



Jageum-Jung improves 2,4-dinitrochlorobenzene-induced atopic dermatitis-like skin lesions in mice and suppresses pro-inflammatory chemokine production by inhibiting TNF- α /IFN- γ -induced STAT-1 and NF κ B signaling in HaCaT cells

Ju-Hye Yang, Hyun Ju Do, Esther Lee, Nam-Hui Yim, Won-Kyung Cho, Kwang-Il Park*, Jin Yeul Ma*

Korean Medicine (KM) Application Center, Korea Institute of Oriental Medicine, 70 Cheomdan-ro, Dong-gu, Daegu 41062, Republic of Korea

ARTICLE INFO

Keywords:

Jageum-Jung
Atopic dermatitis
Dinitrochlorobenzene
Keratinocytes
Mast cells

ABSTRACT

Ethnopharmacological relevance: Jageum-Jung (JGJ) is an oriental herbal formula comprising five herbs (*Melaphis chinensis* Bell, *Cremastra variabilis* Nakai, *Knoxia valerianoides* Thorel, *Euphorbia lathyris* L., and *Moschus moschiferus* L.). It has been used for detoxification and treating cancer and inflammatory diseases in China, Japan, and Korea. However, the mechanism of action of JGJ on keratinocyte inflammatory response is poorly understood.

Aim of the study: In the present study, we investigated the anti-inflammatory mechanism of JGJ and studied the effects of JGJ on atopic dermatitis-like skin lesions in mice.

Materials and methods: We elucidated the anti-inflammatory and anti-inflammatory effects of JGJ on tumor necrosis factor- α /interferon- γ (TNF- α /IFN- γ)-treated human keratinocyte cells, IgE-sensitized RBL-2H3 cells, and 2,4-dinitrochlorobenzene (DNCB)-induced atopic dermatitis (AD)-like mice, respectively.

Results: The results showed that JGJ suppressed the production and mRNA levels of IL-8, IL-6 and, conspicuously, both TARC and RANTES. JGJ inhibited nuclear translocation of the inflammatory transcription factors NF κ B and STAT-1. Moreover, JGJ improved AD-like skin lesions in DNCB-treated mice and inhibited degranulation of mast cell.

Conclusions: The results of this study suggest that JGJ can be considered as a candidate agent for AD treatment.

1. Introduction

Atopic dermatitis (AD) occurs mainly in approximately 20% of children and 3% of adults in developed countries. Most patients with AD are diagnosed within 1 year after birth, of which 80% have symptoms lasting for approximately 5 years (Leung et al., 2004). The incidence of AD has tripled over the past 30 years, and its prevalence continues to increase. AD is a known chronic inflammatory skin disorder, characterized by severe skin lesions, such as rash, erythema, edema, hemorrhage, and lichenification (Werfel, 2009). According to previous studies, atopic skin disease is a type of systemic disease that causes various inflammatory diseases, including allergic diseases and

asthma. AD involves a complex pathogenesis process including multiple causes, such as chemical or environmental agents and abnormal immune responses (Brandt and Sivaprasad, 2011; Kim et al., 2014).

Mast cells are known to produce and secrete inflammatory mediators, such as histamine, growth factors, cytokines, and chemokines, and play a very important role in allergic diseases (Ye et al., 2017). Mast cells express a high-affinity IgE receptor, Fc ϵ RI, on the surface, are activated by reacting immediately with antigen, and act as important effector cells of immediate hypersensitivity. Antigen crosslinking of IgE bound to Fc ϵ RI is required to induce mast cell activation. Mast cells activated by IgE and Fc ϵ RI crosslinking are degranulated, and histamine and allergy mediators are generated and released in this process.

Abbreviations: AD, atopic dermatitis; DAD, diode array UV/VIS detector; DMSO, dimethyl sulfoxide; DNCB, 2,4-dinitrochlorobenzene; DNP-HAS, dinitrophenyl-human serum albumin; ELISA, Enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinases; FBS, fetal bovine serum; HPLC, high-performance liquid chromatography; JAK, Janus tyrosine kinases; JGJ, Jageum-Jung; MAPK, mitogen activated protein kinases; MDC, macrophage-derived chemokine; NO, Nitric Oxide; PBS, phosphate-buffered saline; p-NAG, 4-methyl-umbelliferyl-N-acetyl- β -d-glucosaminide; RANTES, regulated on activation, normal T-cell expressed and secreted; S.E., standard error; TARC, thymus and activation-regulated chemokine

* Corresponding authors.

E-mail addresses: jjuhye@kiom.re.kr (J.-H. Yang), dododo@kiom.re.kr (H.J. Do), lesd514@kiom.re.kr (E. Lee), nhyim23@kiom.re.kr (N.-H. Yim), wkcho@kiom.re.kr (W.-K. Cho), kpark@kiom.re.kr (K.-I. Park), jyma@kiom.re.kr (J.Y. Ma).

<https://doi.org/10.1016/j.jep.2018.04.016>

Received 13 November 2017; Received in revised form 30 March 2018; Accepted 12 April 2018

Available online 14 April 2018

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(Kawakami et al., 2009).

Keratinocytes form the majority of epidermal cells and play a very important role in AD pathogenesis. The keratinocytes play a role in maintaining skin homeostasis through recruiting immune cells, such as neutrophils, monocytes, and T cells, via the production of chemokines and cytokines. Stimulation of keratinocytes with TNF- α and IFN- γ result in the production of inflammatory chemokines and cytokines such as those regulated on activation and normal T-cell expressed and secreted/CCL5 (RANTES); IL-8; IL-6; thymus- and activation-regulated chemokine/CCL17 (TARC); and macrophage-derived chemokine (MDC). These chemokines and cytokines promote the inflammatory response with AD skin lesions and recruit inflammatory cells associated with the development and severity of AD.

Jageum-Jung (JGJ) is an oriental herbal formula comprising five herbs (*Melaphis chinensis* Bell, *Cremastra variabilis* Nakai, *Knoxia valerianoides* Thorel, *Euphorbia lathyris* L., and *Moschus moschiferus* L.) and has been used for detoxification and for treating cancer and inflammatory diseases in China, Japan, and Korea (Jong Won Kim et al., 2009). According to previous studies, JGJ reduced the production of NO and ROS in HepG2 hepatoma cells. In addition, JGJ shows G2/M phase cell cycle arrest inhibitory effect via suppressing the expression of cyclin B1, cdk4 and cyclin E, which are involved in the regulation of the cell cycle, and increasing the expression of Rb, p53, and p21 (Il-dong Park and Chong-kwan, 2002; Park In-Woo et al., 2018; Young-kee Cho et al., 2007).

Corticosteroids are widely used to treat AD; however, repeated administration of corticosteroids leads to serious side effects, including osteoporosis, bone avascular necrosis, skin thinning, and facial edema. Therefore, alternatives with minimal side effects in long-term use have been considered in drug development for chronic diseases, such as AD.

In this study, we clarify the anti-AD and anti-inflammatory effects of JGJ using TNF- α / IFN- γ treated human keratinocyte HaCaT cells and DNCB-treated mice.

