



Topical application of Taglisodog-eum inhibits the development of experimental atopic dermatitis

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ABSTRACT

Aim of study: Taglisodog-eum (托裏消毒飲, Tuo Li Xiao Du Yin), a standardized herbal formula, has been widely used to modulate diverse carbuncles in oriental medicine. However, it is still unclear whether Taglisodog-eum (TSE) can exert a beneficial role in dermatological disease. In this study, we examined the effect of topical application of TSE on experimental atopic dermatitis (AD) and elucidated its action mechanism.

Materials and methods: To test the effect of TSE treatment on IgE production in vitro, U266B1 cells and primary CD19⁺ B cells isolated from AD-induced mice were treated with TSE under LPS/IL-4 stimulation and then IgE level in the culture supernatant was measured by ELISA. To evaluate the effect of TSE treatment on the production of AD related pathogenic cytokines, CD4⁺ T cells isolated from AD-induced mice were treated with TSE under PMA/ionomycin stimulation, then the level of cytokine expression was analyzed by quantitative RT-PCR and ELISA. The effects of TSE on the NFκB promoter activity in T cells and on the expression level of *Aicda* (activation-induced cytidine deaminase) in B cells were examined. To further examine the in vivo efficacy of TSE on AD progression, TSE was topically applied to ears of mice with atopic dermatitis induced by painting of DNCB and house dust mite extract. AD Progression was estimated by following criteria: (a) ear thickness, clinical score, (b) serum total IgE and mite specific IgE level by ELISA, (c) histological examination of ear tissue by H&E staining and (d) cytokine profile of total ear cells and draining lymph node CD4⁺ T cells by quantitative real time PCR and ELISA.

Results: Treatment of TSE to the U266B1 cell line and primary CD19⁺ B cells isolated from AD-induced mice inhibited IgE production. Treatment of TSE down-regulated the expression of several cytokines (IL-4, IL-10, IL-13, IL-17, TNF-α and IFN-γ) in CD4⁺ T cells isolated from AD-induced mice. Topical application of TSE on the ears of AD-induced mice decreased the severity and progression of disease by reducing ear thickness, clinical scores including dryness, edema. TSE treatment reduced the infiltration of lymphocytes to the inflamed site analyzed by histological evaluation. TSE treatment also decreased serum IgE level and expression of AD-associated pathogenic cytokines (IL-4, IL-5 and IL-13) in total ear cells and dLN CD4⁺ T cells by inhibiting the translocation of NFκB into nucleus.

Conclusions: Our study indicates that protective effect of Taglisodog-eum (TSE) in experimental atopic dermatitis is mediated by inhibiting IgE production and the levels of Th2 type cytokines, suggesting the beneficial effect of TSE on modulating atopic dermatitis.

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Abbreviations: TSE, Taglisodog-eum; LPS, Lipopolysaccharide; PMA, Phorbol 12-myristate 13-acetate; AD, Atopic dermatitis; Th1 & Th2, T helper 1 and T helper 2; IL-4, Interleukin 4; IL-5, Interleukin 5; IL-10, Interleukin 10; IL-13, Interleukin 13; IL-17, Interleukin 17; IL-31, Interleukin 31; IFN-γ, Interferon gamma; TNF-α, Tumor necrosis factor alpha; Foxp3, Forkhead box P3; HPRT, Hypoxanthine-guanine phosphoribosyltransferase; DNCB, 2, 4-dinitrochlorobenzene; PBS, Phosphate buffer saline; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; *Aicda*, Activation-induced cytidine deaminase.

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1. Introduction

Taglisodog-eum (托裏消毒飲, Tuo Li Xiao Du Yin), a traditional Korean herbal medicine, comprises eleven component herbs: Ginseng Radix, Astragali Radix, Paeoniae Radix, Angelicae Gigantis Radix, Atractylodis Rhizoma Alba, Citri Unshius Pericarpium, Forsythiae Fructus, Lonicerae Flos, Poria Sclerotium, Angelicae Dahuricae Radix and Glycyrrhizae Radix. Taglisodog-eum has been used to treat pus and skin bumps, and to improve carbuncles by removing inflammation of the lymphatic channels and hypodermic

Table 1
Botanical names and origin of the formula, Taglisodog-eum.

Systematic name (Pharmaceutical name)	Amount (g)	Contents of marker substances
<i>Panax ginseng</i> (Ginseng Radix)	1.25	0.33% of ginsenoside Rg ₁ 0.73% of ginsenoside Rb ₁
<i>Astragalus membranaceus</i> (Astragali Radix)	1.25	
<i>Paeonia lactiflora</i> (Paeoniae Radix)	1.25	2.59% of paeoniflorin
<i>Angelica gigas</i> (Angelica gigantis Radix)	1.25	
<i>Atractylodes macrocephala</i> (Atractylodis Rhizoma Alba)	1.25	
<i>Citrus unshiu</i> (Citri Unshius Pericarpium)	1.25	6.22% of hesperidin
<i>Forsythia viridissima</i> (Forsythiae Fructus)	1.25	
<i>Lonicera japonica</i> (Lonicerae Flos)	1.25	
<i>Poria cocos</i> (Poria Sclerotium)	1.25	
<i>Angelica dahurica</i> (Angelicae Dahuricae Radix)	0.63	0.53% of oxypeucedanin 0.20% of imperatorin 0.12% of isoimperatorin
<i>Glycyrrhiza uralensis</i> (Glycyrrhizae Radix)	0.63	6.10% of glycyrrhizin 3.65% of liquiritigenin
Total amount	12.5	

pustules (Lee and Hong, 2007). Each component of Taglisodog-eum (TSE) has been known to have anti-inflammatory functions (Choi et al., 2007; Kim et al., 2006, 2008). However, limited information is available whether TSE has any immunomodulatory effect on allergic skin disorders.

Atopic dermatitis (AD) is a chronic relapsing skin disease that is manifested by Th2 dominant hyperimmune disorder. Although the exact cause of AD is not completely elucidated, both genetic and environmental factors are known to play crucial role in AD pathogenesis (Novak et al., 2003; Leung et al., 2004; Akdis et al., 2006). The major symptoms of AD are severe scratching, pruritus, dryness and inflammation (Leung, 1995; Leung et al., 2003), which activates keratinocytes to produce proinflammatory cytokines (De Benedetto et al., 2009; Werfel, 2009; Wichmann et al., 2009). Up-regulated proinflammatory cytokines enhance the production of various chemokines (CCL22 and CCL17) and adhesion molecules including ICAM-1, VCAM-1, E-selectin and P-selectin (de Vries et al., 1998; Jung et al., 1996), which recruit pathogenic leukocytes to the skin (Homey et al., 2006). Th2 cells produce IL-4, IL-5, and IL-10 and play major roles in acute atopic dermatitis (Lee et al., 2000; Chen et al., 2004; Tazawa et al., 2004). Enhanced production of IL-4 and IL-13 induces IgE production from B cells and circulating IgE levels in AD patients (Bruynzeel-Koomen et al., 1986; Hajoui et al., 2004). In the later stage of AD where infection mediated inflammation occurs, Th1-type cytokines such as IFN- γ , IL-12 mediate chronic atopic dermatitis (Vestergaard et al., 2001; Leung et al., 2004; Konur et al., 2005). The role of NF κ B transcription factor is also suggested in AD pathogenesis (Hayden et al., 2006; Perkins, 2007). In resting cells, p65/p50 heterodimer, the most abundant NF κ B complex located in the cytoplasm, is bound to inhibitory protein, I κ B. Inflammatory stimulus activates IKK β and triggers I κ B α phosphorylation, which leads to proteosomal degradation of phosphorylated I κ B and translocation of NF κ B complex into nucleus. Binding of NF κ B complex to the promoter of its target genes induces the expression of numerous pro-inflammatory molecules (Li and Verma, 2002).

