Evaluation of the Atopic Dermatitis-mitigating and Anti-inflammatory Effects of Kyung Hee Allergic Disease Herbal Formula (KAHF)

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Abstract The purpose of this study was to investigate the effects of Kyung Hee Allergic Disease Herbal Formula (KAHF) on atopic dermatitis (AD) and its mode of action. Our clinical study showed KAHF reduced Severity Scoring of Atopic Dermatitis (SCORAD) indexes and subjective symptom scores. In parallel, the decreased levels of interferon (IFN)-γ and interleukin (IL)-5 in serum, which contributed to its AD-mitigating effect was observed. To reveal the underlying mechanisms of KAHF in AD, its anti-inflammatory effect on lipopolysaccharide (LPS)-induced responses in RAW 264.7 cells was examined. KAHF was found to significantly inhibit the productions of nitric oxide (NO), prostaglandin E_2 (PGE$_2$), and IL-1β in LPS-stimulated RAW 264.7 macrophages. Consistently, KAHF potently inhibited protein and mRNA expressions of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). Furthermore, KAHF inhibited LPS-induced activation of nuclear factor (NF)-κB. Taken together, our data suggest that KAHF has a beneficial effect on several eicosanoid-related skin inflammations, such as atopic dermatitis.

Keywords: atopic dermatitis, anti-inflammatory, prostaglandin E_2, nuclear factor-κB, cytokine

Introduction

Atopic dermatitis (AD) is a common chronic inflammatory skin disease, which commonly occurs during early infancy and childhood (1). Topical steroids that suppress inflammatory reactions are the mainstream of treatment. However, when treatment is protracted, steroid hormones can induce adverse side effects (2). In addition, the increasing prevalence and the long-standing nature of AD demonstrate the need for the development of complementary and alternative therapies.

Complementary or alternative medicine (CAM) approaches, including the use of traditional herbal medicine (THM), have increased at a remarkable pace in Western countries (3). THM originated more than 2000 years ago and it represents cumulative experience acquired over centuries (4). However, strong scientific evidence that supports the efficacy or reveals the mode of action of THM in AD is lacking.

Kyung Hee Allergic Disease Herbal Formula (KAHF) was designed at the Department of Pediatrics, Hospital of Oriental Medicine, Kyung Hee Medical Center and has been used for years to treat of AD, especially for children. KAHF is composed of *Nei-Xiao-San* and *Da-he-zhong-yin* plus other herbs. *Nei-Xiao-San* and *Da-he-zhong-yin* were originally formulated by the outstanding physician *Gong Zhongxian* described in *Wan-Bing-Hui-Chun* (1587 AD) and by *Zhang Jinyue* as prescribed in *Jing-Yue-Quan-Shu* (1624 AD), respectively.

Complex interactions between immunologic triggers

such as cytokines and T cells are involved in the allergic-inflammatory responses of AD (5). T-Helper type 2 (TH2) cells play a key role in humoral immunity, facilitate immunoglobulin E (IgE) synthesis, and favor the differentiation of eosinophilic granulocytes (6). Predominant secretion of interleukin (IL)-5 contributes to the development and differentiation of eosinophils (7-9). TH1 subtype of T cell contributes to cell-mediated immunity by synthesizing interferon (IFN)-γ, which stimulates major histo-compatibility (MHC) class I and II molecule expressions (7). In addition, TH1 cell promotes macrophage activity, known to play an important role in chronic inflammation, resulting in skin hypertrophy in the chronic stage of AD (10).

The purpose of this study was to evaluate the efficacy of KAHF in AD patients as well as to investigate its mode of mechanism in terms of immunological changes in IL-5 and IFN-γ levels in serum. Additionally, we investigated the macrophage cell-based anti-inflammatory activity of KAHF to clarify the underlying mechanism involved.

