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Genuine traditional Korean medicine, Naju Jjok (Chung-Dae, *Polygonum tinctorium*) improves 2,4-dinitrofluorobenzene-induced atopic dermatitis-like lesional skin

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ABSTRACT

Purpose: Naju Jjok (NJJ, *Polygonum tinctorium*) is a clear heat and release toxin medicinal. It has been used to treat various inflammatory diseases and as a dye in clothing in traditional Korean medicine. However, the effect of NJJ on atopic dermatitis (AD) has not been elucidated. Therefore, we examined whether NJJ would have an inhibitory effect on AD using the mimic AD murine model and *in vitro* model.

Methods: We treated NJJ on 2,4-dinitrofluorobenzene (DNFB)-induced AD-like skin lesions in NC/Nga mice, phorbol myristate acetate/calcium ionophore A23187-stimulated human mast cell line (HMC-1) cells, and anti-CD3/anti-CD28-stimulated splenocytes. Histological analysis, ELISA, PCR, and Western blot analysis were performed.

Results: The oral administration with NJJ suppressed the total clinical severity in DNFB-induced AD-like lesional skin. NJJ significantly suppressed the levels of inflammatory mRNA and protein in AD-like lesional skin. NJJ significantly suppressed the levels of IgE and interleukin-4 in the serum of DNFB-induced AD mice. The expression of mast cells-derived caspase-1 was suppressed by NJJ in AD-like lesional skin. In addition, topical application with NJJ improved clinical symptoms in DNFB-induced AD mice. The topical application with NJJ significantly suppressed the levels of IgE and histamine in the serum of DNFB-induced AD mice. NJJ suppressed the production and mRNA expression of TSLP by blockade of caspase-1 signal pathway in the activated HMC-1 cells. Furthermore, NJJ significantly decreased the production of tumor necrosis factor- α from the stimulated splenocytes.

Conclusions: In conclusion, these results propose curative potential of natural dye, NJJ by showing the scientific evidence on anti-AD effect of NJJ which has been used traditionally.

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Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease associated with cutaneous hyperreactivity to environmental triggers. The AD results from interactions between susceptibility genes, the environment, defective skin barrier function, and immunologic responses (Leung et al. 2004). Clothing can be an effective barrier against flare-inducing factors and persistent scratching, allowing more rapid induction of the eczematous lesions (Ricci et al. 2012). The textile dyes can cause lymphomatoid dermatitis to volunteers

(Narganes et al. 2013). The fluorescent dye in clothing caused the contact Urticaria (Sugiura et al. 2010).

Polygonum tinctorium (Naju Jjok, NJJ) has been used for centuries in traditional Korean medicine (TKM) and as a textile dye traditionally. The TKM describes that the pathological heat toxin in body can induce pain, sore and ulcer, swollen sore, or abscess (Sin 1997), etc. Clear heat and release toxin medicinal have been mostly used at treatment for these diseases. NJJ keeps cold character and has been used to treat sore and ulcer (Sin 1997). It has been observed to have latent-heat-clearing, anti-pyretic, anti-inflammatory and detoxicant actions (Kim et al. 1998). Kim et al. (1998) have reported that NJJ inhibited mast cell-dependent anaphylactic reactions.

The lesional skin in NC/Nga mice of AD murine models are characterized by infiltration of inflammatory cells such as mast cells as well as high expressions of Th2 cytokines and chemokines (Vestergaard et al. 1999). And repeated skin exposure to

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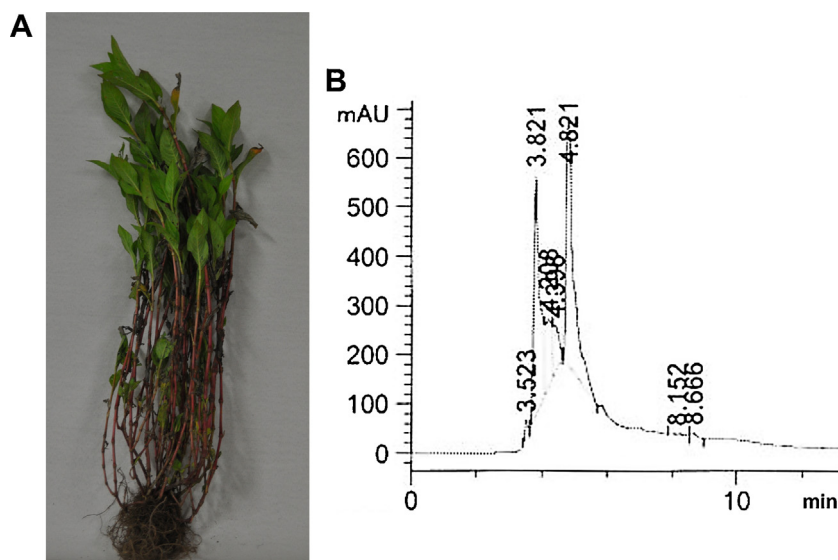