In this study, we examined the immunomodulatory effect of the Taglisodog-eum (TSE) on experimental atopic dermatitis and elucidated its action mechanism. Topical application of TSE suppressed the IgE production from B cells and reduced the levels of pathogenic cytokines in CD4⁺ T cells. Suppression of AD progression by TSE treatment is also associated with reduced IgE production and lymphocytes infiltration. In addition, TSE treatment inhibited NF κ B, p65 promoter activity, its translocation into nucleus and also down-regulated expression of *aicda* expression in B cell lines.

2. Materials and methods

2.1. Standardization of Taglisodog-eum (TSE)

The preparation of Taglisodog-eum (TSE) was performed in compliance with the test guidelines of the Korea Food and Drug Administration (KFDA). The TSE was prepared as described in Table 1. In brief, the 11 herbs used in the TSE were purchased from Gwang Myung Dang pharmaceutical company (Ulsan, Korea), identified by Prof. Bu, department of oriental medicine, Kyunghee University, and authenticated by the Jeonnam Traditional Korean Medical Institute (Jeonnam, Korea) based on the Korean pharmacopoeia guidelines. All herbal voucher specimens (2010-TSE-01~TSE-11) in TSE were deposited at the Department of Herbal Pharmaceutical Development (Nambu University, Gwangju, Korea). They were ground into powder (25 g, 80 mesh), and were extracted with 500 ml of 70% aqueous ethanol at 80 °C. The crude extract was concentrated and lyophilized in vacuo. The weight of the final TSE extract was approximately 16% (4 g) of the starting raw herbs. Each individual herb was tested for heavy metal (Hg, As, Cd) contamination, residual insecticides and microbial limit. Standardization of each herb extract was performed by high performance liquid chromatography (HPLC) analysis. The content of marker substances in herb extract was compared with commercially available indicator chemicals; ginsenoside Rb₁, ginsenoside Rg₁, glycyrrhizin, liquiritigenin and paeoniflorin from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); hesperidin, oxypeucedanin, imperatorin and isoimperatorin from Korea Food and Drug Administration (KFDA, Seoul, Korea). Other chemicals were of analytical grade. A Waters Alliance 2796 system (Waters, MA, US) consisting of quaternary solvent blending, integral in-line degasser, integrated autosampler, column heater, and 996 diode array detector was used to perform HPLC analysis. The dried TSE was kept at 4 °C before use.

2.2. Cytotoxicity examination by WST-1 assay

Cytotoxicity of TSE was conducted using EZ-Cytox cell viability assay kit (Daeil Lab Service Co, Seoul, Korea) by following manufacture's protocol. Briefly, 5×10^3 cells/well isolated from spleen were dispensed in a 96-well plate and incubated for 24 h. Various concentrations of TSE were treated and incubated for 72 h. Using the microplate reader, the absorbance was measured at 450 nm. Data was presented by relative growth inhibition to TSE non-treated cells.

2.3. Animals

Female BALB/c mice (6–8 weeks) were purchased from SLC Inc. (Hamamatsu, Japan). Mice were housed in specific pathogen-free barrier facilities and used in accordance with protocols approved by the Animal Care and Ethics Committees of the Gwangju Institute of Science and Technology (GIST) (permit number: GIST-2011-3).

2.4. Induction of atopic dermatitis

Induction of atopic dermatitis was performed as previously described (Kwon et al., 2010). Briefly, surfaces of both ear lobes of mice were stripped with surgical tape (Nichiban, Tokyo, Japan). After stripping, 20 μ l of 2% 2,4-dinitrochlorobenzene (DNCB) (Sigma Aldrich, St Louis, MO, USA) dissolving in acetone/olive oil solution (acetone: olive oil=1:3) was painted on each ear. After 3 days, 150 μ g of mite extract (Dermatophagoides farinae, GREER source materials, Lenoir, NC, USA) dissolving in PBS containing 0.5% tween 20 was re-painted. Challenge of DNCB and mite extract was repeated once a week alternatively until 5 weeks. After 3 weeks of AD induction, mice were divided into three groups based on AD severity to have similar clinical score then they were daily painted either with 70% ethanol (Cont), 1 mg or 5 mg of TSE/ each ear (TSE-1, TSE-5, respectively) to the both ears for additional 2 weeks with continuous induction of experimental atopic dermatitis.

2.5. Measurement of ear swelling and clinical score

Ear thickness was measured 24 h after application of DNCB or mite extract with a dial thickness gauge (Kori Seiki MFG, Co., LTD., Japan). Representative mouse of each group was photographed to examine the clinical symptoms. Clinical symptoms of each mouse were evaluated as described previously (Matsuoka et al., 2003). Briefly, degree of severity of erythema, edema, excoriation and dryness on the ear surface was scored as 0 (not visible), 1 (mild), 2 (moderate) and 3 (severe), accordingly. Scoring was performed by two independent observers, and the final score was taken as an average of both.

2.6. Histological examination

Excised ears of each group were fixed in 4% paraformaldehyde for 16 h and embedded in paraffin. Then, 6 μ m sections were stained with hematoxylin (Sigma Aldrich, St Louis, MO, USA) and eosin (Sigma Aldrich, St Louis, MO, USA) (H&E). Infiltrated lymphocytes, thickening of the epidermis and fibrosis in the dermis were observed by microscope (100 $\times$, 200 $\times$).

2.7. ELISA

Total IgE levels in the serum were measured using sandwich ELISA kit (BD Biosciences, San Diego, CA, USA) following the manufacturer's protocol. For the detection of mite specific IgE, 1 μ g of mite extract was coated in each 96 well plate, remaining procedures were followed according to the manufacture's protocol. Antigen specific IgE levels were indicated by O.D value. Mean absorbance of antigen coated well minus mean absorbance of non-coated well was used as the O.D value of the mite specific IgE levels. For the detection of IgE production from B cells, Human U266B1 multiple myeloma cells (ATCC TIB-196TM, American Type Culture Collection, USA) or CD19⁺ B cells isolated from AD-induced mice were treated with diverse concentration of TSE and IgE levels were measured by ELISA (BETHYL, Montgomery, TX, USA and BD Biosciences, San Diego, CA, USA, respectively).

For the measurement of cytokine concentration (IL-4, IL-13, IL-17 and IFN- γ) in the culture supernatant, ELISA was performed by using ELISA kits (e-bioscience, San Diego, CA, USA).

