external standard (A) formonetin and (B) AM.

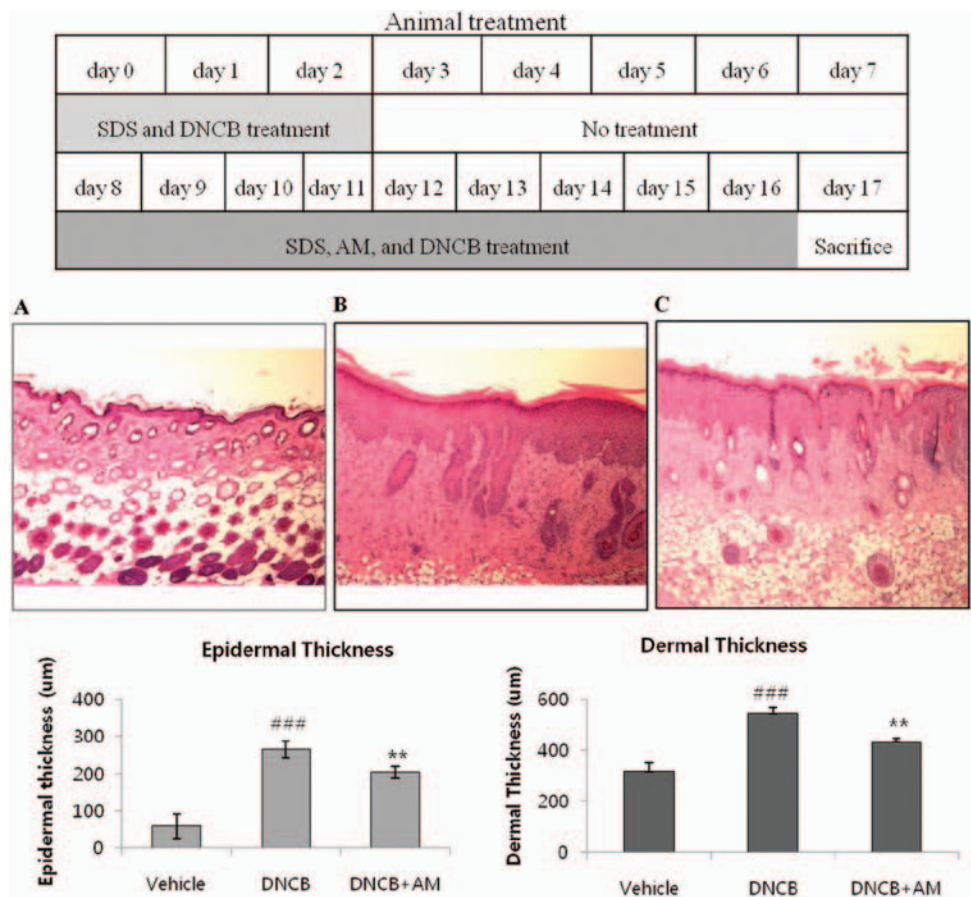


Figure 2. Histopathological findings by hematoxylin/eosin staining of skin sections ($n = 5$, magnification: $\times 200$). No atopic dermatitis induction as (A) normal mice, atopic dermatitis induction as (B) control mice, *Astragalus membranaceus* (AM) topical application following (C) atopic dermatitis induction. Results are presented as mean $\pm$ SD. ###Mean differs significantly between vehicle group and 1-chloro-2,4-dinitrobenzene (DNCB) group ($p < 0.001$). **Mean differs significantly between AM-treated group and DNCB group ($p < 0.01$).

group. Topical AM treatment showed statistically significant inhibition of IL-4, -5, -6, and -13 (Figure 3B).

Discussion and conclusion

This study was performed to examine the effects of AM on DNCB-induced AD. DNCB-sensitized mice develop T-cell-dependent skin inflammation. The DNCB-induced contact allergy is accompanied by changes in cutaneous cytokine production.⁽¹¹⁾ Here, we demonstrated that topical treatment of AM markedly improved the AD skin lesions in BALB/C mice, which were significantly thinner than those in the untreated skin, and the hyperkeratosis disappeared.

NF- κ B is a key transcription factor that regulates the expression of genes involved in immune and inflammatory responses. The activation of the NF- κ B transcription plays an important role in inflammation through its ability to induce the transcription of proinflammatory genes. We found that AM reduced inflammation via suppressing NF- κ B activated by DNCB in AD mice model.

Characteristically, patients with AD have an increased serum IgE level. The serum level is an indicator of

improved AD symptoms.⁽¹²⁾ In the present study, the IgE level tended to decrease in the AM-treated group.

