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Original

An Atopic Preventive Drink (APD) reduces Th2 cytokines in LPS-treated RAW 264.7 cells

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ABSTRACT

We analyzed the effects of an Atopic Preventive Drink (APD) on the regulation of Th2 cytokines using RAW 264.7 macrophage cells. In the evaluation of nitric oxide (NO) production in cells, NO production levels were shown to be elevated only in the APD-treated group in a dose-dependent manner. In the lipopolysaccharide (LPS) with APD-treated group, NO production significantly decreased as APD concentration increased. Further, mRNA expression levels and protein concentrations of pro-inflammatory cytokines in cells were determined. Th2 stimulatory cytokine (IL-1β) and Th2 cytokine (IL-6 and IL-10) levels were significantly reduced in the LPS with APD-treated group compared to the only LPS-treated group. mRNA expression levels of inflammatory-related genes (COX-2 and iNOS) were significantly reduced in the LPS with APD-treated group compared to the only LPS-treated group. These results suggest that APD has an anti-atopic effect by reducing mRNA and proteins expressions of Th2 cytokines and inflammatory-related genes.

Keywords Atopic Preventive Drink, Th2 cytokines, lipopolysaccharides, RAW 264.7 cells

INTRODUCTION

Atopy is a chronic inflammatory disease showing steadily increasing incidence in recent years (Yamamoto et al., 2009). Atopic diseases are mostly exogenous and caused by immune mechanisms associated with Immunoglobulin E (IgE), which is produced by B cells (Tokura, 2010). B cells are regulated by helper T cells (Th), which regulate inflammatory and immune responses (Michele et al., 2006).

Th cells can be classified according to the type of cytokine they produce. Type 1 T helper cells (Th1) produce TNF-α, IFN-γ, and IL-12 while type 2 T helper cells (Th2) produce IL-4, IL-5, IL-6, and IL-10. Upon allergen exposure, naïve T cells (Th0) differentiate into Th1 or Th2, which are involved in immune responses. B cells are stimulated by cytokines produced by Th2. Therefore, the atopic reaction occurs within the dominant environment of Th2 (Grewe et al., 1998; Tokura, 2010). Meanwhile, IL-1β is a cytokine that stimulates Th2, thereby promoting cytokine production by Th2 (Helmby and Grencis, 2004). Therefore, IL-1β and Th2 cytokines can be considered as important markers for the atopic reaction.

In the case of atopic disease, the migration of NF-κB into the nucleus is stimulated due to the ubiquitination of IκB in the macrophage. As a result, the expression of iNOS, the target gene of NF-kB, is stimulated, resulting in the production of large amounts of NO, which results in aggravation of the inflammatory response (Rowe et al., 1995). Thus, an analysis of NO concentration may be a good marker for analyzing atopic response levels.

Atopic Preventive Drink (APD) is a mixture drink composed of Taemyeongcheong extract, which is a Korean traditional health drink (Yi et al., 2015), *Rumex crispus*, *Cordyceps sinensis*, and other herbs (Ryu et al., 2015). APD undergoes a Korean traditional processing method called Beopje (Yi et al., 2015). Beopje is a repeated process involving heating and steaming for a certain time in order to neutralize toxicity and improve efficiency of drug efficacy. Experimental studies on APD have reported effects such as colitis protection, hepatitis protection, and obesity inhibition (Yi et al., 2015).

These results are probably due to regulation of the immune activities of the Beopje-processed ingredients of APD, including herbs and Taemyeongcheong extract. Thus, in this study, we investigated the immune regulatory effects of macrophages on reduction of Th2 cytokines to confirm the anti-atopic effect of an APD sample.

MATERIAL AND METHODS

APD sample preparation

APD were provided by the Gawha wellfood Co. (Jincheon, Chungcheongbuk-do, Korea). APD was composed of the following ingredients: *Rumex crispus* (13.62%), *Camellia Sinensis* (11.14%), *Saururus chinensis* (11.14%), *Viscum album* (11.14%), *Houttuynia cordata* (11.14%), *Atractylodes ovata* (9.90%), *Capsella bursa-pastoris* (8.67%), *Cornus officinalis* (8.67%), *Phyllostachys bambusoides* leaf (7.43%), *Cordyceps sinensis* (6.19%), and Taemyeoncheong extract (Ryu et al., 2015; Yi et al., 2015). Ingredients of APD were processed