Materials and Methods

Chemicals Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Life Technologies Inc. (Grand Island, NY, USA). Cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), β-actin, and peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The enzyme immunoassay (EIA) kits for prostagrandin E_2 (PGE$_2$) and IL-1β were obtained from R&D Systems (Minneapolis, MN, USA). RNA extraction kit was purchased from Intron Biotechnology (Seongnam, Korea). iNOS, COX-2, IL-1β, and β-actin oligonucleotide primers were purchased from

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Bioneer (Seoul, Korea). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), aprotinin, leupeptin, phenyl-methylsulfonylfluoride (PMSF), dithiothreitol, triton X-100, *Escherichia coli* lipopolysaccharide (LPS), and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

KAHF decoction or extract preparation The ingredients of KAHF include Crataegi Fructus (20 g), Kochiae Fructus (20 g), Lithospermi Radix (12 g), Sanguisorbae Radix (12 g), Poncirus Fructus (20 g), Raphani Semen (9 g), Angelicae Dahuricae Radix (6 g), Mori Ramulus (6 g), Hordei Fructus Germiniatus (6 g), Agastachis Herba (4 g), Pinelliae Tuber Hoelen (4 g), Magnoliae Cortex (4 g), Aurantii Nobilis Pericarpium (4 g), Alismatis Rhizoma (4 g), Atractylodes Rhizome (4 g), Cyperi Rhizoma (4 g), Amomi Fructus (4 g), Cnidii Rhizoma (4 g), Scutellariae Radix (4 g), Phellodendri Cortex (4 g), Scirpi Rhizoma (2 g), Zedoariae Rhizoma (2 g), Glycyrrhizae Radix (2 g), Saussureae Radix (2 g), Ephedrae Herba (2 g), Wheat Grains (2 g), Semen Armeniacae Amarum (1 g), Herba Artemisiae Annuae (1 g), and Xanthii Fructus (1 g). These ingredients correspond to parts of the following plants; *Crataegus pinnatifida* Bunge (var. major N.E.Br.) (Rosaceae), *Kochia scoparia* (L.) Schrad. (Chenopodiaceae), *Lithospermum erythrorhizon* Siebold & Zucc. (Boraginaceae), *Sanguisorba officinalis* L. (Rosaceae), *Poncirus trifoliata* (L.) Raf. (Rutaceae), *Raphanus sativus* L. (Brassicaceae), *Angelica gigas* Nakai (Apiaceae), *Morus alba* L. (Moraceae), *Hordeum vulgare* L. (Poaceae), *Agastache rugosa* Kuntze (Lamiaceae), *Pinellia ternata* Ten. ex Breitenb. (Araceae), *Poria cocos* (SCHW.) Wolf. (Polyporaceae), *Magnolia officinalis* Rehder & E.H.Wilson (Magnoliaceae), *Citrus aurantium* L. (Rutaceae), *Alisma orientale* (G.Samuelsson) Juzepczuk (Alismataceae), *Atractylodes japonica* Koidz. (Asteraceae), *Cyperus rotundus* L. (Cyperaceae), *Amomum xanthioides* Wall. (Zingiberaceae), *Cnidium officinale* Makino (Apiaceae), *Scutellaria baicalensis* Georgi (Lamiaceae), *Phellodendron amurense* Rupr. (Rutaceae), *Scirpus fluviatilis* A.Gray (Cyperaceae), *Curcuma zedoaria* Rosc. (Zingiberaceae), *Glycyrrhiza uralensis* Fisch. ex DC. (Leguminosae), *Saussurea lappa* C.B.Clarke (Asteraceae), *Ephedra sinica* Stapf in Farwell (Ephedraceae), *Triticum aestivum* L. (Poaceae), *Prunus armeniaca* L. (Rosaceae), *Artemisia capillaris* Thunb. (Asteraceae), and *Xanthium strumarium* L. (Asteraceae), respectively. In addition, 6 g of Velvet antler glue of Siberian deer (*Cervus elaphus sibiricus*) was included (total volume of KAHF weighed 165 g).

Each component was delivered by the Department of Oriental Pharmacy, Kyung Hee Medical Center and authenticated by Prof. Yook Chang-Soo at the Department of Phrmacognosy, Kyung Hee University. A voucher specimen (#KH-57) has been deposited at the Department of Oriental Pharmacy, Kyung Hee Medical Center. Experienced pharmacists who specialize in herbal medicine at the Department of Oriental Pharmacy in Kyung Hee Medical Center prepared the decoction of herbal formulas according to the following steps 1) every ingredient of KAHF was mixed and packed in one sachet (total amount of 165 g); 2) the number of sachets prescribed was the same with children's age; 3) the pharmacists soaked the