2.8. Isolation of total ear cells and primary CD4⁺ T cells, CD19⁺ B cells

Ears were removed from each group, cut into three pieces and gently stirred in 1.0 mM EDTA in 5% FBS for 20 min at room temperature. Then ear segments were minced, transferred into 50 ml centrifuge tube containing RPMI without serum and vigorously shaken. And the tissues were transferred into RPMI containing 0.5 mg/ml of Collagenase type (Sigma Aldrich, St Louis, MO, USA), incubated for 1 h at 37 $^{\circ}$ C shaking incubator. After incubation and centrifugation, cells were washed with ice-cold PBS then cultured in complete T cell media. For the isolation of T cell or B cells, isolated draining lymph nodes (superficial cervical, axillary, branchial lymph node) from mice were grinded using cell strainer (BD Biosciences, San Diego, CA, USA), then CD19⁺ B cells or CD4⁺ T cells were isolated using isolation bead (Miltenyi Biotech, Germany) according to the manufacture's protocol, respectively. Isolated primary cells were cultured in RPMI 1640 medium (Welgene, Daegu, Korea) supplemented with 10% fetal bovine serum (Hyclone, USA), 3 mM L-glutamine (Sigma Aldrich, St Louis, MO, USA), 100 U/ml penicillin (Sigma Aldrich, St Louis, MO, USA), 100 U/ml streptomycin (Sigma Aldrich, St Louis, MO, USA), non-essential amino acids (Welgene, Daegu, Korea), sodium pyruvate (Welgene, Daegu, Korea), HEPES (Welgene, Daegu, Korea) and 0.05 mM 2-beta-mercaptoethanol (Sigma Aldrich, St Louis, MO, USA).

2.9. RNA isolation and quantitative RT-PCR (qRT-PCR)

For the cytokine analysis, 3 $\times$ 10⁶ cells of CD4⁺ T cells or total ear cells from each group were stimulated with PMA (50 ng/ml) and ionomycin (1 μ M) for 4 h. To evaluate the effect of TSE on the expression of B cell related effector molecules, 2 $\times$ 10⁶ cells of M12 cells were stimulated with TSE (0.5 mg/ml) in 10 μ g/ml of LPS containing complete RPMI media for 4 h. Then mRNA expression levels of cytokines or *Prdm1*, *Pax5* and *Aicda* were analyzed by qRT-PCR. Total RNA was extracted from stimulated cells with TRIzol reagent (Molecular Research center, Cincinnati, OH, USA) according to manufacturer's protocol. For reverse transcription, 1 μ g of total RNA was used and cDNA was generated using oligo(dT) primer (Promega, Madison, WI, USA) and Improm-II reverse transcriptase (Promega, Madison, WI, USA) in a total volume of 20 μ l. The mRNA level was determined using 1 μ l of cDNA by quantitative real time PCR (qRT-PCR) with SYBR using a protocol provided by the manufacturer (MJ research chromo4). Mouse HPRT primer was used for qRT-PCR to normalize the amount of cDNA used for each condition. The primer sequences of cytokines were listed in Table 2.

2.10. Immunoblotting

EL4 T cells (1 $\times$ 10⁷) were treated with TSE (0.5 mg/ml) or same volume of 70% ethanol as a control for 24 h then stimulated with PMA/ionomycin for 0, 1, 2 h, respectively. Nuclear extract was prepared as previously described (Lahiri and Y, 2000). Cells were washed with ice cold PBS and resuspended in cold buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 2 mM MgCl₂, 1 mM DTT and 0.5 mM PMSF) containing protease inhibitors and 0.4% NP-40. The cells were allowed to swell by incubation on ice for 15 min, and then homogenate was centrifuged for 3 min. The nuclear pellet was resuspended in cold buffer B (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 10% glycerol, 2 mM MgCl₂, 1 mM

Table 2
Primer sequences used for qRT-PCR.

Genes	Sequences (5'–3')	Tm (°C)
HPRT	F: TTATGGACAGGACTGAAAGAC R: GCTTTAATGTAATCCAGCAGGT	53.9 54.4
IL-4	F: ACAGGAGAAGGGACGCCAT R: GAAGCCGTACAGACGAGCTCA	55.4 55.4
IL-5	F: AGCACAGTGGTGAAGAGAC R: TCCAATGCATAGCTGGTGATT	55.4 54.4
IL-10	F: ATAAGTGCACCCACTTCCCA R: TCATTTCGATAAGGCTTGG	55.4 53.4
IL-13	F: GCAACATCACAGGACCAGA R: GTCAGGGAATCCAGGCTAC	55.9 59.5
IL-17 A	F: TTCATCTGTCTCTGATGCT R: TTGACCTTCACATTCTGGAG	53.9 53.3
IL-31	F: ACAACTATAGCATAAAGCAGGC R: GATTCATCAGTATTTCCAGGCA	54.4 54.4
IFN- γ	F: GAGCCAGATTATCTCTTCTACC R: GTTGTGACCTCAAAGTGG	56.6 53.4
TNF- α	F: CCCTCACACTCAGATCATCTTCT R: GCTACGACGTGGGCTACAG	58.4 59.2
Foxp3	F: TTCCTTCCAGAGTTCTTCC R: CTCAAATTCATCTACGCTCCA	55.4 53.9
AID	F: ACGGATCTTCTATTCTTCCCT R: AAGATCCTTTATTGAAATCACCCA	54.9 53.6

DTT and 0.5 mM PMSF) containing protease inhibitors. The tube was mixed thoroughly and placed on a rotatory shaker for 2 h and then nuclear extract was centrifuged for 5 min. The supernatant containing nuclear proteins was used for immunoblotting. Nuclear extract (30 μ g) were subjected to 10% SDS-PAGE and electrotransferred onto PVDF (polyvinylidene fluoride) membranes for immunoblot analysis. The membranes were incubated with anti-p65 (Abcam, Cambridge, CB4 0FL, UK) or anti-Lamin B (Santa Cruz, Santa Cruz, CA, USA) antibodies. Blots were developed using HRP (horseradish peroxidase) conjugated secondary antibody (Sigma Aldrich, St Louis, MO, USA) and the ECL western blot detection kit (Amersham Pharmacia Biotech, Arlington Height, IL, USA).

2.11. Transfection and luciferase assay

Mouse NF κ B (p65) plasmid was transiently transfected into EL4 T cells by Geneexpresso (Excellgen, Rockville, MD, USA) according to the manufacturer's protocol. After 19 h, cells were pre-treated with indicated amount of TSE (0.25, 0.5 mg/ml) for 1 h and then stimulated with PMA/ionomycin for additional 4 h. Luciferase activity measured by dual luciferase assay system (Promega, Madison, WI, USA) was expressed relative to expression of the co-transfected Renilla luciferase promoter activity (phRL-null; Promega Madison, WI, USA) to control for transfection efficiency.

2.12. Statistical analysis

A Student's *t*-test was used to calculate the statistical significance of the experimental data. The level of significance was set at **P* < 0.05, ***P* < 0.01 and ****P* < 0.001. Significance was only indicated when appropriate.

3. Results

3.1. Quantitative analysis of marker substances in herbs by HPLC

To ensure the quality and purity of each preparation of TSE, HPLC analysis was performed by measuring the content of known

active compounds of 9 marker substances of five herbs of TSE by following the Korean pharmacopoeia guidelines (9th edition). Ginsenoside Rg1 and ginsenoside Rb1 in Ginseng Radix were quantified by HPLC-DAD using a C18 column and isocratic elution with water and acetonitrile (4:1 for ginsenoside Rg1, 7:3 for ginsenoside Rb1). The amount of ginsenoside Rg1 and ginsenoside Rb1 in Ginseng Radix were calculated as 0.33% and 0.73%, respectively. The contents of marker substances in Paeoniae Radix, Citri Unshius Pericarpium, Angelicae Dahuricae Radix, and Glycyrrhizae Radix were calculated as 2.59% (paeoniflorin), 6.22% (hesperidin), 0.53% (oxyeucedanin), 0.20% (imperatorin), 0.12% (isoimperatorin), 6.10% (glycyrrhizin), and 3.65% (liquiritigenin), respectively, (Table 1 and Fig. 1). This result indicates that the content of these 9 compounds in the TSE showed the upper value of the contents criterion in Korean pharmacopoeia guidelines (9th edition).