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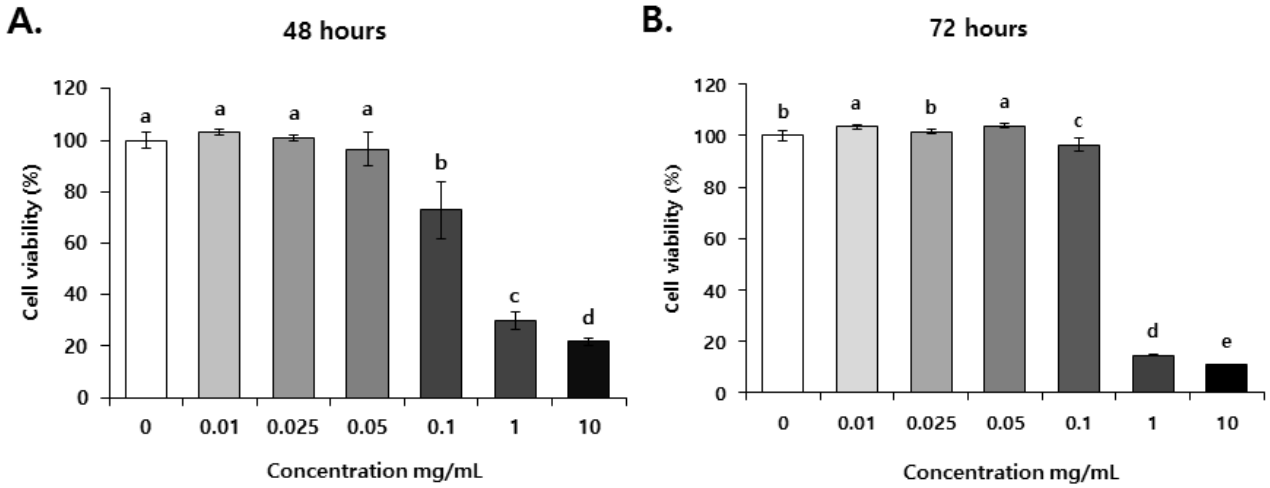

Fig. 1. Effect of APD on viability of RAW 264.7 cell line. The cells were induced with various concentrations (0, 0.01, 0.025, 0.05, 0.1, 1, 10 mg/mL) for 48 (A) and 72 (B) hours. Data were presented as the means ± SDs. [a-e] means with the different letters on the bars are significantly different ($p < 0.05$) by Duncan's multiple range tests.

by the Korean traditional processing method Beopje (Yi et al., 2015). Prepared APD was freeze-dried at -80° C and stored at -4° C for until further experiments.

Cell culture

RAW 264.7 macrophages were purchased from the Korean Cell Line Bank (KCLB), Seoul, Korea. The RAW 264.7 cells were cultured in Dulbeco's Modified Eagle's Media (DMEM), supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin solution, and incubated at 37℃ with 5% carbon dioxide. Cultured cells were refed two to three times a week.

Cell viability analysis (MTT assay)

Cultured RAW 264.7 cells were plated in 96-well plates to a concentration of 1.5×10^4 cells/mL per well for 24 h and live cells were counted by using a cell counter (Luna automated cell counter; Logos Biosystems, Gyunggi-do, Korea). After the culture, medium was removed and new medium with various concentrations of samples and LPS (2 μg/mL) were added to each well, followed by incubation for 48 and 72 h. After incubation, medium was removed, and MTT solution prepared at a concentration of 500 μg/mL with PBS was mixed with medium and incubated for 4 h. After incubation, the formazan crystals were dissolved in DMSO, and absorbance was measured at 550 nm by using an Wallac Victor3 1420 Multilabel Counter (Perkin-Elmer, Wellesley, MA) (Skehan et al., 1990).

NO concentration

The nitrite concentration was measured by the Griess method (Khan et al., 2009). Cultured RAW 264.7 cells were plated in 96-well plates to a concentration of 1.5×10^4 cells/mL for 24 h and live cells were counted by using a cell counter (Luna automated cell counter; Logos Biosystems, Gyunggi-Do, Korea). After incubation, DMEM without FBS and penicillin-streptomycin was treated for starvation for 24 h. After the culture, the medium was removed. New medium with various concentrations of samples and LPS (2 μg/mL) was then added to each well, followed by incubation for 48 and 72 h. Thereafter, cell media were collected, treated with the same amount of griess reagent, and absorbance measured at 550 nm with an Wallac Victor3 1420 Multilabel Counter (Perkin-Elmer, Wellesley, MA).