total sachets altogether in 7,000 mL water for 30 min, then boiled for 30 min and concentrated to 3,600 mL; 4) while hot, the decoction was divided and packed into 45 small retortable pouches in equal amount (80 mL); 5) the decoctions were stored in a cool place; 6) administration was required 3-6 times/day before or during the meals. For *in vitro* study, the same mixture (total amount of 1,650 g) was extracted twice in boiling water (1,500 mL) for 2 hr. These extracts were filtered and evaporated in a rotary vacuum evaporator and then finally lyophilized with a freezing dryer. A 537.54 g (yield: 32.58%) of extract powder was obtained. The sample was dissolved in phosphate buffered saline (PBS) and sterilized by passing through 0.22-μm syringe filter.

Patients Ten children with AD diagnosed according to Hanifin criteria (11) attending the Department of Pediatrics, Hospital of Oriental Medicine, Kyung Hee University participated in this study. Parents of children or patients were informed about the detail of study and gave written informed consent. This study was approved by the Kyung Hee University Ethical Committee. Patients were excluded if they had concurrent systemic illness except allergic diseases; if they had a history of sensitivity to herbal medicine; if they had received systemic steroids, adreno corticotropic hormone (ACTH), and immuno-suppressant; if they had psoralen ultraviolet A (UVA) treatment in the 8 weeks prior to the start of the study; if they had abnormal renal and liver function at baseline. Children and parents were instructed to maintain their current diet and treatments during the study.

During the initial visit, a detailed medical history including diet, emollient, and drug use as well as the measurement of serum total IgE and eosinophil counts in the peripheral blood were taken. Patients were supplied with KAHF for 45 days. One pouch of KAHF decoction (approximately 80 mL) was drunk daily. Assessment of AD severity, laboratory safety assays (AST, ALT, BUN, and creatinine), and serum cytokine levels were investigated at the first visit and the end of the treatment period. Parents were asked to keep a diary to record any troublesome symptom.

Assessment of AD severity Severity of AD was assessed by Severity Scoring of Atopic Dermatitis (SCORAD) index (12).

Serum total IgE analysis and eosinophil counts in the peripheral blood Serum IgE taken from venous blood was analyzed by using Abbott IgE EIA kit (Abbott Laboratories, Chicago, IL, USA). The number of eosinophil in the peripheral blood was counted by an automated hematology analyzer (Neu-Bauer chamber) and expressed as numbers/mm^3.

Serum cytokine analysis Serum IL-5 and IFN-γ were quantified using EIA kits according to manuals provided by the manufacturers (R&D Systems, Minneapolis, MN, USA).

Cell culture and sample treatment RAW 264.7 murine macrophage cell line was obtained from the Korean Cell

Line Bank (Seoul, Korea). These cells were grown at 37°C in DMEM medium containing 10% heat-inactivated FBS, penicillin (100 units/mL), and streptomycin sulfate (100 μg/mL) in a humidified atmosphere of 5% CO_2. The cells were incubated with KAHF at various concentrations (50, 100, 200, and 400 μg/mL) or positive chemicals and stimulated with LPS 1 μg/mL.

MTT assay for cell viability Cell respiration as an indicator of cell viability was determined on the basis mitocondrial-dependent reduction of MTT to formazan as described in our previous study (13).

Nitrite, PGE₂, and IL-1β assay The nitrite which accumulated in culture supernatant was measured as an indicator of nitric oxide (NO) production according to the Griess reagent and as described in our previous study (13). PGE₂ and IL-1β levels in macrophage culture medium were quantified using EIA kits according to the manufacture's instructions (R&D Systems).

Western blot analysis and reverse transcription-polymerase chain reaction (RT-PCR) Western blot analysis was performed by using anti-iNOS, anti-COX-2, and anti-β-actin antibody as previously described (13). Total mRNA was isolated using Easy Blue® kits (Intron Biotechnology) and subsequent procedures were done according to the manufacturer's instructions and as described in our previous study (13).

Electrophoretic mobility shift assay (EMSA) EMSA was performed as previously described (13).

Statistical analysis In clinical study, statistical significance was calculated by Wilcoxon Signed Ranks test. All calculations were performed by SPSS for windows, version 8.0 (SPSS Inc., Chicago, IL, USA). All comparisons were 2-tailed, differences were considered statistically significant for values of $p<0.05$. *In vitro* study, statistical analysis was performed by analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Figures in this article were obtained from at least 3 independent experiments with similar patterns. Results are expressed as the means± standard deviation (SD).