2. Materials and methods

2.1. Cell culture and reagents

The human keratinocyte cell line HaCaT was provided by Dr. Misun Won, Korea Research Institute of Bioscience and Biotechnology. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin under a humidified atmosphere of 95% air and 5% CO₂ at 37 °C. Complete cell culture medium, FBS, and antibiotics were purchased from Lonza (Basel, Switzerland). Recombinant human TNF- α , IFN- γ , and ELISA kits for RANTES, TARC, IL-6, and IL-8 were obtained from BioLegend (San Diego, CA, USA). Primary and secondary antibodies used in western blot analyses were purchased from Cell Signaling Technology (Boston, MA, USA). All other chemicals were of reagent grade. Gallic acid, methylgallate, ellagic acid, muscone, and 1,2,3,4,6-penta-O-galloyl- β -D-glucose (PGG) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Militarin, ingenol, and eudesmin were purchased from Faces Biochemical Co., Ltd. (Wuhan, China). The purities of these eight reference standards were > 98%, and their structures are shown in Fig. 7. Acetonitrile of HPLC grade was purchased from Fisher (Pittsburgh, PA, USA) and trifluoroacetic acid (TFA) from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water was prepared using Puris-Evo UP Water system with Evo-UP Dio VFT and Evo-ROP Dico20 (Mirae ST Co., Ltd., Anyang, Gyeonggi-do, Korea).

2.2. Preparation of JGJ

JGJ was obtained from the Chung Shin herbal medicine shop (Daggu, Korea) and prepared as per a recipe described previously (Young-kee Cho et al., 2007). All constituents of JGJ have been recognized as standard products by the Korea Ministry of Food and Drug Safety. JGJ

powder was dissolved in dimethyl sulfoxide and centrifuged at 14,000 rpm for 10 min. The resulting supernatant was filtered through a 0.2 mm syringe membrane filter and then stored at 4 °C prior to its use for in vitro assays. For in vivo assays, JGJ powder was dissolved in a solution of 0.5% carboxymethyl cellulose (CMC) and 0.9% NaCl. For HPLC analysis of JGJ, the standard stock solutions were prepared by dissolving accurately weighed in 100% methanol (1 mg/mL). JGJ was extracted in 100% methanol (10 mg/mL) by ultrasonic for 30 min. All working solutions were filtered through 0.2 μ m syringe membrane filter from Whatman Ltd (Maidstone, UK) before injection into the HPLC-DAD system.

2.3. Cell-counting kit (CCK) assay

Cytotoxicity was analyzed using a CCK. CCK assay was performed as described previously (Yang et al., 2016a). Briefly, cells were allowed to attach to plates, treated with 50, 100, and 200 μ g/mL JGJ, and incubated at 37 °C in a 5% CO₂ incubator for 24 h. CCK solutions were added to each well, and the plates were incubated for 1 h. Optical density was measured at 570 nm using an ELISA plate reader (Infinite M200, Tecan, Männedorf, Switzerland).

2.4. Measurement of cytokine and chemokine production

HaCaT cells (1×10^6 cells/well) were seeded in six-well plates. After 18 h, the cells were treated with JGJ for 1 h at 37 °C in a 5% CO₂ incubator. After treatment, TNF- α /IFN- γ (10 ng/mL each) was added, and the incubation continued for another 24 h. Culture medium was then harvested, and chemokine levels in supernatants were measured using ELISA kits as per the manufacturer's instructions. The inhibitory effect of JGJ was determined based on absorbance at 450 nm, which was measured using an ELISA reader.

2.5. RNA isolation, reverse transcription, quantitative real-time PCR (Q-PCR)

Total RNA was isolated from HaCaT cells using the RNA-Spin total RNA extraction kit (iNtRoN, Daejeon, Korea) as per the manufacturer's instructions. Total RNA was reverse transcribed into cDNA using the AccuPower CycleScript RT premix (Bioneer). cDNA was amplified with AccuPower 2 $\times$ Greenstar qPCR Mastermix (Bioneer) using the CFX96 Touch™ Real-Time PCR Detection Systems (Bio-Rad Laboratories Inc., Irvine, CA, USA). For measuring TARC, RANTES, IL-8, and MDC mRNA, PCR conditions employed were those as previously described (Yang et al., 2015). Relative expression was calculated using the software CFX Manager™ (Bio-Rad Laboratories Inc., Irvine, CA, USA). Individual transcripts in each sample were assayed thrice and normalized to β -actin mRNA levels.

2.6. Western blot analysis

Protein expression was assessed using western blot analysis. Proteins were extracted in radio-immunoprecipitation assay lysis buffer (Millipore, Bedford, MA, USA) containing protease and phosphatase inhibitor cocktail (Roche, Basel, Switzerland). Cell lysates were then separated via electrophoresis in sodium dodecyl sulfate-polyacrylamide gels, and the separated proteins were electrotransferred onto PVDF membranes. The blots were blocked with 3% BSA and incubated with primary antibodies at 4 °C overnight and then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Specific proteins were detected using Super Signal West Femto Chemiluminescent Substrate (Thermo Scientific, Rockford, USA).

2.7. Immunocytochemistry (ICC) analysis

HaCaT cells were pretreated with JGJ for 1 h and stimulated with TNF- α /IFN- γ (10 ng/mL each) for 20 min. ICC analysis was performed following a previous method (J.H. Yang et al., 2017). The fixed cells were incubated with anti-p65 and anti-signal transducer and activator of transcription (STAT)-1 antibodies (mouse monoclonal antibodies; Cell Signaling Technology, MA, USA) and the cells were incubated with secondary Alexa-Fluor-488- and Alexa-568-labeled antibodies (goat anti-mouse; Life Technologies, CA, USA). Nuclei were stained using DAPI solution for 10 min at room temperature. All samples were then observed using a confocal microscope (FV3000 FLUOVIEW, Olympus, Tokyo, Japan).

2.8. Animals

Male BALB/c mice (5 weeks old) were purchased from Samtako BioKorea (Osan, Korea) and observed daily for 1 week during quarantine and acclimation. The mice were then divided into five groups ($n = 5$ per group): (1) negative control (vehicle), (2) DNCB + vehicle (control), (3) DNCB + 50 mg/kg JGJ, (4) DNCB + 100 mg/kg JGJ, and (5) 1 mg/kg dexamethasone. All groups were maintained under standard conditions of temperature ($22.5^\circ\text{C} \pm 0.5^\circ\text{C}$), humidity ($42.6\% \pm 1.7\%$), lighting (12 h, 8:00 a.m.–8:00 p.m., 290 lx), ventilation (10–15 times per hour), and diet (Teklad Global Diets, Harlan Laboratories Inc., USA). This study was conducted as per guidelines listed in the Pharmaceutical Affairs Act of the Korea Food and Drug Association and approved by the Institutional Animal Care and Use Committee of Korea Conformity Laboratories (IA11-00920).

2.9. RBL-2H3 cell culture and β -hexosaminidase release assay

RBL-2H3 cells were obtained from the Korean Cell Line Bank (Seoul, Korea) or the American Type Culture Collection (Manassas, VA, USA). IgE-sensitized cells were treated with JGJ for 30 min and then stimulated with 0.1 $\mu\text{g/mL}$ dinitrophenyl-human serum albumin (DNP-HSA) for 4 h. The culture medium of IgE-sensitized RBL-2H3 cells treated with JGJ were mixed with 25 μL of the 4-methyl-umbelliferyl-N-acetyl- β -D-glucosaminide (10 mM, p-NAG) in sodium citrate buffer (0.1 M, pH 4.5) and then incubated for 1 h at 37°C according to a previous report (Park et al., 2016). The absorbance was measured at 405 nm using a microplate reader.