3.2. Effect of TSE treatment on cytokine expression in CD4⁺ T cells and IgE production in CD19⁺ B cells

To define the optimal concentration at which TSE does not induce cell damage, we performed the cytotoxicity test using total splenocytes. TSE treatment up to 1 mg/ml did not induced cell death (Fig. 2A) rather slightly increased in high concentration of TSE (over 0.5 mg/ml). However this could be an artifact of WST-1 assay which analyze the cell viability based on the color changes, since high concentration of TSE shows yellow color pigmentation. Indeed when cell viability was measured by trypan blue staining, 1 mg/ml of TSE did not induce the cell proliferation (data not shown). So based on these experiments, we concluded that high concentration of TSE does not cause the cell proliferation and used suboptimal concentration of TSE (0.25 or 0.5 mg/ml) in all the in vitro experiments. First, we investigated the effect of TSE treatment on IgE production in human B cell line, U266B1 as well as in CD19⁺ B cells isolated from AD-induced mice. TSE treatment markedly decreased IgE production from B cell lines (Fig. 2B) as well as primary CD19⁺ B cells (Fig. 2C) in a dose dependent manner. Next, we evaluated the effect of TSE treatment on the expression of key regulatory cytokines associated with the development of atopic dermatitis including Th2 type (IL-4, IL-5, IL-10 and IL-13), Th1 type (IFN- γ and TNF- α), Th17 (IL-17) cytokine as well as Foxp3, a marker of regulatory T cell in CD4⁺ T cells from AD-induced mice. TSE treatment significantly decreased mRNA expression of AD-associated pathogenic cytokines (IL-4, IL-5, IL-13, INF- γ , IL-17, IL-10 and TNF- α) and Foxp3 expression level was shown a tendency to increase in a dose dependent manner (Fig. 2D). In addition, TSE treatment also reduced the protein level of IL-4, IL-17 and IFN- γ in the T cell culture supernatant (Fig. 2E). Collectively, these data indicate that TSE treatment could inhibit the production of AD-associated pathogenic molecules produced by CD4⁺ T cells and CD19⁺ B cells.

3.3. Amelioration of AD symptoms by topical application of TSE

Down-regulation of IgE production and expression of pathogenic cytokines by TSE treatment in vitro led us to test whether topical application of TSE could also suppress the progression of ongoing atopic dermatitis. Experimental AD was induced on the both ears of BALB/c mice as depicted in Fig. 3A. AD symptoms including erythema, horny substance, dryness, and scaling were evidently seen in control group (Fig. 3B). However, TSE treatment significantly reduced those AD symptoms in both TSE-1 and TSE-5 (Fig. 3B) groups. In addition, in agree with phenotypical observation, TSE treatment significantly decreased ear thickness (Fig. 3C) as well as clinical score (Fig. 3D) compared with control treatment in a dose dependent manner. To further evaluate the AD

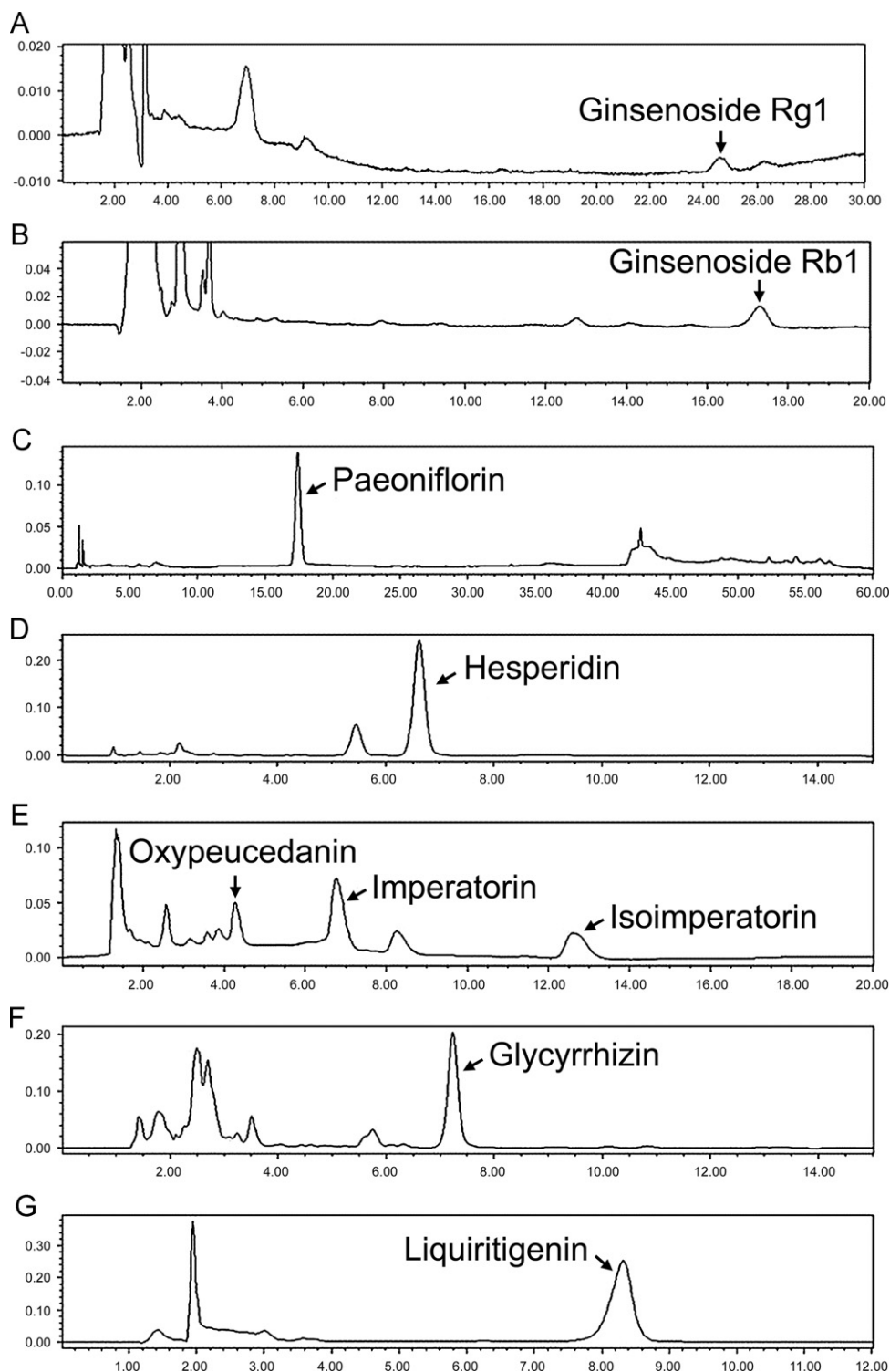


Fig. 1. HPLC chromatograms of marker substances in Taglisodog-eum. HPLC analysis was performed on Phenomenox Gemini-Nx C18 column (4.6 × 250 mm, 5 μm). The optimum mobile phase and wavelength for detection were water and acetonitrile for (A) ginsenoside Rg1 (4:1, 203 nm), (B) ginsenoside Rb1 (7:3, 203 nm), (C) paeoniflorin (gradient, 10% – 50% ACN, 230 nm); water and methanol for (D) hesperidin (6:4, 280 nm), (E) for oxypeucedanin, imperatorin, isoimperatorin (3.5:6.5, 254 nm); aqueous acetic acid and acetonitrile for (F) glycyrrhizin (3:2, 254 nm), (G) liquiritigenin (7.5:2.5, 276 nm). Data are representative of three independent experiments.

symptoms, we performed histological analysis on the ears taken from different treatment groups. Compared with control, the numbers of infiltrated lymphocytes in ear regions were significantly reduced in TSE-1 as well as TSE-5, which was well reflected by reduced thickness of epidermis (Fig. 3E).