Results and Discussion

Clinical results Several studies support the belief that atopic states are characterized by enhanced serum IgE levels and peripheral eosinophilia. Activated serum IgE

Table 1. Baseline characteristics of subjects with atopic dermatitis[1]

Gender (n, male/female)	4/6
Age (year)	9.77±3.92
Onset of AD (year)	2.35±2.49
Past history of allergic disease (n)	10
Family history of allergic disease (n)	9
Serum total IgE (IU/mL), mean (minimum-maximum)	628 (32-2,130)
Eosinophil counts in peripheral blood (/mm³), mean (minimum-maximum)	494 (100-2,270)

[1]Data including diet, emollient use, and drug use as well as the measurement of serum total IgE and eosinophil counts in the peripheral blood of 10 children diagnosed as atopic dermatitis according to Hanifin criteria were analyzed.

production and its distribution systemically through the circulatory system, and promoted eosinophil infiltration into inflamed tissues are the hallmarks of the allergic inflammation (8,16). In addition, the roles of IgE and of eosinophil-mediated allergic inflammation in AD have been revealed in connection with biphasic T cell response in skin (5,17,18). TH2 cytokines are markedly upregulated in lymphocytes infiltrating acute AD lesions and this upregulation promotes eosinophil influx and infiltration (19-21). However, in chronic AD, eosinophil and macrophage infiltration is associated with a switch to TH1 cellular response where cells primarily express IFN-γ, which suggests that the maintenance of chronic inflammation is associated with IFN-γ up-regulation (22,23). In the present study, the chronic AD was present in the subjects (Table 1). Considering these together with characteristic sudden and repetitious relapses in AD, it is reasonable to speculate that both TH2 cytokines (IL-5) and TH1 (IFN-γ) play critical roles in AD. Similarly, children with AD were found to show increased cytokine secretion such as IL-5 and IFN-γ in serum (24). Further, recent evidences indicate that TH2 and TH1-type cytokines in AD are not mutually inhibitory, and suggest that both TH2 and TH1 cells contribute to the pathogenesis of AD (25,26). These findings also support the importance of IL-5 and IFN-γ as key pathogenic factors in the occurrence of AD.

The present study demonstrates that enhanced serum total IgE levels and eosinophilia beyond the normal range (27,28) may be associated with a systemic allergic-inflammatory response (Table 1). Clinical improvement was observed in 10 children as suggested by significant reductions of SCORAD and subjective symptom scores. In parallel, marked decrease was observed in serum IL-5 and

Table 2. Outcome measures of atopic dermatitis severity (SCORAD index), subjective symptom severity of sleep loss and pruritis, and serum cytokine level (IL-5 and IFN-γ) at baseline and end-point

	Baseline	End-point	p value[1]
SCORAD index[2]	44.10±19.11	33.00±17.08	0.028
Subjective symptoms (sleep loss & pruritis)	12.40±5.02	8.50±5.87	0.034
IL-5 (pg/mL)	6.77±1.54	5.86±0.68	0.047
IFN-γ (pg/mL)	15.00±2.48	13.06±1.10	0.008

[1]All comparisons were 2-tailed, differences were considered statistically significant for values of $p<0.05$.
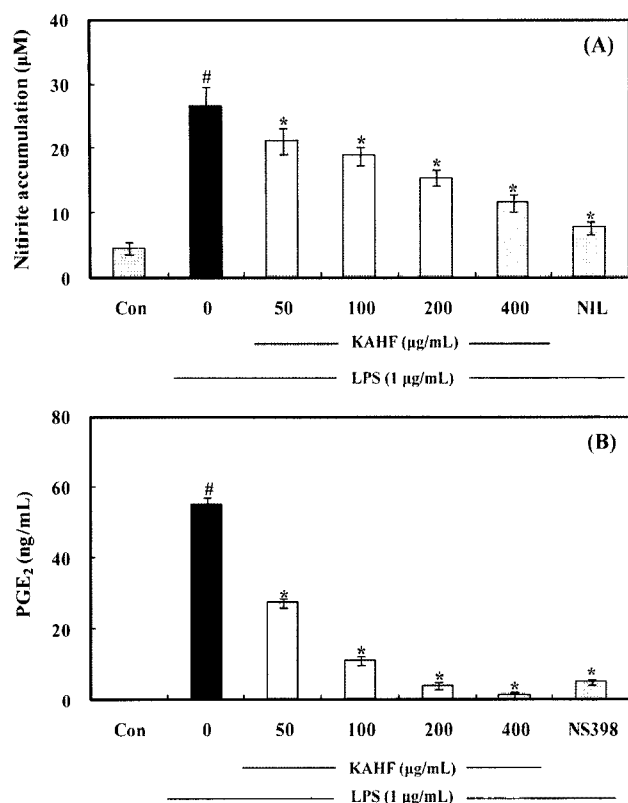[2]Severity Scoring of Atopic Dermatitis index.