2.10. Induction of AD and drug treatment

The AD-like mouse model involved induction as described previously (Yang et al., 2016b). DNCB dissolved in an acetone:olive oil mixture (3:1 vol/vol) was applied to the dorsal skin and both ears of BALB/c mice to induce AD-like symptoms and skin lesions. JGJ dissolved in saline + 0.5% CMC (10-mL/kg body weight) was orally administered by gavage at a dose of 50 or 100 mg/kg thrice a week for 4 weeks (days 0–24), and dexamethasone (1 mg/kg; Sigma-Aldrich) was used as a control drug.

2.11. Ear thickness measurements and histopathological analysis

Ear thickness in each mouse was measured using a micrometer (Mitutoyo, Kawasaki, Japan). To minimize variation, a single investigator performed all measurements. Dorsal skin lesion samples from each mouse were fixed with 4% paraformaldehyde overnight at room temperature. Hematoxylin and eosin staining and analysis of fixed samples were performed by a commercial company (Garam Meditech, South Korea). All samples were observed using a microscope (Nikon Eclipse Ti, Nikon, Tokyo, Japan), and data are representative of five observations.

2.12. Chromatographic conditions

The analytical HPLC data were obtained using a Dionex Ultimate 3000 system equipped with binary pump, auto-sampler, column oven, and diode array UV/VIS detector (DAD). The output signal of the detector was recorded using a Chromeleon software for HPLC system. Chromatographic separation was achieved on a Xbridge[®] C₁₈ column (5 μm , 4.6×250 mm, Waters Co., Milford, MA, USA) using 0.1% TFA water (v/v); solvent A and acetonitrile; solvent B as mobile phase at a flow rate of 1 mL/min. The HPLC elution condition was optimized as follows: 0–5 min, 5% B; 5–20 min, 5–15% B; 20–50 min, 15–30% B; 50–70 min, 30–70% B. The column oven and auto-sampler injection volume were set at 40°C and 10 μL , respectively. The detected wavelength was set at 280 nm and total run time was 70 min.

2.13. Statistical analysis

Data were analyzed using the software GraphPad Prism ver 5.0 (GraphPad Software, San Diego, CA, USA). Data are represented as means $\pm$ S.E. and evaluated using Student's *t*-test or analysis of variance. A *p*-value of < 0.05 was considered statistically significant. All experiments were performed at least in triplicate.

3. Results

3.1. Effects of JGJ on HaCaT cell viability

As shown in Fig. 1, JGJ showed some cytotoxic effect on HaCaT cells at a concentration of 200 $\mu\text{g/mL}$ but not at 100 $\mu\text{g/mL}$. Therefore, the experiment was conducted with the highest concentration selected as 100 $\mu\text{g/mL}$ in the next test.

3.2. JGJ inhibits production of chemokines and cytokines in TNF- α - and IFN- γ -treated human keratinocytes

To assess the anti-inflammatory effects of JGJ in AD, inflammatory cytokines and chemokines produced upon TNF- α /IFN- γ co-stimulation in HaCaT human keratinocytes were measured. Pro-inflammatory chemokine and cytokines, including RANTES, TARC, IL-8, and IL-6, have been found to be produced or secreted from numerous inflammatory diseases, including AD (Tapia et al., 2007). The cells were pretreated with JGJ for 1 h, and then stimulated with TNF- α /IFN- γ for 24 h; subsequently, the supernatants were analyzed for RANTES, TARC, IL-6, and IL-8 using ELISA. We found that specific cytokines or chemokines were excessively produced after simultaneous TNF- α /IFN- γ stimulation and were dose-dependently inhibited by JGJ. The results showed that JGJ suppressed the production of IL-8, IL-6 and, conspicuously, both TARC and RANTES in a dose-dependent manner (Fig. 2A and B).

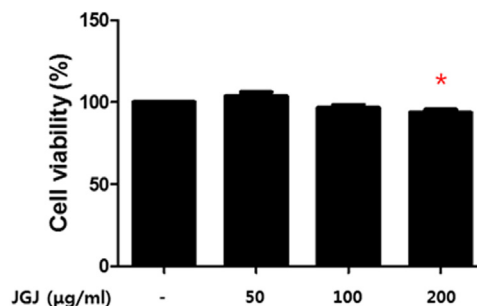


Fig. 1. Cell toxicity of JGJ in HaCaT cells. Cytotoxicity of JGJ was determined using CCK-8 assays. After HaCaT cell attachment in 96-well plates, indicated concentrations of JGJ were added and the plates incubated for 24 h. Data are represented as mean $\pm$ S.E. of three independent experiments. JGJ, Jageum-Jung; CCK, cell-counting kit; S.E., standard error.

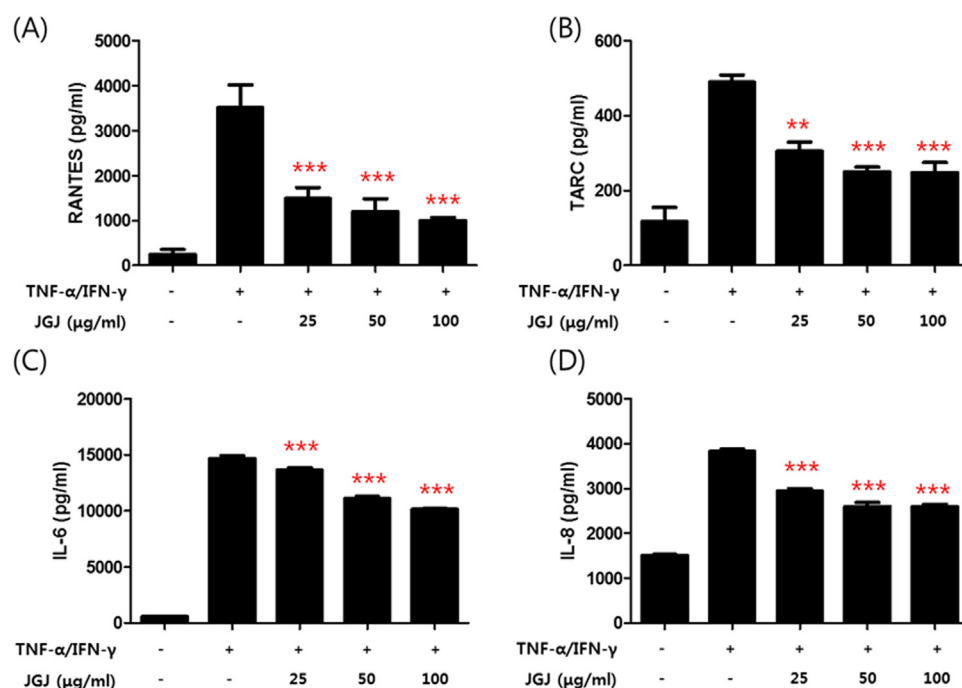


Fig. 2. Effect of JGJ on the production of inflammatory mediators from HaCaT cells stimulated with TNF-α / IFN-γ. Production of (A) RANTES, (B) TARC, (C) IL-6, and (D) IL-8 was determined using culture supernatants of HaCaT cells stimulated for 24 h with TNF-α/IFN-γ. Cells were pretreated with indicated concentrations of JGJ (25, 50, and 100 μg/mL) for 1 h and then stimulated with TNF-α/IFN-γ (10 ng/mL each) for 24 h. Data are represented as mean ± S.E. of three independent experiments. ***p < 0.001 compared with the TNF-α/IFN-γ-treated control group. JGJ, Jageum-Jung; TNF, tumor necrosis factor; IFN, interferon; RANTES, regulated on activation, normal T-cell expressed and secreted/CCL5; TARC, thymus- and activation-regulated chemokine/CCL17; IL, interleukin; S.E., standard error.