Real-Time quantitative Polymerase Chain Reaction (RT-qPCR) assay

To determine the mRNA expression levels of inflammatory cytokines in RAW 264.7 murine macrophages, we performed RT-qPCR. Cultured RAW 264.7 cells were plated in 6-well plates to a concentration of 1.5×10^6 cells/mL for 24 h and live cells were counted by using a cell counter (Luna automated cell counter; Logos Biosystems, Gyunggi-do, Korea). After the culture, medium was removed. New medium with 0.1 mg/mL of samples and LPS (2 μg/mL) was then added to each well, followed by incubation for 48 and 72 h. After incubation, medium was removed, and total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The isolated RNA was dissolved in DEPC water and quantified using NanoDrop ND-1000 (NanoDrop Technologies Inc., Wilmington, DE). Dissolved RNA was used to synthesize first-strand cDNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Synthesized cDNA was amplified using a thermal cycler BioRad CFX-96 real time system (BioRad, USA) under the following conditions: 50 cycles of 95℃ for 15 s and 60℃ for 40 s. Amplified products were detected by measuring the fluorescence produced by the FIREPol PCR Mix (Solis BioDyne, Tartu, Estonia). Primers for 18s rRNA, IL-1β, IL-6, IL-10, iNOS, and COX-2 were purchased from Bioneer Corp. (Daejeon, Korea). PCR was performed using the following primers: 18s rRNA forward 5'-TCG AGG CCC TGT AA TTG GAA-3' and reverse 5'-CCC TCC AAT GGA TCC TCG TT-3', IL-1β forward 5'-AAGGGCTGCTTCCAAAC-3' and reverse 5'-CTC CAC AGC CAC AAT GA-3', IL-6 forward 5'-ATG AAG TTC CTC TCT GCA A-3' and reverse 5'-AGT GGT ATC CTC TGT GAA G-3', IL-10 forward 5'-CCA AGC CTT ATC GGA AAT GA-3' and reverse 5'-TTT TCA CAG GGG AGA AAT CG-3', iNOS forward 5'- ATG GCT TGC CCC TGG AA-3' and reverse 5'-TAT TGT TGG GCT GAG AA-3', COX-2 forward 5'-GGC AGC AAA TCC TTG C-3' and revers 5'-TAT TGT TGG GCT GAG AA-3'.

Enzyme-Linked Immunosorbent Assay (ELISA)

Cultured RAW 264.7 cells were plated in 6-well plates to a concentration of 1.5×10^6 cells/mL for 24 h and live cells were counted by using a cell counter (Luna automated cell counter; Logos Biosystems, Gyunggi-do, Korea). After the culture,

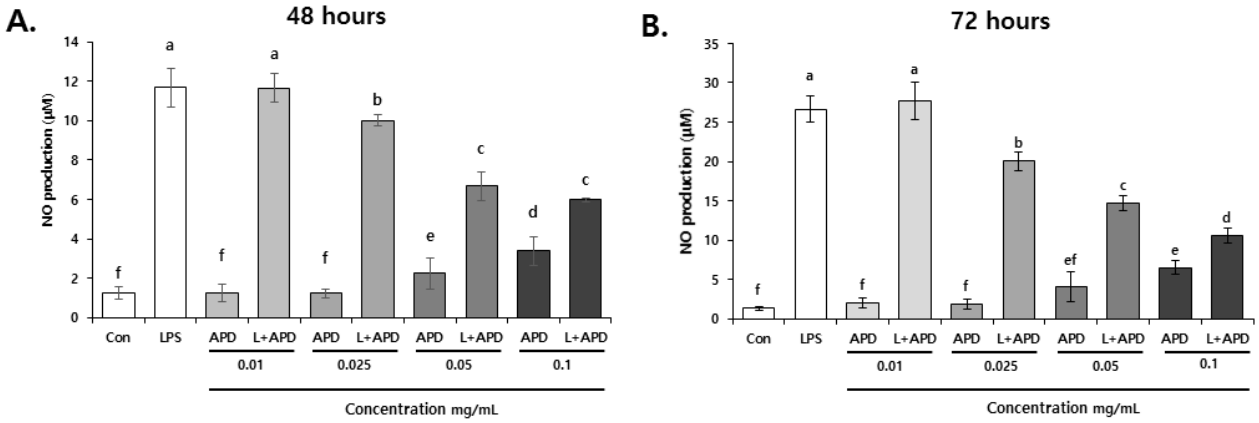

Fig. 2. Effect of APD on NO production in LPS-stimulated RAW 264.7 cell line. Con, cells treated with media; LPS, cells treated with 2 μg/mL LPS; APD, cells treated with 0.1 mg/mL APD; L+APD, cells treated with 2 μg/mL LPS and 0.1 mg/mL APD. The cells were induced with various concentrations of APD (0.01, 0.025, 0.05, 0.1, 1, 10 mg/mL) for 48 (A) and 72 (B) hours. Data were presented as the means ± SDs. [a-f] means with the different letters on the bars are significantly different ($p < 0.05$) by Duncan's multiple range tests.