Fig. 1. Effect of Kyung Hee Allergic Disease Herbal Formula (KAHF) on nitrite (A) and prostaglandin E₂ (PGE₂) (B) productions on lipopolysaccharide (LPS)-induced RAW 264.7 cells. Results are expressed as the means±SD (*$p<0.05$: compared to LPS-treated group).
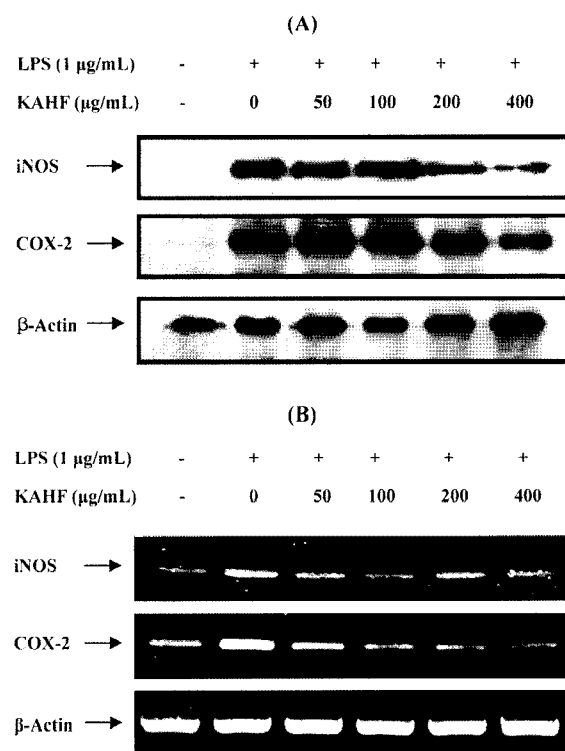

Fig. 2. Effect of Kyung Hee Allergic Disease Herbal Formula (KAHF) on lipopolysaccharide (LPS)-induced inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) protein (A) and mRNA (B) expressions in RAW 264.7 Cells. Data represent 1 of 3 similar results.

IFN-γ levels after KAHF administration (Table 2), which may have contributed to the AD-mitigating effect of KAHF. Two children complained about the taste, but no evidence of liver or kidney toxicity was observed (data not shown).

Inhibitory effect of KAHF on LPS-induced NO and PGE₂ production To explore the underlying mechanisms involved, we turned our attention to the anti-inflammatory effect of KAHF on macrophages, one of the most important effecter cells in the immune system (29). Macrophages are also one of the predominant cell types in chronic AD and are known to play a role in the chronic inflammatory responses of the skin (30,31). With regard to *in vitro* studies, PGE₂ was found to promote IgE production by B lymphocytes, indicating the importance of PGE₂ in the pathologies of various IgE-mediated allergic diseases (32). Furthermore, PGE₂ was found to stimulate the synthesis of TH2 cytokines (33), which are important for the initiation and maintenance of allergic response (10). NO not only aggravates erythema and edema in AD by stimulating vasodilation (34), but also plays an important regulatory role in immune response, such as, by regulating the expansion of the TH1 cell population (35).