3.3. JGJ inhibits mRNA expression of chemokines in TNF-α- and IFN-γ-treated human keratinocytes

The effects of JGJ on mRNA expression of IL-8, RANTES, TARC, and MDC from HaCaT cells was quantified using qPCR. We found that the mRNA level of the inflammatory mediators increased by stimulation with TNF-α/IFN-γ was significantly reduced (Fig. 3).

3.4. JGJ inhibits p38 phosphorylation in TNF-α/IFN-γ-stimulated HaCaT cells

Immunoblotting was used to determine the effects of JGJ on the phosphorylation of MAPK in HaCaT cells (Fig. 4). Results showed that the levels of phosphorylated p38 MAPKs were reduced because of JGJ treatment in TNF-α/IFN-γ-stimulated HaCaT cells but the treatment did not affect total p38 expression (Fig. 4A and B).

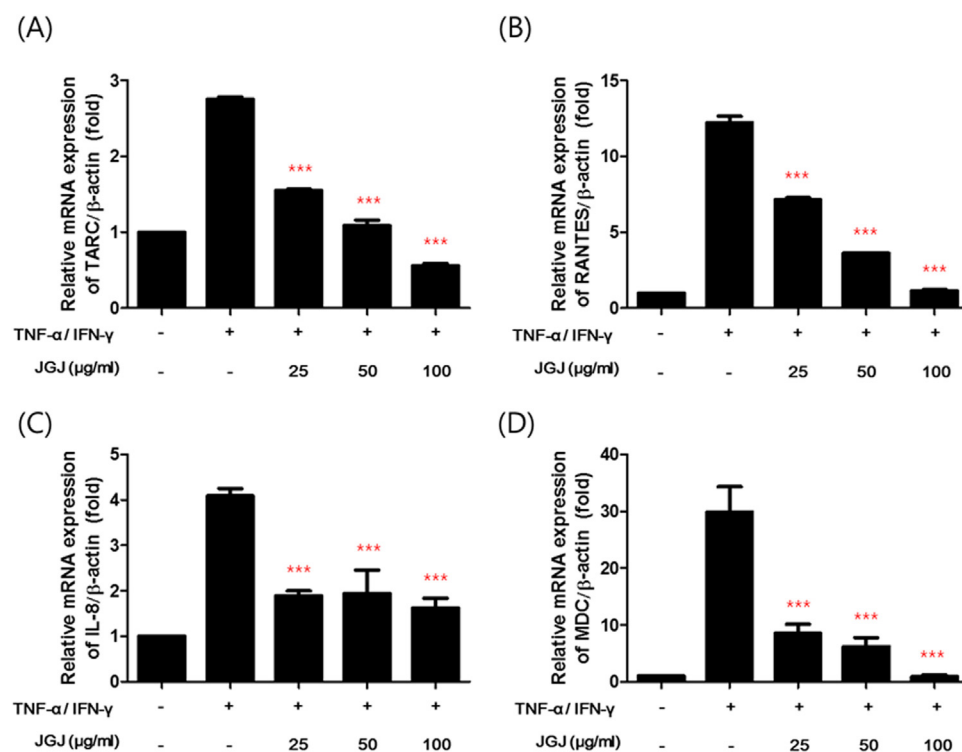


Fig. 3. Effect of JGJ on the mRNA expression of inflammatory mediators from HaCaT cells stimulated with TNF-α / IFN-γ. HaCaT cells were pretreated with JGJ for 1 h and then stimulated with TNF-α/IFN-γ (10 ng/mL each) for 6 h. Total mRNA was extracted, and levels of (A) TARC, (B) RANTES, (C) IL-8, and (D) MDC-1 were determined using real-time quantitative PCR. Data are represented as mean ± S.E. of three independent experiments. ***p < 0.001 compared with the TNF-α/IFN-γ-treated control group. JGJ, Jageum-Jung; TNF, tumor necrosis factor; IFN, interferon; RANTES, regulated on activation, normal T-cell expressed and secreted/CCL5; TARC, thymus- and activation-regulated chemokine/CCL17; IL, interleukin; MDC-1, mediator of DNA damage checkpoint protein 1; S.E., standard error.

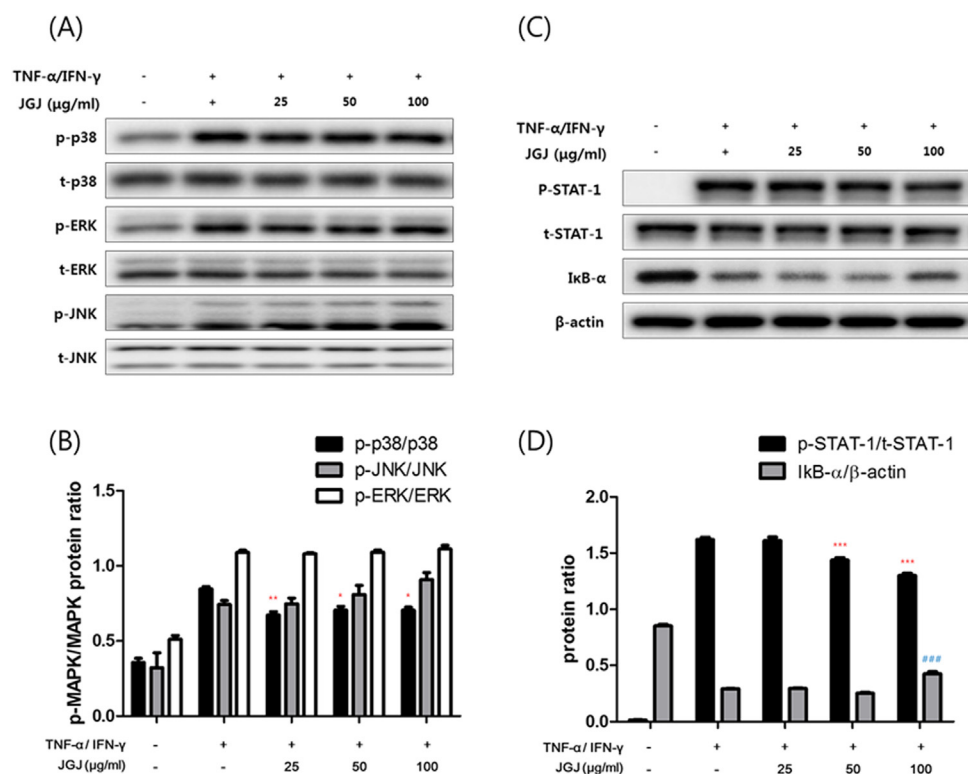


Fig. 4. Effects of JGJ on MAPK, STAT-1, and IκB-α degradation from HaCaT cells stimulated with TNF-α/IFN-γ. HaCaT cells were pretreated with JGJ for 1 h and then stimulated with TNF-α/IFN-γ (10 ng/mL each) for 30 min. Levels of phosphorylation and expression were determined by western blot using the indicated antibodies. (A) Effects of JGJ on MAPK phosphorylation in HaCaT cells. (B) Effects of JGJ on STAT-1 activation and IκB-α degradation. Blots were quantified using densitometry, and normalized to total protein and β-actin. Bars represent quantitative densities of bands. Relative protein abundance was calculated for the indicated ratios. Data are represented as mean ± S.E. of three independent experiments. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, and ###*p* < 0.001 compared with the TNF-α/IFN-γ-treated control group. JGJ, Jageum-Jung; TNF, tumor necrosis factor; IFN, interferon; MAPK, mitogen-activated protein kinase; STAT-1, signal transducer and activator of transcription 1; S.E., standard error.