Fig. 1. (A) Feature of the whole plant of NJJ. (B) HPLC chromatogram of NJJ.

2,4-dinitrofluorobenzene (DNFB) caused AD-like lesional skin (Kim et al. 2010a,b). Mast cells play an important role in both adaptive and innate immunity and increase in immunological skin diseases, including contact dermatitis and AD (Navi et al. 2007). Thymic stromal lymphopoietin (TSLP) is an important immunologic factor involved in the pathogenesis of AD (Iyengar et al. 2013). And the expression of TSLP was increased in lesional skin of patients with acute and chronic AD (Zhu et al. 2011). TSLP was expressed in activated mast cells and triggered allergic inflammation (Liu 2006). Caspase-1 is the best characterized inflammatory caspase (Song et al. 2012). Receptor interacting protein2 (RIP2) is a caspase recruitment domain-containing kinase that interacts with caspase-1 and plays an important role in NF- κ B activation (Song et al. 2012). TSLP was mediated by receptor interacting protein2 (RIP2)/caspase-1 signal pathway in mast cells (Moon and Kim 2011).

The effect of NJJ on AD has not yet been clarified. Therefore, we studied the effect of NJJ on DNFB-induced AD-like lesional skin of NC/Nga mice, phorbol myristate acetate and calcium ionophore A23187 (PMACI)-activated human mast cell line (HMC-1) cells, and anti-CD3 and anti-CD28-stimulated splenocytes.

Materials and methods

Reagents

We purchased Isocove's Modified Dulbecco's Medium (IMDM) from Gibco BRL (Grand Island, NY, USA); PMA, calcium ionophore A23187, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Sigma Chemical Co. (St. Louis, MO, USA); TSLP antibodies from R&D Systems (Minneapolis, MN, USA); RIP2, caspase-1, and actin antibodies from Santa Cruz Biotechnology (Santacruz, CA, USA); DNFB from Sigma Chemical Co. (St. Louis, MO, USA); IgE, IL-4, IL-6, and TNF- α antibodies from BD Pharmingen (Torreyana Road, San Diego, CA, USA). NJJ has been cultivated traditionally in Naju, republic of Korea. We were provided with whole plant of NJJ (Fig. 1A) from Naju city. For all experiments, the whole plant of NJJ was extracted with distilled water (DW) at 80°C for 3 h. The filtered crude extracts were lyophilized and reduced to powder. The yield of dried extract from starting materials was about 18% (w/w). And then the powder of NJJ was dissolved in DW and filtered with

0.22 μ m syringe filter. We made experiments with the filtered NJJ extract.

HPLC analysis

NJJ extract was passed through a 0.2 μ m membrane filter, and 20 μ l aliquots of the filtrate were injected into the HPLC. Hyper-sil GOLD C18 (250 mm $\times$ 4.6 mm; particle size 5 μ m) was used as an analytical column. The mobile phases consisted of solvent A (DI water) and solvent B (acetonitrile). The analysis was carried out at a flow rate of 0.7 ml/min. The injection volume was 10 μ l. Constituents were detected on UV 230 nm (Fig. 1B).

Animals

Male NC/Nga mice were obtained from Charles River Laboratories International, Inc. (Yokohama, Japan). And the animals were maintained under conventional condition and performed under approval from the animal care committee of Kyung Hee University [Protocol Number. KHUASP (SE)-11-009]. Mice were sacrificed with CO₂ inhalation.