medium was removed. New medium with 0.1 mg/mL of samples and LPS (2 μg/mL) was then added to each well, followed by incubation for 48 and 72 h. After incubation, the medium was collected and analyzed using a Wallac Victor3 1420 Multilabel Counter (Perkin-Elmer, Wellesley, MA). Concentrations of IL-1β and IL-6 were measured using appropriate ELISA kits (BioLegend, San Diego, CA). This experiment was performed according to the manufacturer's suggested method.

Statistical Analysis

RT-qPCR results were presented as means ± SEs, and other results were presented as means ± SDs. One-way analysis of variance (ANOVA) and Duncan's multiple range tests were used to determine the significances of intergroup differences. Student's t-test was used to determine significance of the difference between the two groups, and analysis was performed using SPSS v23 statistical analysis software (SPSS Inc.). Values of $p < 0.05$ were considered statistically significant.

RESULTS

Effects of APD on survival of RAW 264.7 cells

In the case of treatment for 48 h (Fig. 1A), there was no significant difference in cell survival at APD concentrations of 0.01 to 0.05 mg/mL versus the untreated control group, and it was confirmed that cell viability was 72.9 ± 11.2% at a concentration of 0.1 mg/mL. On the other hand, cells treated for 72 h (Fig. 1B) at concentrations of 0.01 and 0.05 mg/mL showed a slight increase in cell viability. For the groups treated with 0.1 mg/mL of APD, survival rate slightly decreased to 96.3 ± 2.6%.

Effects of APD on NO production in LPS-stimulated RAW 264.7 cells

NO production levels of the LPS-treated group were significantly elevated compared to the untreated control after 48 and 72 h of treatment ($p < 0.05$) (Fig. 2A, B.). In the case of the APD without LPS treatment groups, levels of NO production increased in a dose-dependent manner. On the other hand, levels of NO production in the LPS with APD-treated group decreased from 11.6 ± 0.72 μM to 5.6 ± 0.09 μM after 48 h and from 27.7 ± 2.4 μM to 10.6 ± 0.9 μM after 72 h as APD concentration increased.

Effects of APD on mRNA expression levels of Th2 cytokines in RAW 264.7 cells

Cells were treated with each sample for 48 h to evaluate gene expression. Gene expression of IL-1β (Fig. 3A.), a cytokine that stimulates Th2 cells (Helmby and Grencis, 2004), was significantly down-regulated in the APD with LPS-treated group compared to the LPS group after 48 h of incubation ($p < 0.05$). Likewise, in the LPS group, expression levels of IL-6 and IL-10, a Th2 cytokine (Fig. 3B, C.), were significantly elevated compared to the controls ($p < 0.05$). On the other hand, the APD and LPS-treated group showed significantly decreased Th2 cytokine expression levels compared to the LPS group ($p < 0.05$).