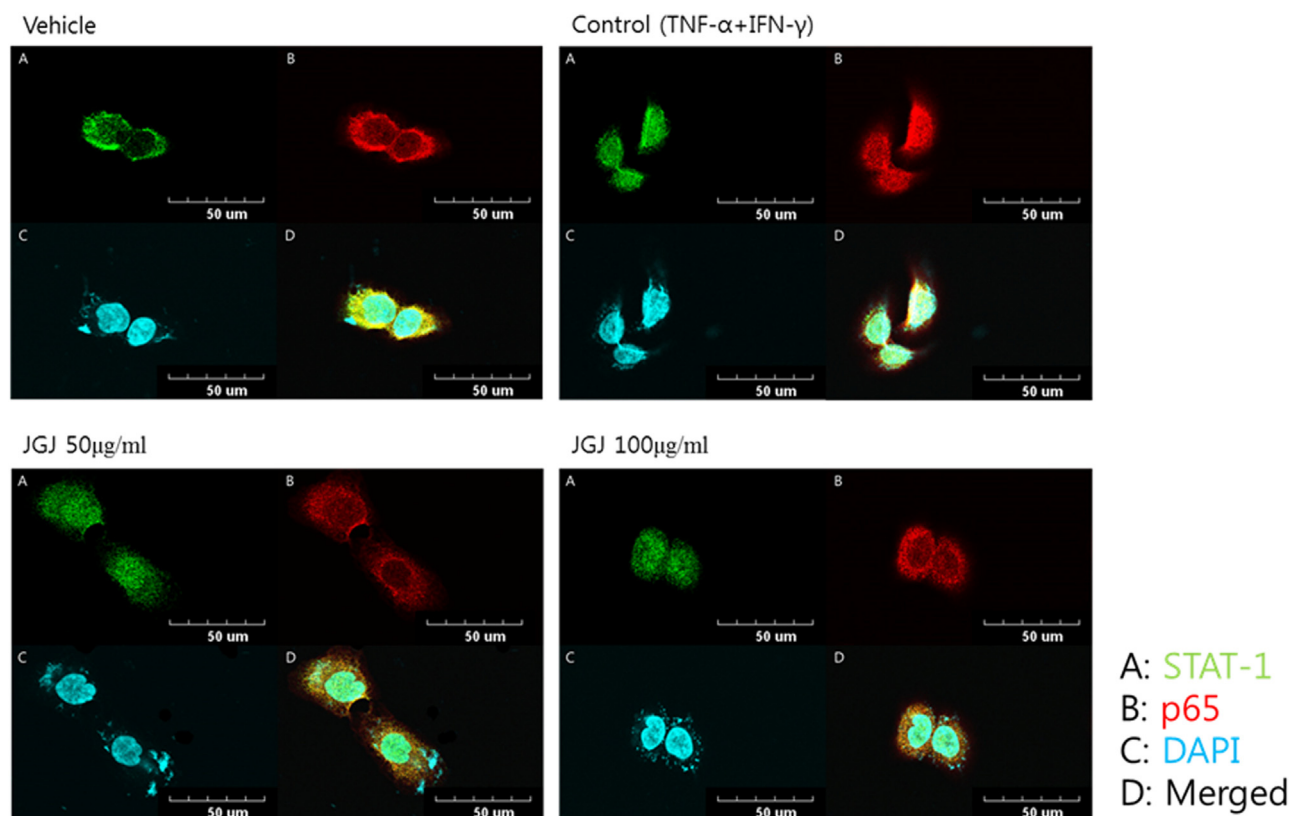


Fig. 5. Effects of JGJ on STAT-1 and NFκB p65 translocation from HaCaT cells stimulated with TNF-α/IFN-γ. Confocal microscopy analysis of NF-κB p65 and STAT-1 in HaCaT cells. Cells were pretreated with JGJ (50 and 100 μg/mL) for 1 h and stimulated with TNF-α/IFN-γ for 20 min. HaCaT cells were triple stained for STAT-1 (green, A), NF-κB p65 (red, B), and DAPI (blue, C). Merged images (Merged, D) indicates colocalization. Vehicle (DMSO); Control (vehicle + TNF-α/IFN-γ). JGJ, Jageum-Jung; TNF, tumor necrosis factor; IFN, interferon; STAT-1, signal transducer and activator of transcription 1; DMSO, dimethyl sulfoxide; DAPI, 4',6-diamidino-2-phenylindole; S.E., standard error.