Sensitization with DNFB

For active sensitization, 100 μ l 0.15% DNFB dissolved in acetone was topically challenged to the shaved abdominal skins of NC/Nga mice. A week later, the shaved dorsal skins of NC/Nga mice were challenged with 50 μ l 0.15% DNFB. At that time, NJJ (1, 10, and 100 mg/kg) was orally administered to DNFB-challenged mice. The same volume of acetone was challenged to the shaved dorsal skin and saline was orally administered as an unchallenged vehicle group. In the same way, NJJ (100 μ g/ml) or saline were topically applied to DNFB-challenged mice. Dorsal skin samples and serum were obtained 4 h after the last DNFB challenge. After anesthetization, blood was withdrawn from the heart of mouse into syringes. And then, serum was prepared by centrifugation at 3400 rpm at 4°C for 10 min.

Histological analysis

Formaldehyde (10%)-fixed dorsal skin samples were embedded in paraffin, cut into 4- μ m-thick sections. After dewaxing and dehydration, sections were stained with hematoxylin and eosin (H&E)

to visualize epidermal thickness and count the number of inflammatory cells.

Reverse transcription (RT)-PCR analysis

Using an easy-BLUETM RNA extraction kit (iNtRON Biotech, Republic of Korea), we isolated the total RNA from HMC-1 cells in accordance with the manufacturer's specifications. We performed RT-PCR with the following primers: TSLP (5' TGC AAG TAC TAG TAC GGA TGG GG C 3'; 5' GGA CTT CTT GTG CCA TTT CCT GAG 3'); IL-4 (5' ACG GAG ATG GAT GTG CCA AA 3'; 5' CGA GTA ATC CAT TTG CAT GA 3'); IFN- γ (5' TAC TGC CAC GGC ACA GTC ATT GAA 3'; 5' GCA GCG ACT CCT TTT CCG CTT CCT 3'); IL-6 (5' CGG GAT CCA TGT TCC CTA CTT CAC AA 3'; 5' CCC AAG CTT CTA CGT TTG CC 3'); TNF- α (5' TAC AGG CTT GTC ACT CGA AT 3'; 5' ATG AGC ACA GAA AGC ATG AT 3'); thymus and activation-regulated chemokine/CCL17 (TARC) (5' CAG GAA GTT GGT GAG CTG GTA TA 3'; 5' TTG TGT TCG CCT GTA GTG CAT A 3'). Reference gene GAPDH (5' GGC ATG GAC TGT GGT CAT GA 3'; 5' TTC ACC ACC ATG GAG AAG GC 3') was used to verify that equal amounts of RNA. The annealing temperature was 62 °C for TSLP, IL-4, and IFN- γ ; 50 °C for IL-6; 60 °C for TNF- α and GAPDH; 55 °C for TARC. Products were electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide. The levels of each mRNA were normalized to the level of the GAPDH.

Cytokines assay

The levels of TSLP, IL-4, IL-6, IgE, and TNF- α were determined using a sandwich ELISA method according to the manufacturer's instructions (R & D system Inc., Minneapolis, MN, USA; Pharmingen, San Diego, CA, USA). The single cells (2.5×10^6 /ml) from the spleen of normal NC/Nga mouse were stimulated with immobilized anti-CD3 (2 μ g/ml) and soluble anti-CD28 antibodies (4 μ g/ml) or treated with NJJ in 24-well plates. A cell supernatant was measured by ELISA method.

Analysis of confocal laser-scanning microscope

Dorsal skin samples were immediately fixed with 4% formaldehyde and embedded in paraffin. After dewaxing and dehydration, sections were blocked with fetal bovine serum (FBS) followed by 1 h of incubation and then stained with anti-rabbit caspase-1 and FITC-conjugated anti-rabbit IgG, (Santacruz Biotechnology), PE-conjugated anti-mouse c-Kit antibodies (BD Pharmingen). 4',6-Diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA) containing mounting medium was used to counterstain the nuclei. The images of stained cells and specimens were randomly visualized using a confocal laser-scanning microscope.

Histamine assay

The histamine contents from serum were measured by the OPA spectrofluorometric procedure. The fluorescent intensity was measured at 440 nm (excitation at 360 nm) in spectrofluorometer.

HMC-1 cells culture

HMC-1 cells were cultured as previously described (Song et al. 2012).

MTT assay

Cell viability was measured by a MTT assay. Briefly, 500 μ l of HMC-1 cell (4×10^5) suspension was treated with NJJ for 2 h and

stimulated with PMACI for 8 h and then harvested. The cell suspension containing MTT solution (5 mg/ml) was incubated at 37 °C for an additional 4 h. After washing the supernatant out, the insoluble formazan product was dissolved in DMSO. Then, the optical density of 96-well culture plate was determined using an ELISA method reader at 540 nm.