3.4. Reduction of IgE levels by TSE treatment

Since increased serum IgE level is associated with clinical symptoms of AD (Akdis et al., 2006; Werfel, 2009), we tested whether improved AD symptom by TSE treatment is also related

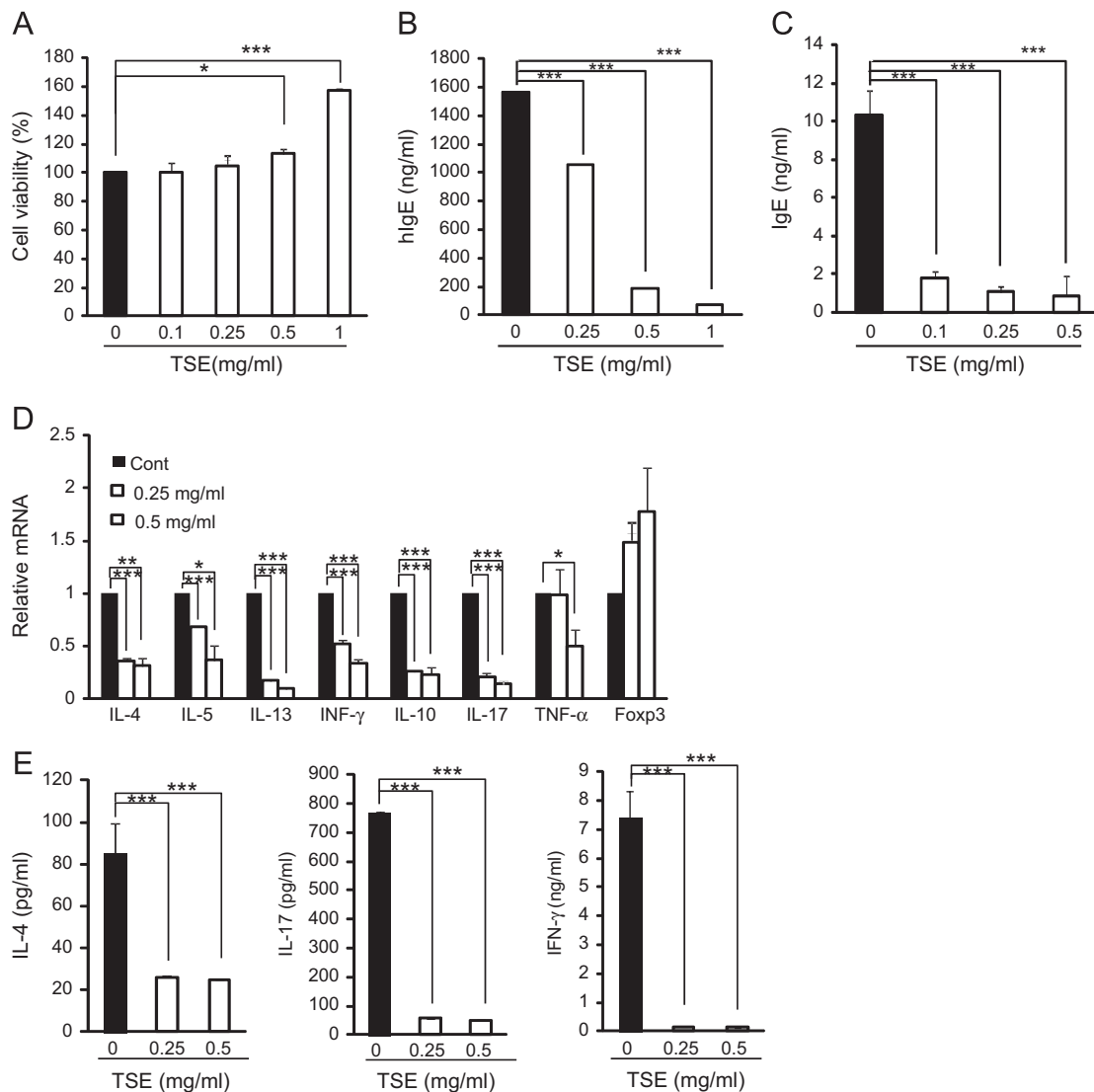


Fig. 2. Inhibitory effect of Taglisodog-eum on the production of IgE and cytokine expression. (A) Mouse splenocytes were incubated with various concentration of Taglisodog-eum (TSE) for 72 h. Cell viability was estimated with WST-1 assay. (B) Human B cell line, U266B1 was stimulated with LPS (10 μ g/ml), IL-4 (5 ng/ml) in the presence of indicated concentration of TSE for 72 h then human IgE (hIgE) levels in the culture supernatant was measured by ELISA. (C) Draining lymph node CD19⁺ B cells isolated from AD-induced mice were stimulated with LPS (10 μ g/ml)/IL-4 (5 ng/ml) in the presence of various concentration of TSE for 72 h. Mouse IgE levels in the B cell culture supernatant were measured by ELISA. (D) Draining lymph node CD4⁺ T cells from AD-induced mice were stimulated with PMA (50 ng/ml)/ionomycin (1 μ M) in the presence of TSE (0.25 mg/ml or 0.5 mg/ml) for 4 h. Relative expression of cytokines of TSE treated samples was compared with control samples by qRT-PCR. Expression level of HRPT was used as an internal control. (E) CD4⁺ T cells from AD-induced mice were stimulated with PMA/ionomycin for 72 h in the presence of TSE (0.25 mg/ml or 0.5 mg/ml), then protein level of each cytokine was analyzed by ELISA. Same volume of 70% alcohol was treated as a control. Error bars indicate SD. One (*), two (**), three (***) indicates $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively. Data are representative of three independent experiments.

with changes in serum IgE levels. After 5 weeks of AD induction, sera were obtained from each group, then total IgE and mite specific IgE levels were measured by ELISA. Compared with control, topical application of 1 mg or 5 mg of TSE significantly decreased total IgE (Fig. 4A) as well as mite specific IgE levels (Fig. 4B). Next, we compared the effect of TSE treatment on IgE production by primary B cells. CD19⁺ B cells isolated from each treatment group were stimulated with LPS plus IL-4 and then IgE levels in the culture supernatant were analyzed by ELISA. As shown in Fig. 4C, B cells from TSE-1 or TSE-5 group produced much less amount of IgE (TSE-1; 31 ng/ml, TSE-5; 37 ng/ml) than that of the control group (83 ng/ml). Next, to investigate the underlying mechanism of TSE-induced down-regulation of IgE production in B cells, we examined whether TSE treatment could affect the mRNA expression pattern of *Prdm1* (encoding Blimp1), *Pax5* (encoding Pax5) and *Aicda* (encoding activation-induced cytidine deaminase) which are crucial

factors for B cell development and class switching, respectively. Treatment of TSE to M12 cells under LPS stimulation did not alter the mRNA expression of *Prdm1* and *Pax5* while it significantly decreased the *Aicda* expression (Fig. 4D). These results indicate that topical application of TSE inhibits the IgE production from the activated B cells through down regulation of *Aicda* expression.