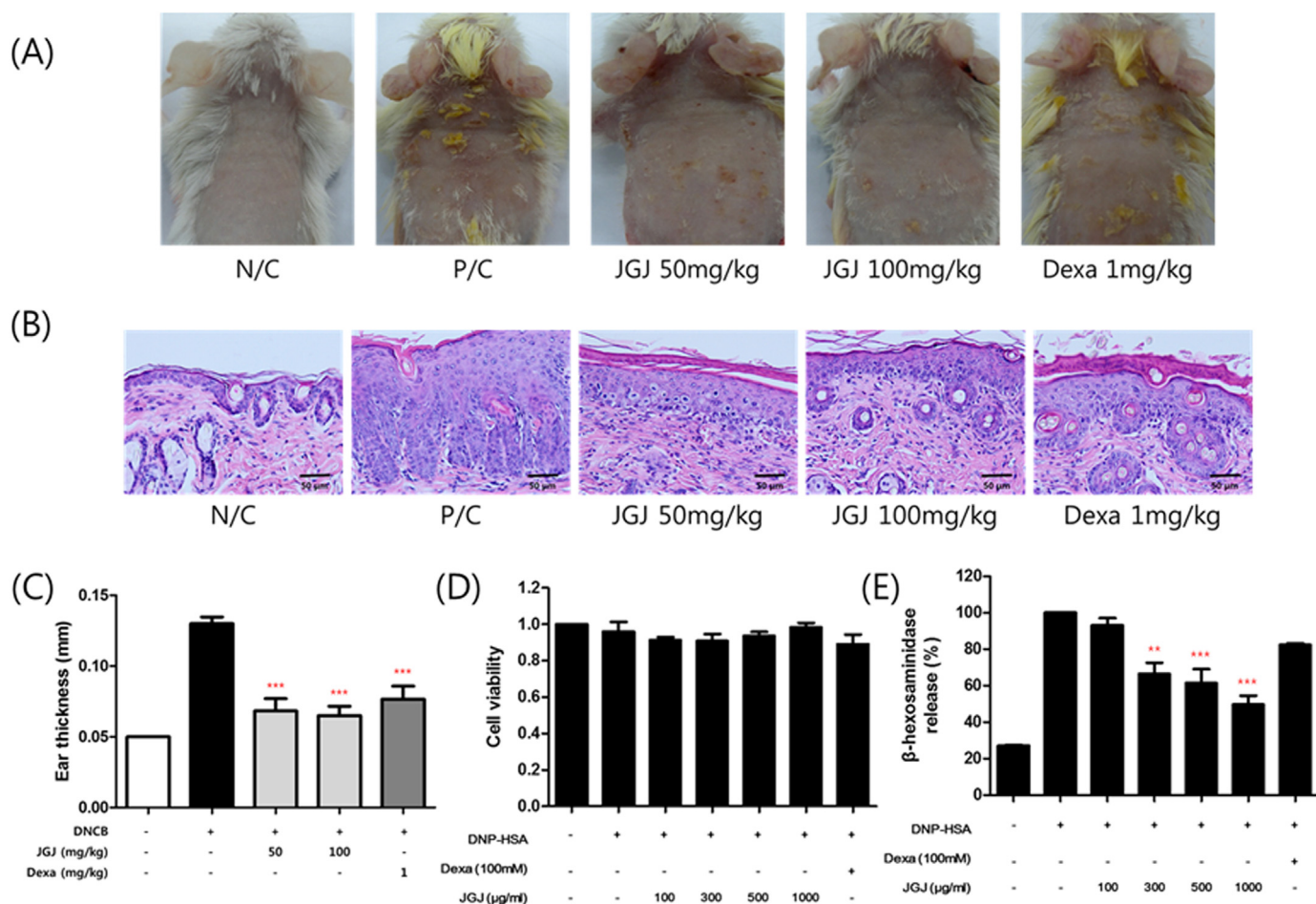


Fig. 6. Effects of JGJ on DNCB-induced AD-like symptoms and degranulation of mast cells. (A) Histopathological analyses for AD severity in DNCB-induced AD-like skin lesions in mic. (B) H&E staining of the dorsal skin for oral administration of JGJ or dexamethasone. (C) Ear thickness in mice with DNCB-induced AD-like skin lesions. (D) Effect of JGJ on viability of RBL-2H3 mast cells. (E) Release of β -hexosaminidase in RBL-2H3 mast cells. Cells were treated with JGJ for 30 min and then with DNP-HSA (0.1 μ g/mL) for 4 h. Data are represented as mean $\pm$ S.E. of least three independent experiments; data were tested using analysis of variance with Bonferroni's post-hoc testing. ** $p < 0.01$ and *** $p < 0.001$ compared with the DNP-HSA-treated group.

3.5. JGJ inhibits NF κ B and STAT-1 activation in TNF- α /IFN- γ -stimulated HaCaT cells

I κ B- α is a regulatory protein that binds to NF- κ B protein and blocks the activation signal to sequester it in the inactive state from the cytoplasm and inhibits NF- κ B. When I κ B- α is degraded, NF- κ B is activated and the inflammatory signaling pathway proceeds. We evaluated I κ B- α degradation and STAT-1 activation. As shown in Fig. 4C and D, activation of STAT-1 was increased by treatment with TNF- α / IFN- γ , but in the group treated with JGJ, the activation of STAT-1 was significantly decreased. I κ B- α was significantly degraded by treatment with TNF- α /IFN- γ ; however, pretreatment with JGJ reduced this degradation.

3.6. JGJ inhibits NF κ B-p65 and STAT-1 translocation in TNF- α /IFN- γ -stimulated HaCaT cells

We investigated the ability of JGJ to inhibit translocation factors in TNF- α /IFN- γ -stimulated HaCaT cells. We performed immunofluorescence microscopy to confirm whether treatment with JGJ could inhibit nuclear translocation of the inflammatory transcription factors NF κ B and STAT-1. TNF- α /IFN- γ markedly increases the translocation of NF κ B and STAT-1 into the nucleus and strengthens the fluorescence intensity (Red, NF κ B; Green, STAT-1) in the nucleus (Fig. 5). The results clearly suggested that the NF κ B and STAT-1 signaling pathway is affected by JGJ and that JGJ can therefore be used as an anti-AD agent.

3.7. JGJ inhibits mast cell degranulation and attenuates DNCB-induced AD-like skin lesions in mice

To investigate the effect of JGJ on AD-like skin lesions induced by DNCB, we repeatedly exposed the dorsal skin region of the BALB/c mouse and ear skin area to DNCB. We observed significantly improved skin conditions in JGJ-treated mice compared to control group mice (Fig. 6A). JGJ reduced epidermal thickness of dorsal skin and thickness of ear skin increased by DNCB dose dependently (Fig. 6B and C).

Furthermore, we analyzed β -HEX release to determine the effect of JGJ on mast cell degranulation and measured cell viability in RBL-2H3 cells (Fig. 6D and E). As shown in Fig. 6E, JGJ potently reduced β -HEX release and reduced degranulation response dose dependently in RBL-2H3 cells. These results showed that JGJ has a beneficial effect that can reduce symptoms of AD-like skin inflammation in mice.

3.8. HPLC analysis of JGJ

According to the maximum absorption of the standards, UV detector was set at 280 nm for HPLC analysis of eight standard compounds, including gallic acid (1), methylgallate (2), militarin (3), ellagic acid (4), PGG (5), ingenol (6), muscone (7), and eudesmin (8). The HPLC chromatograms of standard mixture and JGJ extract are presented in Fig. 7. The mixed standards were indicated at the retention time of 4.57 (1), 15.19 (2), 21.99 (3), 25.46 (4), 29.57 (5), 53.01 (6), 57.51 (7), and