Effects of APD on production of Th2 cytokines in RAW 264.7 cells

Similar to the gene expression experimental results, IL-1β

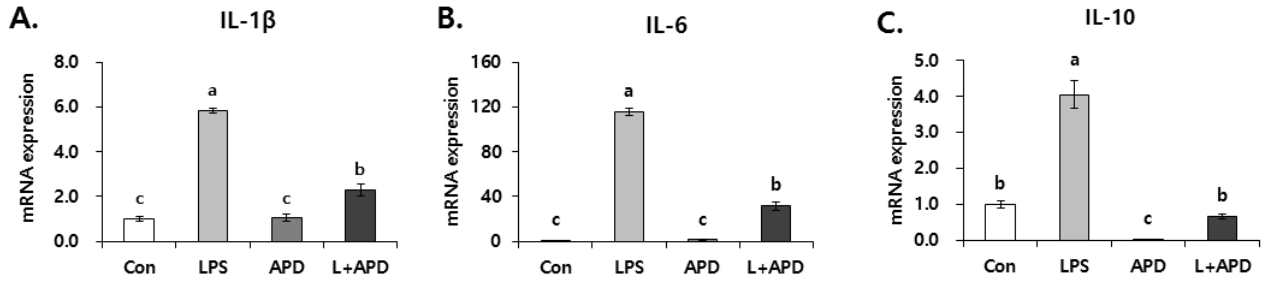

Fig. 3. Effect of APD (0.1 mg/mL, 48 h) on mRNA expression of Th2 cytokines in RAW 264.7 cell line. Con, cells treated with media; LPS, cells treated with 2 μg/mL LPS; APD, cells treated with 0.1 mg/mL APD; L+APD, cells treated with 2 μg/mL LPS and 0.1 mg/mL APD. The mRNA expression levels were calculated based on mRNA 18s rRNA, which was used as a control gene. [a-c] means with the different letters on the bars are significantly different ($p < 0.05$) by Duncan's multiple range tests.

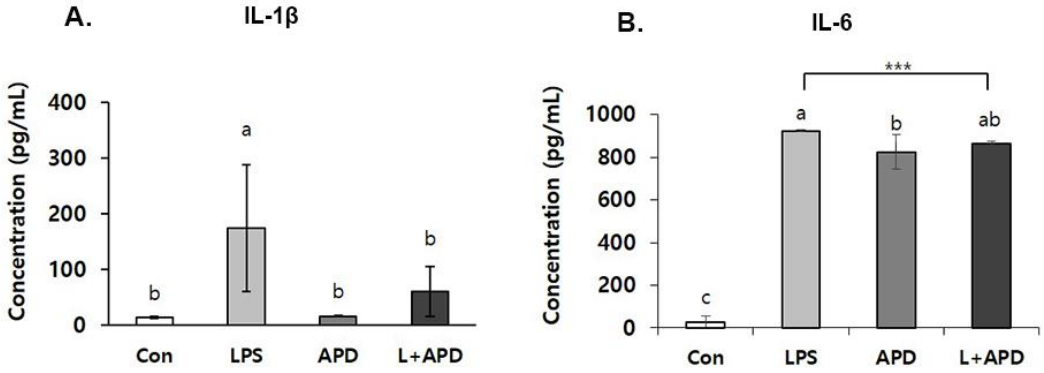

Fig. 4. Effect of APD (0.1 mg/mL, 48 h) on Th2 cytokine concentrations in cell media of RAW 264.7 cell line. Con, cells treated with media; LPS, cells treated with 2 μg/mL LPS; APD, cells treated with 0.1 mg/mL APD; L+APD, cells treated with 2 μg/mL LPS and 0.1 mg/mL APD. [a-c] means with the different letters on the bars are significantly different ($p < 0.05$) by Duncan's multiple range tests. *** means with the significant difference $p < 0.001$ by Student's *t*-test.

concentration of the only LPS-treated group was 174.3 ± 113.7 pg/mL, which was significantly higher than those of the untreated control group (14.5 ± 1.1 pg/mL) and the only APD-treated group (16.4 ± 1.1 pg/mL) after 48 h of incubation (Fig. 4A, B.). The IL-1β concentration of the APD-treated group with LPS was 61.0 ± 45.3 pg/mL, which was lower than that of the LPS group ($p < 0.05$).

The concentration of IL-6 in the only LPS group was 923.1 ± 3.9 pg/mL, which was significantly higher than that of the untreated control group (27.3 ± 27.1 pg/mL). The concentration of IL-6 in the APD-treated group without LPS was 823.5 ± 80.4 pg/mL while that in the APD-treated group with LPS was 862.9 ± 12.0 pg/mL, which were slightly lower compared to the LPS group ($p < 0.05$).

Effects of APD on mRNA expression levels of inflammatory genes in RAW 264.7 cells

In RAW 264.7 cells treated with only LPS, expression levels of COX-2 and iNOS were elevated compared to the control after 48 h ($p < 0.05$), whereas mRNA expression levels in the APD-treated group without LPS were significantly reduced. In the LPS with APD-treated groups, expression levels were significantly reduced compared to the LPS group (Fig. 5 A, B.).

DISCUSSION

In this study, we investigated the ability of APD to inhibit atopic symptoms and regulate inflammatory processes. NO is produced by immune cells such as macrophages, dendritic cells, and neutrophils, and NO plays important roles in regulation of cytokines and proliferation of Th cells (Bogdan, 2001). Therefore, NO is used as a marker of atopic inflammation. In this study, NO production was elevated in the APD-treated group without LPS depending on concentration. Thus, inflammation was induced in the APD-treated group, whereas it was reduced in the APD and LPS-treated group. These results suggest that the inflammatory responses were partially regulated by APD.