Quantitative real time-polymerase chain reaction (PCR) analysis

Quantitative real time-PCR was performed using a SYBR Green master mix and the detection of mRNA was analyzed using an ABI StepOne real time-PCR System (Applied Biosystems, Foster City, CA, USA). Primer sequences for the reference gene GAPDH and gene TSLP were as follows: GAPDH (5' TCG ACA GTC AGC CGC ATC TTC TTT 3'; 5' ACC AAA TCC GTT GAC TCC GAC CTT 3'); TSLP (5' TAT GAG TGG GAC CAA AAG TAC CG 3'; 5' GGG ATT GAA GGT TAG GCT CTG G 3'). Typical profile times used were the initial step, 95 °C for 10 min followed by a second step at 95 °C for 10 s for 40 cycles with a melting curve analysis. The level of target mRNA was normalized to the level of the GAPDH and compared to the control. Data were analyzed using the $\Delta\Delta$ CT method.

Western blot analysis

Western blot analysis was performed as previously described (Song et al. 2012).

Statistical analysis

The results shown are a summary of the data from at least-three experiments and are presented as the mean $\pm$ SD. Statistical evaluation of the results was performed by an independent *t*-test and ANOVA with Tukey post hoc test. The results were considered significant at a value of $p < 0.05$.

Results

Oral administration of NJJ improves clinical symptoms in DNFB-induced AD mice

We studied whether the oral administration of NJJ could suppress the clinical symptoms in DNFB-induced AD mice. The noticeable erythema, dryness, and erosion were present in lesional skin of DNFB-induced AD mice; whereas the oral administration of NJJ markedly improved these phenotypes in AD-like lesional skin (Fig. 2A). And we investigated thickness of epidermis by H&E staining. The thickness of epidermis was suppressed in lesional skin of NJJ-administered and DNFB-challenged group compared with that of the DNFB-challenged group (Fig. 2A). Furthermore, NJJ significantly suppressed the infiltration of inflammatory cells ($p < 0.05$, Fig. 2B). However, NJJ did not significantly suppress the scratching behavior (data not shown).

Oral administration of NJJ suppresses the levels of TSLP in lesional skin of DNFB-induced AD mice

As oral administration of NJJ improved clinical symptoms, we studied the inhibitory effect of NJJ on the levels of TSLP which is a trigger factor of AD (Iyengar et al. 2013). The mRNA expression and protein level of TSLP increased by DNFB challenge were suppressed by the oral administration of NJJ (Fig. 3A and B). In addition, the mRNA expressions of IL-4, IFN- γ , IL-6, TNF- α , and TARC were suppressed by NJJ (Fig. 3A and B). The protein levels of IL-4 and IL-6 were significantly suppressed by NJJ ($p < 0.05$, Fig. 3B). NJJ significantly suppressed the levels of serum IgE and IL-4 ($p < 0.05$,