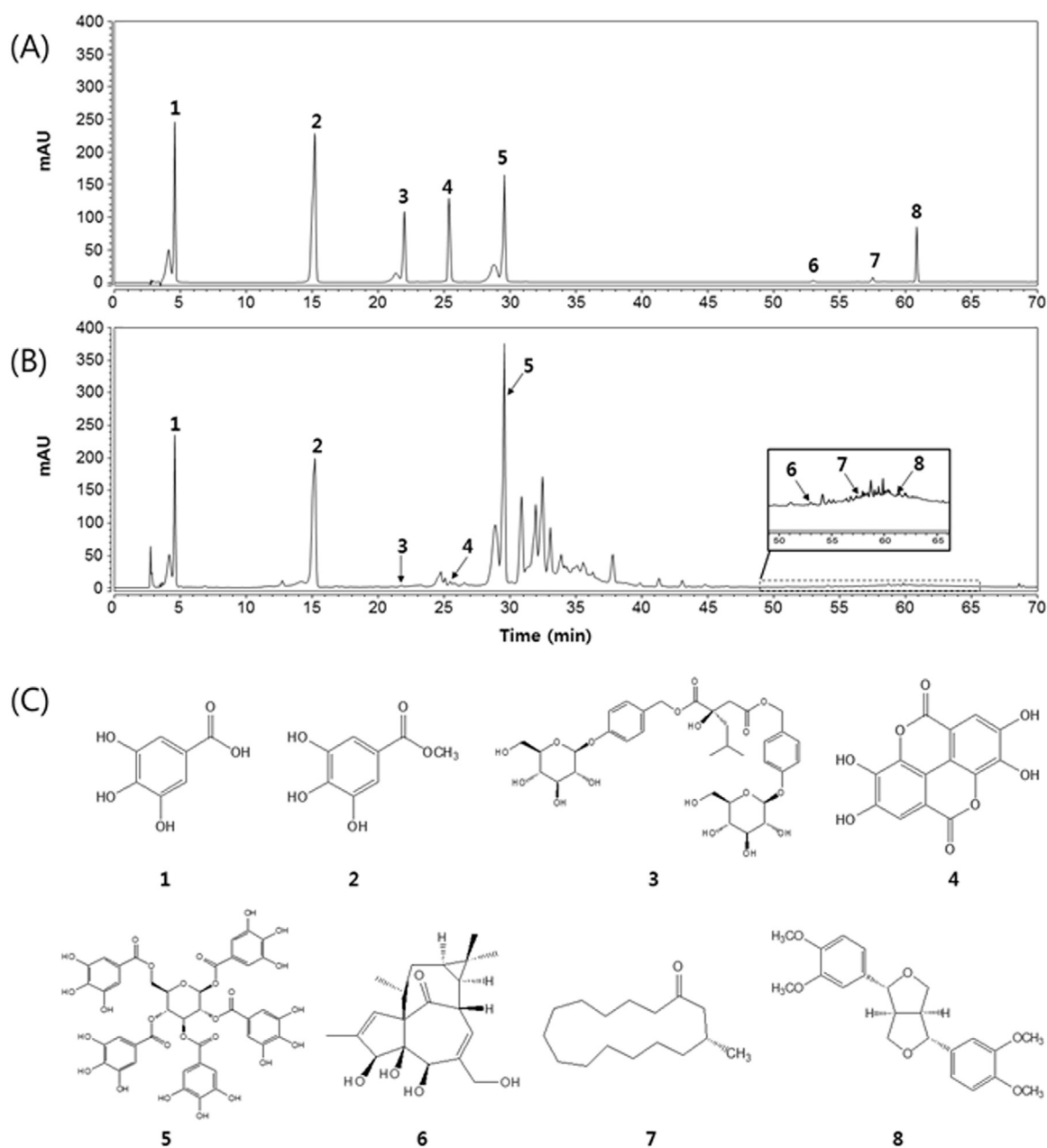


Fig. 7. Measurement of representative components of JGJ by HPLC analysis. HPLC chromatograms of eight standard compounds mixture (A) and JGJ (B) at 280 nm. 1, gallic acid; 2, methylgallic acid; 3, militarin; 4, ellagic acid; 5, PGG; 6, ingenol; 7, muscone; 8, eudesmin.

60.84 min (8). Under the same conditions, the components in JGJ were observed at 4.57 (1), 15.19 (2), 21.81 (3), 25.46 (4), 29.56 (5), 52.98 (6), 57.46 (7), and 60.84 min (8) by comparing ultraviolet (UV) spectral data with the standard compounds.

4. Discussion

AD is an acute and chronic inflammatory skin disease with high prevalence and recurrence rate. In acute phase, levels of infiltrated helper T (Th) cells and associated cytokines are increased, accompanied by IgE hyper-production due to the release of histamines and several pro-inflammatory mediators that cause pruritus in inflammatory skin lesions.

Keratinocytes and mast cells play central roles in AD. Keratinocytes are initiated by stimuli mechanical injuries such as scratching behavior and actively participate in the immune response of the skin by producing various solubility factors. Among the solubility factors,

keratinocyte-derived chemokines and cytokines are known to be directly related to the pathogenesis of AD (Asahina and Maeda, 2017). Mast cells are mainly observed in the epidermis of atopic patients, particularly in skin connective tissues and around capillaries (Imayama et al., 1995). High-affinity receptors for IgE (FcεRI) are expressed in mast cells and agglutinate or crosslink upon IgE binding and induce activation of cells. Mast cells are closely related to AD pathogenesis in the majority of patients with AD, characterized by increased IgE, increased sensitization to allergens, and massive secretion of inflammatory mediators from mast cells (Kawakami et al., 2009).

Pro-inflammatory mediators, such as cytokine, chemokine, IL-8, TARC, IL-6, RANTES, and MDC, play important roles in the progression of inflammation due to the activation of monocyte. These common mediators activate and recruit immune cells at the site of chronic inflammation and are associated with high levels of cytokine and chemokine expression in patients with AD that Th2 lymphocytes infiltration in skin lesions (Jeong et al., 2010; Lee et al., 2008; Pham et al.,

2017; Tanaka et al., 2006; Yang et al., 2015).

Cytokines such as TNF- α and IL-1 bind to the endothelial receptors and activate cell signaling, including the NF- κ B pathway, in resident cells such as keratinocytes, mast cells, and DCs (Leung et al., 2004). I κ B- α is a well-known regulatory protein that inhibits NF- κ B by masking the nuclear localization signal of NF- κ B protein and sequestering it from the cytoplasm to an inactive state. When I κ B- α is degraded, NF- κ B is activated and inflammatory signaling pathway proceeds.

IFN- γ is a potent inducer of the synthesis of cytokines and chemokines of keratinocytes and expresses high-affinity receptors of IFN- γ on the surface of human keratinocytes. In addition, activated keratinocytes synthesize adhesion molecules with IFN- γ (Pastore et al., 1998). IFN- γ has high-affinity receptors, such as IFN- γ R1 and IFN- γ R2, and binds to the Janus tyrosine kinases JAK1 and JAK2 immediately bound with the IFN- γ receptor. JAK1 and JAK2 induce docking and phosphorylation of STAT-1 and phosphorylate IFN- γ receptor. STAT-1 phosphorylation has been confirmed to play an important role in IFN-mediated innate immunity against microbial infections, particularly inflammatory reactions. In addition, IFN- γ is reported to stimulate the activation of MAPK and the activation of NF- κ B (Kang et al., 2015).

For the past few decades, anti-histamine agents, immunosuppressive agents, moisture care therapy, corticosteroids, or localized immunoregulatory agents have been used in treating AD, and whole body corticosteroid treatment is often chosen for severe cases of AD. However, topical corticosteroids and long-term use of oral have been reported to cause drug intolerance and side effects. Recently, a new local calcineurin inhibitor was used in treating AD, but it was not effective. Accordingly, further studies are needed to develop safer and more efficacious therapies against AD (Z. Yang et al., 2017).

In conclusion, JGJ improved AD-like skin lesions in DNCB-treated mice and inhibited degranulation of mast cell and production of pro-inflammatory chemokine by inhibiting TNF- α /IFN- γ -induced STAT-1 and NF κ B signaling in HaCaT cells. These results demonstrate that JGJ can be used as a candidate agent for treating AD.

Conflict of interest

The authors declare that there are no conflicts of interest.

Author contribution

J.Y.M. and K.I.P. developed the study design and revised the manuscript. J.H.Y. participated in the study design, performed the experiments, analyzed the data and wrote the draft manuscript H.J.D., E.L., N.H.Y. and W.K.C. performed the experiments and analyzed the data.

Acknowledgements

This work was supported by grants (No. K18101 and K18102) from the Ministry of Education, Science and Technology (MEST) and the Daegu Medi-city Fund (No. G17060).

References

Asahina, R., Maeda, S., 2017. A review of the roles of keratinocyte-derived cytokines and chemokines in the pathogenesis of atopic dermatitis in humans and dogs. *Vet. Dermatol.* 28 (1) (16–e15).