In this study, mRNA expression levels and concentrations of Th2 stimulatory (IL-1β) and Th2 (IL-6 and IL-10) cytokines were reduced in the LPS with APD-treated group compared to the only LPS-treated group. According to another study on allergic diseases such as atopy, elevated expression of inflammatory Th2 cytokines was shown to be associated with increased IgE production levels compared to the non-allergic control group, leading to itching, erythema, and urticaria (Won et al., 2011). Meanwhile, IL-1β functions as a stimulator of Th2

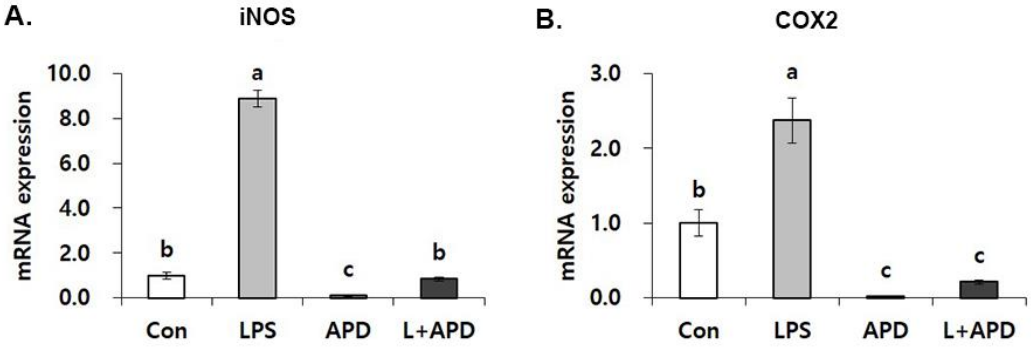

Fig. 5. Effect of APD (0.1 mg/mL, 48 h) on mRNA expression of inflammatory-related genes in RAW 264.7 cell line. Con, cells treated with media; LPS, cells treated with 2 μg/mL LPS; APD, cells treated with 0.1 mg/mL APD; L+APD, cells treated with 2 μg/mL LPS and 0.1 mg/mL APD. The mRNA expression levels were calculated based on mRNA 18s rRNA, which was used as a control gene. [a-c] means with the different letters on the bars are significantly different ($p < 0.05$) by Duncan's multiple range tests.

proliferation (Nakae et al., 2003). Thus, atopic inflammationcan be inhibited by regulating Th2-stimulated cytokine production, thereby decreasing Th2 cytokine production.

In atopic dermatitis, NF-κB regulates genes coding for inducible enzymes, further exacerbating the atopic reaction (Bruch-Gerharz et al., 1998). COX-2 and iNOS are well known inflammatory-related factors that play important roles in producing NO and prostaglandin, respectively (Park et al., 2014).Thus, measurement of iNOS and COX-2 inhibition can be used as a test for anti-inflammatory activity. In fact, patients with atopic dermatitis are known to have significantly increased NOS and COX-2 levels compared to normal subjects (Redington et al., 2001). Therefore, the NF-κB pathway is activated, and as a result, expression of iNOS and COX-2 as well as various inflammatory substances such as cytokines is induced, which appears to exacerbate atopic diseases. In this study, the group treated with APD and LPS showed significantly decreased iNOS and COX-2 mRNA expression levels compared to the LPS group. These results suggest that APD seemed to inhibits the atopic reaction by reducing iNOS and COX-2 levels.

Cordyceps sinensis, one of the major ingredients of APD, is known to strong high anti-inflammatory effects (Yang, et al., 2011). According to another study, Cordymin, a peptide purified from *Cordyceps sinensis*, has anti-inflammatory effects (Qian, et al., 2012). *Rumex crispus*, another major ingredient of APD, has been shown to inhibit LPS-induced NO production and expression of COX-2 and iNOS genes (Im et al., 2014). In addition, *Camellia sinensis* is known to have high anti-immunological activity (Huynh, 2017). This herb also has high concentrations of Epigallocathechin gallate (EGCG) compared to other herbs. According to prior study results, EGCG has anti-inflammatory properties stemming from biochemically active compounds (Singh et al., 2011). It is concluded that APD has been shown to have anti-atopic efficacy by regulating Th2 cytokines, which stimulate the atopic reaction and inflammatory-related genes.

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CONFLICT OF INTEREST

The authors have no conflicting financial interests.

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