3.5. Down-regulation of pathogenic cytokine expressions by TSE treatment

Dysregulated cytokine expression also mediates the AD pathogenesis (Homey et al., 2006; Leung et al., 2004). To elucidate the effect of TSE treatment on cytokine network, changes in expression pattern of several cytokines were analyzed. Ear total cells and draining lymph node CD4⁺ T cells isolated from each treatment group were stimulated with PMA/ionomycin for 4 h and the expression level of

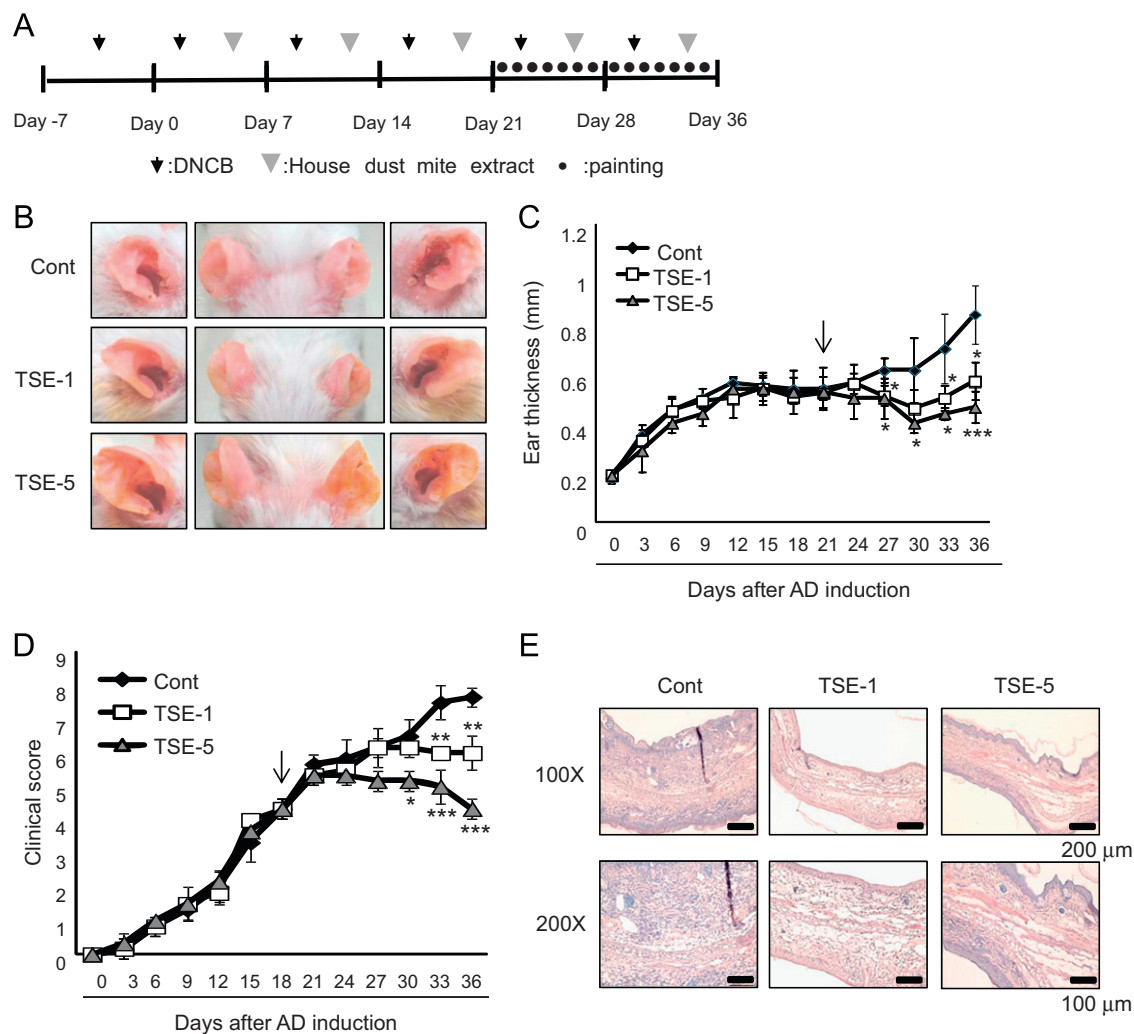


Fig. 3. Suppressive effect of Taglisodog-eum on the clinical symptoms of atopic dermatitis. (A) Experimental AD was induced in BALB/c mice for 3 weeks. Then TSE (1 mg or 5 mg/each ear) or 70% ethanol (same volume of TSE) was painted every day for additional 2 weeks. (B) Representative ear pictures of control group or TSE treated group were shown. (C) Progression of AD was assessed by measuring ear thickness 24 h after each DNCB or mite extract treatment. (D) Clinical score (ear swelling, dryness and edema) was measured once a week. Arrow indicates the starting point of TSE treatment. (E) Ears excised from each group were fixed with 4% para-formaldehyde for 24 h and embedded in paraffin. Paraffin embedded ears were sectioned into 6 μm and stained with hematoxylin and eosin (H&E). Infiltrated lymphocytes and thickness of epidermis were observed under the microscope (100× and 200×). Error bars indicate SD. One (*), two (**), three (***) indicates $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively. Data are representative of three independent experiments.

cytokines was compared between them. TSE treatment significantly reduced the levels of AD-associated pathogenic cytokines such as IL-4, IL-5, IL-13 and IL-17 in the ear residual total cells (Fig. 5A) as well as in CD4⁺ T cells (Fig. 5B). Interestingly, inhibitory effect of TSE was in a dose dependent manner only in the ear residual total cells not in CD4⁺ T cells. Significant decrease of TNF- α , IFN- γ and IL-31 expression levels was mainly observed in ear total cells from TSE-5 group compared with control (Fig. 5A). In consistent with mRNA results, TSE treatment significantly reduced IL-4 and IL-13 protein levels in CD4⁺ T cell culture supernatant compared with control (Fig. 5C). These results suggest that topical application of TSE improved the AD symptoms by down-regulating the expression of pathogenic cytokines in the inflamed tissues as well as in draining lymph node CD4⁺ T cells.

3.6. Down-regulation of NF κ B (p65) activity and nuclear translocation by TSE treatment

Treatment of TSE significantly down-regulated the expression levels of IL-4, IL-5, IL-13, IL-17, IL-31 and TNF- α (Fig. 5). Since NF κ B is one of the common transcription factors involved in expression of

those cytokines (Perkins, 2007; Dong et al., 2010), we tested whether TSE treatment could reduce the transactivity of NF κ B (p65) by performing NF κ B (p65) luciferase-based reporter assay. EL4 T cells were transfected with NF κ B (p65) reporter construct (Rhee et al., 2007; Lin et al., 2009) and then stimulated with PMA/ionomycin in the presence of different concentration of TSE. Indeed, TSE treatment significantly down-regulated the NF κ B (p65) activities in a dose dependent manner (Fig. 6A). We also tested whether TSE treatment could reduce the nuclear translocation of NF κ B (p65). EL4 cells were stimulated with PMA/ionomycin in the presence of TSE (0.5 mg/ml) and nuclear levels of NF κ B (p65) were measured by immunoblotting analysis. Interestingly TSE treatment significantly inhibited translocation of endogenous NF κ B (p65) into nucleus (Fig. 6B). These data indicate that TSE treatment down-regulates NF κ B (p65) transactivity as well as nuclear translocation in T cells.