- Brandt, E.B., Sivaprasad, U., 2011. Th2 cytokines and atopic dermatitis. *J. Clin. Cell. Immunol.* 2 (3).
- Il-dong Park, C.-G.S., Chong-kwan Cho, 2002. A literal study of anti-tumor effects of Jakeumjung. 大田大學校 韓醫學研究所. 韓醫學編 10(2), 73–81.
- Imayama, S., Shibata, Y., Hori, Y., 1995. Epidermal mast cells in atopic dermatitis. *Lancet* 346 (8989), 1559.
- Jeong, S.I., Choi, B.M., Jang, S.I., 2010. Sulforaphane suppresses TARC/CCL17 and MDC/CCL22 expression through heme oxygenase-1 and NF- κ B in human keratinocytes. *Arch. Pharmacol. Res.* 33 (11), 1867–1876.
- Jong Won Kim, S.H.J., Jung, Heung Sik, Kang, Kyung Hwa, Lee, In. Sun, Lee, Sang Hoon, Kim, Kyu Kon, Lee, Young Tae, 2009. HyungSang medical approach to detoxification. *Korean J. Orient. Physiol. Pathol.* 23 (2), 278–287.
- Kang, N.J., Koo, D.H., Kang, G.J., Han, S.C., Lee, B.W., Koh, Y.S., Hyun, J.W., Lee, N.H., Ko, M.H., Kang, H.K., Yoo, E.S., 2015. Dieckol, a component of *Ecklonia cava*, suppresses the production of MDC/CCL22 via Down-regulating STAT1 pathway in interferon-gamma stimulated HaCaT human keratinocytes. *Biomol. Ther.* 23 (3), 238–244.
- Kawakami, T., Ando, T., Kimura, M., Wilson, B.S., Kawakami, Y., 2009. Mast cells in atopic dermatitis. *Curr. Opin. Immunol.* 21 (6), 666–678.
- Kim, G.D., Lee, S.E., Park, Y.S., Shin, D.H., Park, G.G., Park, C.S., 2014. Immunosuppressive effects of fisetin against dinitrofluorobenzene-induced atopic dermatitis-like symptoms in NC/Nga mice. *Food Chem. Toxicol.* Int. J. 66, 341–349.
- Lee, J.S., Kim, I.S., Ryu, J.S., Yun, C.Y., 2008. House dust mite, *Dermatophagoides pteronissinus* increases expression of MCP-1, IL-6, and IL-8 in human monocytic THP-1 cells. *Cytokine* 42 (3), 365–371.
- Leung, D.Y., Boguniewicz, M., Howell, M.D., Nomura, I., Hamid, Q.A., 2004. New insights into atopic dermatitis. *J. Clin. Invest.* 113 (5), 651–657.
- Park In-Woo, K.H.-Y., Jin-Hong, Cheon, Yin-Hao, Shih, Bok-Suk, Oh, S.R., Yoon, D.Y., 2018. Case series study about the effect of Jageum-Jung on atopic dermatitis. *J. Pediatr. Korean Med.* 32 (1), 11–18.
- Park, K.I., Kim, D.G., Yoo, J.M., Ma, J.Y., 2016. The herbal medicine KIOM-MA128 inhibits the antigen/IgE-mediated allergic response in vitro and in vivo. *Molecules* 21 (8).
- Pastore, S., Corinti, S., La Placa, M., Didona, B., Girolomoni, G., 1998. Interferon-gamma promotes exaggerated cytokine production in keratinocytes cultured from patients with atopic dermatitis. *J. Allergy Clin. Immunol.* 101 (4 Pt 1), 538–544.
- Pham, T.H., Kim, M.S., Le, M.Q., Song, Y.S., Bak, Y., Ryu, H.W., Oh, S.R., Yoon, D.Y., 2017. Fargesin exerts anti-inflammatory effects in THP-1 monocytes by suppressing PKC-dependent AP-1 and NF- κ B signaling. *Phytomedicine: Int. J. Phytother. Phytopharm.* 24, 96–103.
- Tanaka, K., Roberts, M.H., Yamamoto, N., Sugiura, H., Uehara, M., Hopkin, J.M., 2006. Upregulating promoter polymorphisms of RANTES relate to atopic dermatitis. *Int. J. Immunogenet.* 33 (6), 423–428.
- Tapia, B., Morel, E., Martin-Diaz, M.A., Diaz, R., Alves-Ferreira, J., Rubio, P., Padial, A., Bellon, T., 2007. Up-regulation of CCL17, CCL22 and CCR4 in drug-induced maculopapular exanthema. *Clin. Exp. Allergy: J. Br. Soc. Allergy. Clin. Immunol.* 37 (5), 704–713.
- Werfel, T., 2009. The role of leukocytes, keratinocytes, and allergen-specific IgE in the development of atopic dermatitis. *J. Invest. Dermatol.* 129 (8), 1878–1891.
- Yang, J.H., Hwang, Y.H., Gu, M.J., Cho, W.K., Ma, J.Y., 2015. Ethanol extracts of *Sanguisorba officinalis* L. suppress TNF- α /IFN- γ -induced pro-inflammatory chemokine production in HaCaT cells. *Phytomedicine: Int. J. Phytother. Phytopharm.* 22 (14), 1262–1268.
- Yang, J.H., Yoo, J.M., Cho, W.K., Ma, J.Y., 2016a. Anti-inflammatory effects of *Sanguisorbae Radix* water extract on the suppression of mast cell degranulation and STAT-1/Jak-2 activation in BMMC and HaCaT keratinocytes. *BMC Complement. Altern. Med.* 16, 347.
- Yang, J.H., Yoo, J.M., Cho, W.K., Ma, J.Y., 2016b. Ethanol extract of *sanguisorbae radix* inhibits mast cell degranulation and suppresses 2,4-dinitrochlorobenzene-induced atopic dermatitis-like skin lesions. *Mediat. Inflamm.* 2016, 2947390.
- Yang, J.H., Yoo, J.M., Lee, E., Lee, B., Cho, W.K., Park, K.I., Yeul Ma, J., 2017a. Anti-inflammatory effects of *Perillae Herba* ethanolic extract against TNF- α /IFN- γ -stimulated human keratinocyte HaCaT cells. *J. Ethnopharmacol.* 211, 217–223.
- Yang, Z., Liu, M., Wang, W., Wang, Y., Cao, B., Gao, Y., Chen, H., Li, T., 2017b. Pseudolaric acid B attenuates atopic dermatitis-like skin lesions by inhibiting interleukin-17-induced inflammation. *Sci. Rep.* 7 (1), 7918.
- Ye, J., Piao, H., Jiang, J., Jin, G., Zheng, M., Yang, J., Jin, X., Sun, T., Choi, Y.H., Li, L., Yan, G., 2017. Polydatin inhibits mast cell-mediated allergic inflammation by targeting PI3K/Akt, MAPK, NF- κ B and Nrf2/HO-1 pathways. *Sci. Rep.* 7 (1), 11895.
- Young-kee Cho, J.-Y.J., Shin, Yong-jeon, Seol, Jae-Kyun, Rhee, Jae-hwa, Won, Jin-hee, Dept, Goo Moon, 2007. Induction of apoptosis and cell cycle arrest by Jageum-Jung in HepG2 hepatoma cells. *Korean J. Orient. Int. Med.* 28 (4), 694–708.