Thus, the effect of KAHF on NO and PGE₂ synthesis in RAW 264.7 macrophages was studied and KAHF was found to have a dose-dependent inhibitory effect on LPS-induced NO production. L-N⁶-(1-Iminoethyl)lysine (L-NIL) 10 μM, a competitive inhibitor of iNOS, was used as

a positive inhibitor. Similarly, the production of PGE₂ was also significantly inhibited by KAHF in a dose-dependent manner as shown in Fig. 1B. Additionally, cell viability was determined by the MTT assay to exclude the possibility that the inhibitory effect of KAHF was due to cytotoxicity. The viability of LPS-induced RAW 264.7 cells treated with or without KAHF at different concentrations was >94% (data not shown).

Inhibitory effect of KAHF on iNOS and COX-2 To elucidate the mechanism involved in the inhibitions of NO and PGE₂ generation by KAHF in LPS-induced macrophages, we further studied the effects of KAHF on iNOS and COX-2 protein and gene expression. In LPS-stimulated macrophages, the expression of iNOS was markedly increased and KAHF significantly and dose-dependently inhibited iNOS protein and mRNA induction (Fig. 2A and 2B). A similar pattern was observed when the effect of KAHF on LPS-induced COX-2 expression was examined (Fig. 2A). Under the same conditions, COX-2 mRNA levels were also significantly reduced in a similar pattern (Fig. 2B). These findings suggest that the inhibition of NO and PGE₂ production by KAHF is due to the suppression of the LPS-induced expressions of iNOS and COX-2 mRNA, respectively.

Inhibition of LPS-induced IL-1β production by KAHF Since KAHF was found to be a potent inhibitor of pro-inflammatory mediators, we further investigated the effect of KAHF on LPS-induced IL-1β release by EIA and RT-

(A)

KAHF (μg/mL)

LPS (1 μg/mL)

(B)

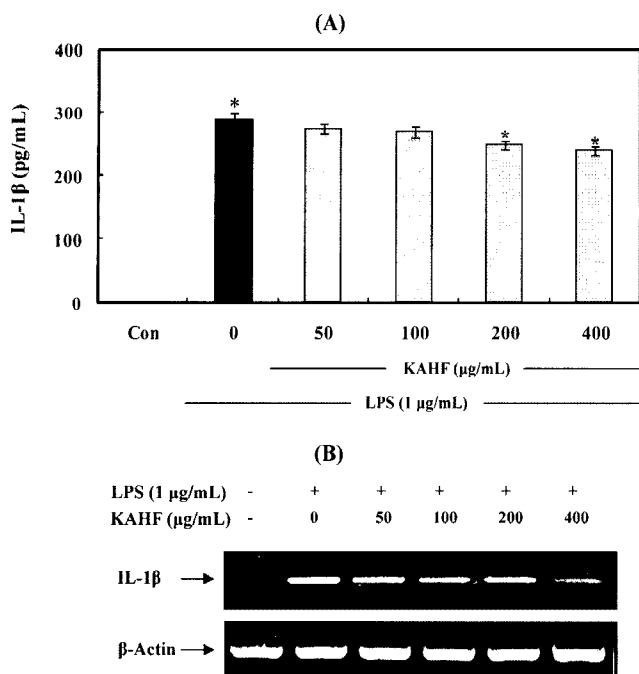| LPS (1 μg/mL)	-	+	+	+	+	+
KAHF (μg/mL)	-	0	50	100	200	400

IL-1β →

β-Actin →

Fig. 3. Effect of Kyung Hee Allergic Disease Herbal Formula (KAHF) on lipopolysaccharide (LPS)-induced interleukin (IL)-1β release (A) and mRNA expression (B) in RAW 264.7 cells. Results are expressed as the means±SD ($*p<0.05$: compared to LPS-treated group).

LPS (1 μg/mL)	-	+	+	+	+	+
KAHF (μg/mL)	-	0	50	100	200	400

p65 →

Free probe →

Fig. 4. Inhibition of nuclear factor-κB (NF-κB)-DNA binding by Kyung Hee Allergic Disease Herbal Formula (KAHF). Nuclear extracts were prepared and analyzed for NF-κB binding to DNA by electrophoretic mobility shift assay (EMSA). The arrow indicates the position of NF-κB. Data represent 1 of 2 similar results.

PCR. IL-1β is one of the pro-inflammatory cytokines that regulates multiple aspects of immune and inflammatory responses and enhances the activation and proliferation of T cells (36). Critically, IL-1β is known to stimulate IFN-γ production via IL-12 activation (37). In the present study, pretreatment of RAW 264.7 cells with KAHF for 1 hr decreased both IL-1β production and mRNA expression in a dose-dependent manner (Fig. 3A and 3B).