Previous studies of AD have suggested that skin lesions accompanied by topical eosinophilia and systemic IgE elevation are associated with the expression of Th2-type cytokines (IL-4, -5, and -13).⁽¹³⁾ Indeed, the predominant expression of Th2 cytokines has been confirmed in both acute and chronic AD lesions compared with the uninvolved skin of patients with AD and in healthy control subjects.⁽¹⁴⁾ Th1 cells characteristically produce IL-13, a central effector cytokine involved in the development of inflammatory tissue diseases.⁽¹⁵⁾ Th2 cells produce IL-4, which promotes B cell proliferation, IgE class switching, and then augments IgE secretion.⁽¹⁶⁾ Furthermore, IL-5 and -6, which are Th2-type cytokines, enhance IL-4-induced IgE production.⁽¹⁷⁾ Here, we examined the therapeutic effects of topical AM on expression of various cytokines in topical DNCB-treated BALB/c mice as a model of human AD. In the present study, AM suppressed the expression of cytokines IL-4, -5, -6, and -13, indicating that AM is associated with a Th2-mediated pathway.

The plasma TNF- α concentration is increased in AD and the magnitude of the increase is correlated with the severity of dermatitis. TNF- α is produced in keratinocytes,

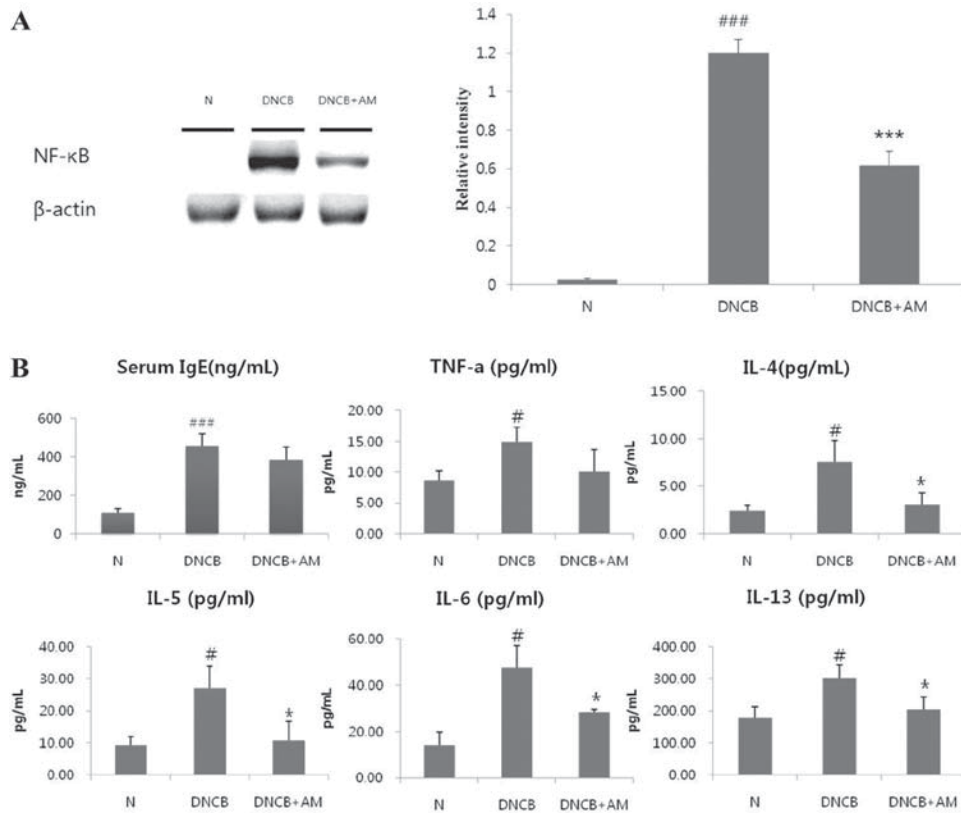


Figure 3. Effects of *Astragalus membranaceus* (AM) on nuclear factor- κ B (NF- κ B) activation, immunoglobulin G (IgE), and cytokines expression. (A) Band image for expression of NF- κ B protein. (B) Serum IgE and cytokines expression. Results are presented as mean $\pm$ S.D. ###Mean differs significantly between vehicle group and 1-chloro-2,4-dinitrobenzene (DNCB) group ($p < 0.05$ and 0.001 , respectively). *Mean differs significantly between AM-treated group and DNCB group ($p < 0.05$).

and may play a regulatory role in dermal-epidermal interactions during wound healing, inflammation, and epidermal growth and differentiation.⁽¹⁸⁾ We also found that AM treatment decreased the level of TNF- α expression.

These data support previous study showing that oral treatment of aqueous extract of AM inhibited dermatitis. Lee et al. reported oral administration of AM ameliorates AD-like skin lesions in ear and dorsal skin and IFN- γ *in vivo*.⁽¹⁹⁾ Based on the results, we demonstrate topical treatment of ethanol extract of AM might be more effective on AD.

In conclusion, topical treatment of AM is effective for treating AD via regulation of NF- κ B activation and cytokine production. AM may be an alternative or complementary therapeutic option for treating patients with AD. Additional detailed studies are required to clarify the molecular mechanisms of action of AM in AD.

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Declaration of interest

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