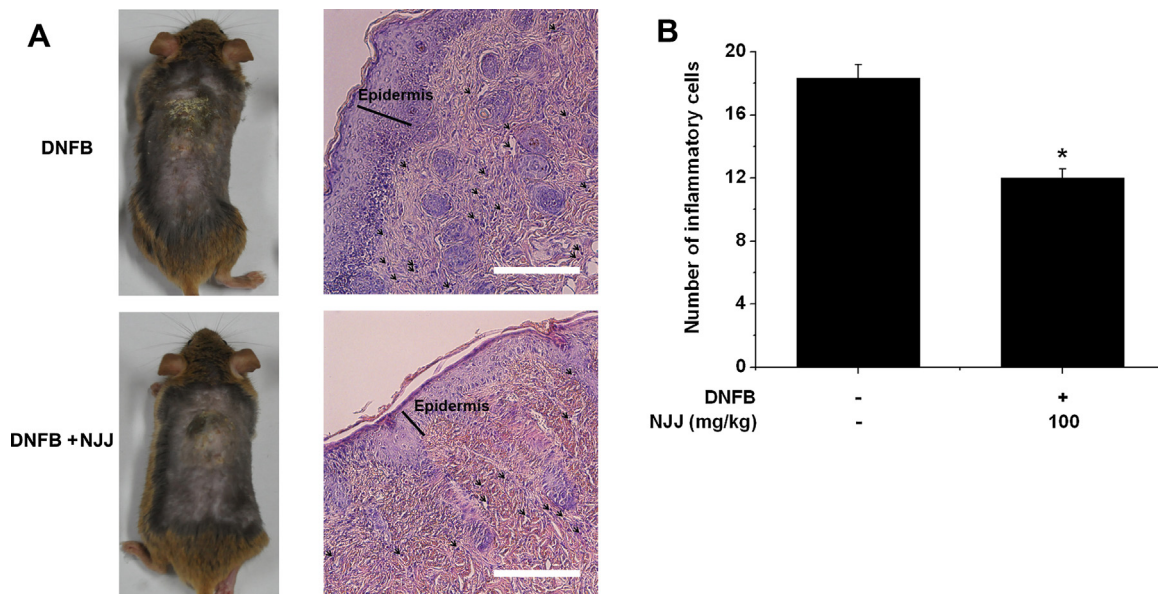


Fig. 2. Oral administration of NJJ improves clinical symptoms in DNFB-induced AD mice. NJJ (100 mg/kg) was orally administered to DNFB-challenged mice. The clinical features were observed 4 h after the last DNFB challenge. Histological analysis of lesional skin was examined by H&E staining for (A) epidermal thickness and (B) inflammatory cells. Representative photomicrographs were examined at 400 $\times$ magnification. The inflammatory cells were indicated by arrows. Five tissue sections ($1.5 \times 10^3 \mu\text{m}^2$) per mouse were randomly selected and counted. DNFB, saline-orally administered and DNFB-challenged mice; DNFB + NJJ, NJJ-orally administered and DNFB-challenged mice. * $p < 0.05$, significantly different from unchallenged vehicle group. $n = 5$.

Fig. 3C). However, NJJ did not significantly suppress the level of serum histamine (data not shown).

Oral administration of NJJ suppresses the expression of caspase-1 in lesional skin of DNFB-induced AD mice

As TSLP was mediated through the activation of caspase-1 in the HMC-1 cells (Moon and Kim 2011), we studied whether NJJ could regulate the level of caspase-1 in AD-like lesional skin. NJJ significantly suppressed the mast cells-derived caspase-1 expression in AD-like lesional skin (Fig. 4).

Topical application with NJJ improves clinical symptoms in DNFB-induced AD mice

Furthermore, we applied NJJ topically to DNFB-challenged mice, as NJJ has been used as a textile dye traditionally. As shown in Fig. 5A, the topical administration of NJJ improved the clinical symptoms in DNFB-challenged NC/Nga mice. And the thickness of epidermis was also suppressed in lesional skin of NJJ-applied and DNFB-challenged group compared with that of the DNFB-challenged group. NJJ significantly suppressed the infiltration of

inflammatory cells ($p < 0.05$, Fig. 5B). And NJJ significantly suppressed the levels of serum IgE and histamine ($p < 0.05$, Fig. 5C). However, NJJ did not significantly suppress the scratching behavior (data not shown).

NJJ suppresses the level of TSLP in the activated HMC-1 cells

We studied whether NJJ could regulate the levels of TSLP in mast cells by using human mast cell line, HMC-1 cells. First, NJJ did not affect the cell viability of HMC-1 cells (Fig. 6A). In the activated HMC-1 cells, the production and mRNA expression of TSLP were increased; whereas NJJ (100 $\mu\text{g}/\text{ml}$) suppressed the production and mRNA expression of TSLP increased in the activated HMC-1 cells ($p < 0.05$, Fig. 6B and C). NJJ (100 $\mu\text{g}/\text{ml}$) had no effect on the production of TSLP from the inactivated HMC-1 cells.