Inhibitory effect of KAHF on NF-κB-DNA binding
To further investigate the mechanism underlying the KAHF-mediated inhibition of the transcriptions of iNOS, COX-2, and IL-1β, we focused on NF-κB, a well-known transcription factor of iNOS, COX-2, and IL-1β (14,15). NF-κB is an important modulator of allergic inflammation, playing a central role in the progression and maintenance of AD and in the transactivation of genes encoding cytokine and adhesion molecules in T and B cell development (38). Therefore, the inhibition of the NF-κB pathway is expected to have a substantial therapeutic potential for the treatment of several inflammatory diseases including AD (39).

EMSA analyses demonstrated a reduction in NF-κB-DNA binding activity in nuclear extracts obtained from LPS-activated RAW macrophages treated with KAHF (50, 100, 200, and 400 μg/mL) (Fig. 4). Moreover, the extent of this reduction was in a similar range to reductions observed in iNOS and COX-2 expressions at the protein and mRNA levels.

Taken all together, these findings suggest that the anti-inflammatory characteristics of KAHF may underlie its immunomodulatory and AD-mitigating effects.

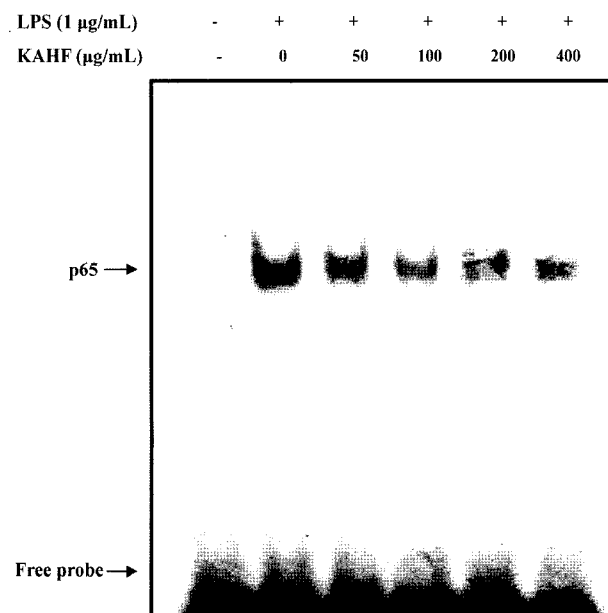Some herbs in KAHF possess anti-inflammatory properties. Lithospermi Radix (root of *Lithospermum erythrorhizon*

Siebold. et Zuccarinii) has an anti-inflammatory effect as demonstrated by its inhibitory effect on NO and tumor necrosis factor (TNF)-α release from macrophages via inhibition of NF-κB activation (40). Shikonin, one of the active components of the roots Lithospermi Radix, significant dose-dependent inhibition of TNF-α promoter activation (41). In addition, Kochiae Fructus (the fruits of *Kochia scoparia*) inhibited LPS-induced NO, PGE₂, and TNF-α release by blockading NF-κB activation (42). Kochiae Fructus is also known to have a peripheral anti-nociceptive effect mediated by its anti-inflammatory action (43,44). Generally, herbal formulas are mixtures of several medicinal herbs. Since all components are boiled together during decoction, interactions are likely to occur. Therefore, the effects of KAHF may differ from the combined effects of individual herbs. However, it is noteworthy that the water extract of KAHF, a widely used herbal formula in the clinical setting, also exerted an anti-inflammatory effect. Concerning interactions between herbs in a traditional formula, a multi-targeting theory was proposed, i.e., in combination, multiple components are expected to repeatedly interact with the patho-physiological mechanisms of a disease while maximizing therapeutic effects via synergistic actions and ameliorating potential adverse effects. This multi-targeting theory may help explain the effectiveness of herbal formulas for the treatment of heterogenous disorders (45-47) including AD.

Based on the observed AD-mitigating and immunomodulatory effects of KAHF in association with its anti-inflammatory property, we propose that KAHF be considered for clinical use as a treatment for AD in children. Further research on the underlying mechanism of KAHF and methodologically sound, well-controlled clinical trials are required to investigate its clinical efficacy.

Acknowledgments

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