4. Discussion

Treatment of inflammatory disease by oriental herbal extracts has been widely used as a folk remedy (Sheid et al., 2009). Herbal

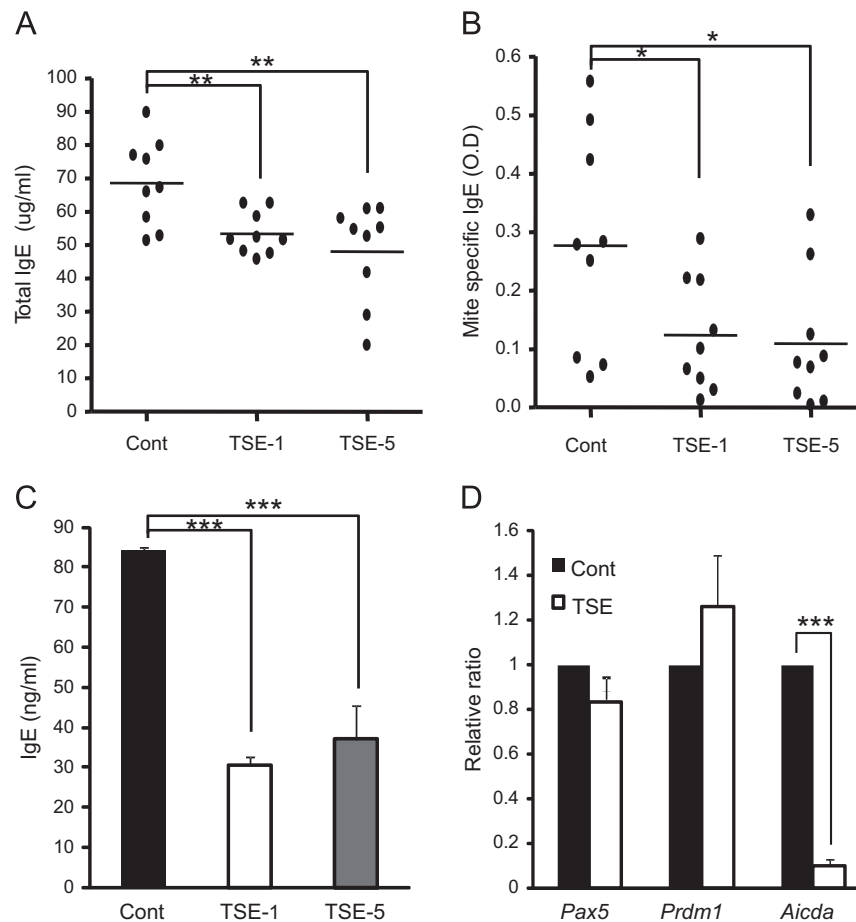


Fig. 4. Inhibitory effect of Taglisodog-eum on the IgE levels and *Aicda* expression. Serum total IgE levels (A) and mite specific IgE levels (B) were measured with ELISA. (C) B cells isolated from draining lymph node of each group were stimulated with LPS (10 μ g/ml)/IL-4 (5 ng/ml) for 72 h then IgE levels in the culture supernatant were measured by ELISA. (D) M12 cells were stimulated with LPS in the presence of TSE (0.5 mg/ml) for 4 h then expression of *PRDM1*, *PAX5* and *Aicda* was measured by qRT-PCR. Error bars indicate SD. One (*), two (**), three (***) indicates $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively. Data are representative of three independent experiments.

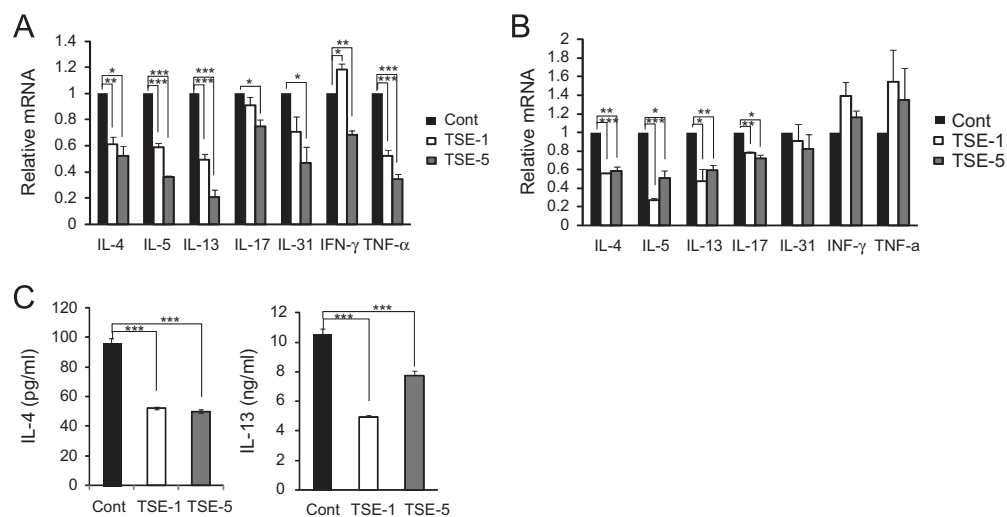


Fig. 5. Effects of Taglisodog-eum treatment on the expression levels of cytokines. Total ear cells (A) and draining lymph node CD4⁺ T cells (B) from each treatment group were stimulated with PMA (50 ng/ml)/ionomycin (1 μ M) for 4 h. Relative expression of cytokines of TSE treated samples was compared with control samples by qRT-PCR. The expression level of HPRT was used as an internal control. (C) Draining lymph node CD4⁺ T cells from each group were stimulated with PMA/ionomycin for 72 h then IL-4 and IL-13 levels were measured by ELISA. Error bars indicate SD. One (*), two (**), three (***) indicates $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively. Data are representative of three independent experiments.

extracts have diverse functions such as anti-inflammatory, antiviral and anti-cancer effects with less side effects (Lee and Lau, 2011; Ye et al., 2011). However the underlying effector

mechanism of herbal extracts on immune modulation is still not clear. In the present study, we demonstrated the potent immunomodulatory effect of Taglisodog-eum (TSE) in experimental

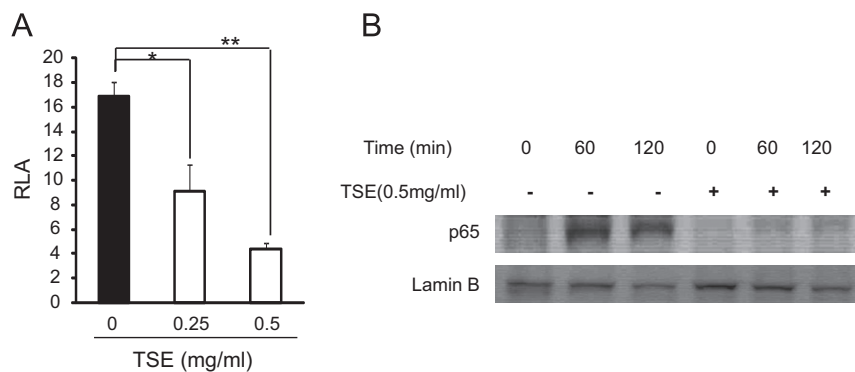


Fig. 6. Inhibition of NF κ B (p65) activity and nuclear translocation by Taglisodog-eum treatment. (A) NF κ B (p65) promoter containing reporter vector was transiently transfected into EL4 T cells. After 19 h cells were pre-treated with various concentration of TSE for 1 h then stimulated with PMA/ionomycin for 4 h. NF κ B promoter activity was analyzed as a relative ratio to the activity of a co-transfected Renilla luciferase plasmid (hr-Luci) as a control for transfection efficiency. Relative luciferase activity (RLA) of TSE treated sample was expressed as a fold difference relative to that of the control. (B) EL4 T cells were pre-treated with TSE (0.5 mg/ml) or left without treatment for 24 h then stimulated with PMA/ionomycin for indicated time. The level of nuclear p65 was measured by mouse anti-p65 antibody. The expression level of Lamin B was used as a loading control. Error bars indicate SD. One (*), two (**), three (***) indicates $p < 0.05$, $p < 0.005$ or $p < 0.001$, respectively. Data are representative of three independent experiments.