NJJ suppresses the expressions of RIP2 and caspase-1 in the activated HMC-1 cells

Next, we studied the signal pathway to regulate the production of TSLP by NJJ. PMACI increased the expressions of RIP2 and

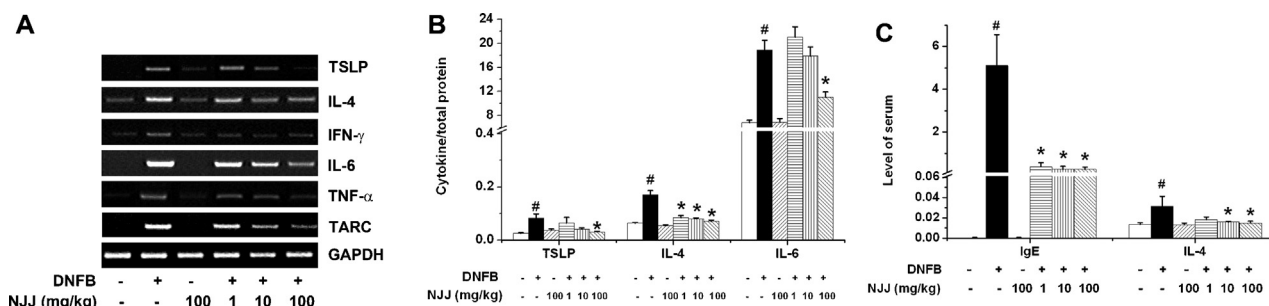


Fig. 3. Oral administration of NJJ suppresses the levels of TSLP in lesional skin of DNFB-induced AD mice. NJJ (1, 10, and 100 mg/kg) was orally administered to DNFB-challenged mice. (A) The mRNA expressions were analyzed with RT-PCR analysis. (B) The protein expressions from the lesional skin homogenate were analyzed with ELISA. (C) The levels of serum IgE ($\mu\text{g}/\text{ml}$) and IL-4 (ng/ml) were analyzed with ELISA. * $p < 0.05$, significantly different from unchallenged vehicle group. * $p < 0.05$, significantly different from control group (DNFB).