atopic dermatitis. TSE is a mixture of 11 herbal medicines as listed in Table 1. Topical application of TSE reduced atopic symptoms such as ear swelling, dryness, edema and lymphocytes infiltration to inflamed site ears (Fig. 3). Therapeutic effect of TSE on pathogenesis of atopic dermatitis is mediated by down-regulation of serum IgE (Fig. 4) and pathogenic cytokines as well (Fig. 5). Underlying mechanism of TSE effectiveness is associated with the inhibition of NF κ B (p65) transactivity mediated down-regulation of pathogenic cytokine expressions and the reduction of *Aicda*-mediated IgE production (Fig. 6).

One of the main characteristics of atopic dermatitis is increased IgE level in the serum (Bruynzeel-Koomen et al., 1986; Wollenberg and Bieber, 2000). Dendritic cells uptake outer allergens, process them and present antigens on the contact of TCR/MHC class interactions with precursor T helper cells. Under this microenvironment, naive CD4⁺ T helper cells differentiated into Th2 cells and produce cytokines such as IL-4 and IL-13. These cytokines in turn induce class switching into IgE producing plasma cell (Eberl, 2010; Akdis and Akdis, 2012). In this study, we have found that TSE treatment significantly reduced total IgE and mite specific IgE levels in the serum (Fig. 4). To elucidate the underlying mechanism of TSE mediated IgE down-regulation, we measured the mRNA expression levels of B cell development and differentiation related molecules such as *Prdm1* (Blimp-1) and *Pax5* (Pax5) and class switching factor, *Aicda* (activation-induced cytidine deaminase). TSE treatment did not alter the expression of *Prdm1* and *Pax5* while significantly decreasing *Aicda* level (Fig. 6C). Especially, *Aicda* is involved in the initiation of three separate immunoglobulin diversification processes; somatic hypermutation (SHM), class switch recombination (CSR) and gene conversion (GC) (Kinoshita and Honjo, 2001; Chaudhuri and Alt, 2004). Our results suggest that reduced IgE levels by TSE treatment is mediated by the inhibition of *Aicda*-mediated class switching. Since IgE has high affinity to Fc ϵ R1, binding of small amount of IgE can stimulate mast cells to release many inflammatory cytokines and mediators such as IL-4, IL-5, IL-13, histamine and PGE2 (Prostaglandin E2) (Bischoff, 2007; Galli et al., 2008). Resultant histamine from these processes can act on a variety of cells and at different levels. Histamine can facilitate the recruitment of inflammatory cells by direct chemotaxis of additional dendritic cells, eosinophils and mast cells to the inflamed sites via action at H₄ receptors or H₁ receptors (Thurmond et al., 2008). Decreased IgE levels by TSE treatment could reduce a histamine release from mast cells and eventually decreased recruitment of inflammatory cells to ears. We also found that

treatment of TSE to human mast cell line, HMC-1 significantly decreased histamine release (data not shown). Interestingly down regulated IgE levels are well correlated with decreased expression of IL-4, IL-5 and IL-13 in draining lymph node CD4⁺ T cells and total ear cells from TSE treated groups (TSE-1, TSE-5) compared with that of control group (Fig. 5). Expression of IL-31, IFN- γ and TNF- α was significantly reduced in total ear cells especially in TSE-5 group. Although IL-31 is mainly produced by activated CD4⁺ T cells (Dillon et al., 2004), mast cells and eosinophils also produce IL-31 (Niyonsaba et al., 2010). Down-regulated IL-31 in ear total cells but not in CD4⁺ T cells indicates that TSE treatment may reduce IL-31 production by non-CD4⁺ T cells such as mast cells and eosinophils. Upregulated IL-31 level in AD patients is correlated with IL-4 and IL-13 levels (Neis et al., 2006) and heterodimeric IL-31 receptor (IL-31 receptor A and oncostatin M receptor b) are highly expressed in the adult dorsal root ganglia, where sensation of itching is initiated (Bando et al., 2006). IL-31 transgenic mouse shows spontaneous atopic dermatitis symptoms (Dillon et al., 2004) and treatment of anti-IL-31 antibody inhibits the scratching behavior in NC/Nga mouse (Grimstad et al., 2009). These reports strongly suggest that IL-31 has crucial function for the induction of itching which is the main symptom of atopic dermatitis. Reduced scratching behavior of TSE-treated groups compared to control group (data not shown) is also related with down-regulated IL-31 expression. In this study, we found that TSE treatment also reduced expression of proinflammatory cytokines such as IFN- γ and TNF- α . Expression of IFN- γ and TNF- α are up-regulated in the skin lesions of AD patients (Lee et al., 2000; Vestergaard et al., 2001). High dose of TSE treatment (TSE-5) significantly decreased expression of IL-31, IFN- γ and TNF- α from total ear cells but not from draining lymph node CD4⁺ T cells. Low dose of TSE (TSE-1) failed to reduce the expression of IL-31 and IFN- γ while reducing the TNF- α level. This result suggests that high concentration of TSE could affect the cytokine expression from residual non-CD4⁺ T cells from inflamed site, which could lead to better effect on improvement of ear thickness, clinical score as well as total serum IgE levels.

To examine whether therapeutic effect of TSE treatment on atopic dermatitis is related with generation of regulatory T cells, we also measured the expression levels of Foxp3, IL-10 and TGF- β . Although TSE treatment did not significantly increase the Foxp3 expression level slightly increased its expression (Fig. 2B). In addition, CD4⁺ T cells isolated from TSE-1 and TSE-5 did not show any significant alterations in the expression levels of IL-10 and TGF- β , Foxp3 expression was slightly increased

(data not shown). Further optimization of doses and ways of TSE treatment as well as composition of it may further induce Foxp3 expression with better therapeutic efficacy. In addition, even though we mainly demonstrated the anti-inflammatory/allergic effects of TSE, a mixture of 11 herbs, we also found that one of the single components of TSE has also potent anti-inflammatory effect as well. We also generated a modified TSE that contains 9 herbs without yellow color pigmentation. Currently, we are evaluating a therapeutic effect of modified TSEs in experimental AD model.

5. Conclusion

We have demonstrated that Taglisodog-eum (TSE) treatment effectively suppresses the development of experimental atopic dermatitis by down-regulating pathogenic cytokines as well as IgE levels. Underlying mechanism of this therapeutic effect of TSE is mainly mediated by reduction of NF κ B (p65) transactivity in T cells and by reduction of *Aicda*-mediated IgE class switching in B cells, respectively. Our study extends the understanding of the pharmacological mechanisms underlying the immunomodulatory activity of Taglisodog-eum (TSE) observed in oriental medicine. Thus our results indicate that Taglisodog-eum (TSE) could be employed as an effective supplement to treat diverse symptoms of atopic dermatitis.

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