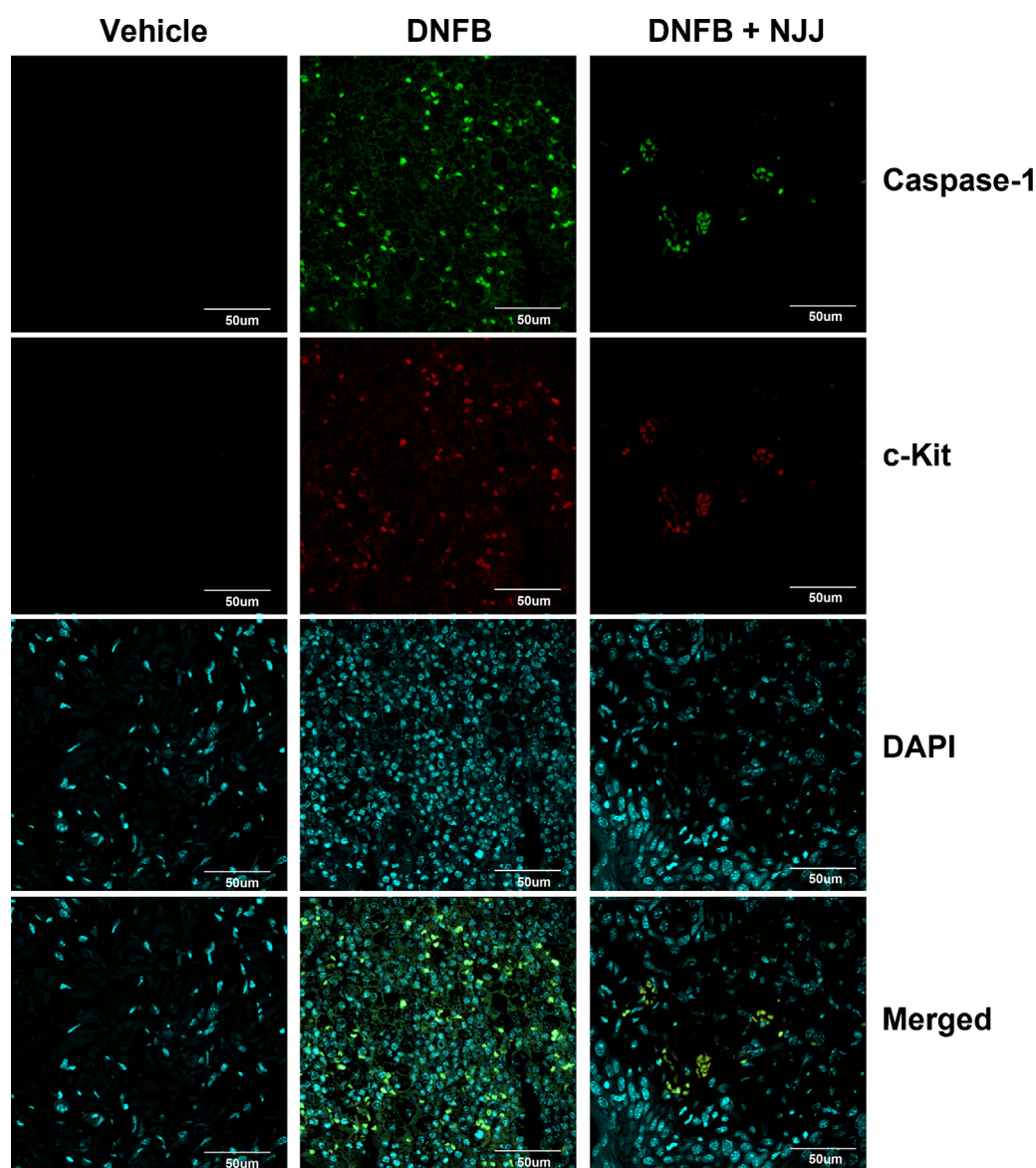


Fig. 4. Oral administration of NJJ suppresses the expression of caspase-1 in lesional skin of DNFB-induced AD mice. NJJ (100 mg/kg) was orally administered to DNFB-challenged mice. The caspase-1⁺ (FITC) and c-Kit⁺ (PE) cells from DNFB-induced lesional skin were examined with a confocal laser-scanning microscope. Mast cells are identified as c-Kit⁺ cells. The merged image indicated the mast cells and caspase-1 colocalization. Representative photomicrographs were examined at 60× magnification (scale bar = 50 µm).

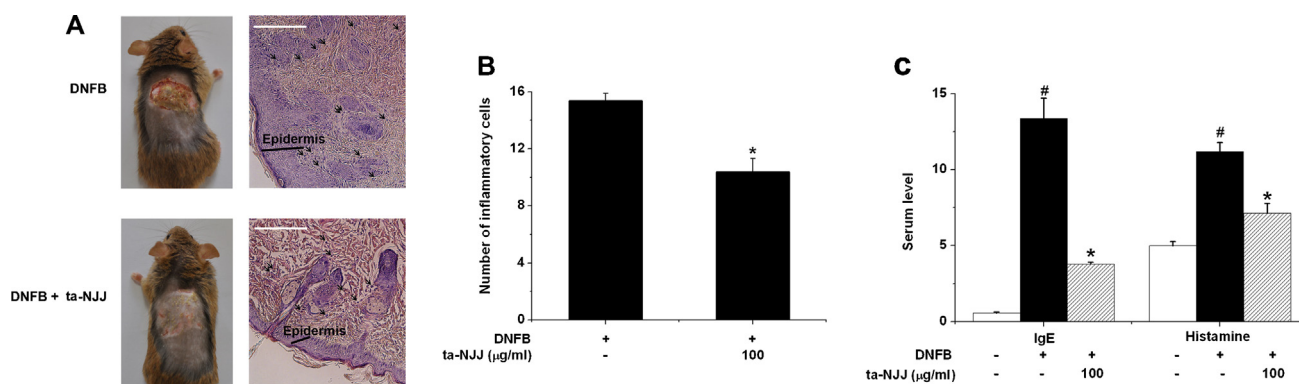


Fig. 5. Topical application with NJJ improves clinical symptoms in DNFB-induced AD mice. NJJ (100 µg/ml) or saline were topically applied to DNFB-challenged mice. The clinical features were observed 4 h after the last DNFB challenge. Histological analysis of lesional skin was examined by H&E staining for (A) epidermal thickness and (B) inflammatory cells. Representative photomicrographs were examined at 400× magnification. The inflammatory cells were indicated by arrows. Five tissue sections ($1.5 \times 10^3 \mu\text{m}^2$) per mouse were randomly selected and counted. DNFB, saline-applied and DNFB-challenged mice; DNFB + ta-NJJ, NJJ-topically applied and DNFB-challenged mice. (C) The level of serum IgE (ng/ml) was analyzed with ELISA. The intensity of serum histamine was assayed as described in "Materials and Methods" section. # $p < 0.05$, significantly different from unchallenged vehicle group. * $p < 0.05$, significantly different from control group (DNFB). $n = 3$.

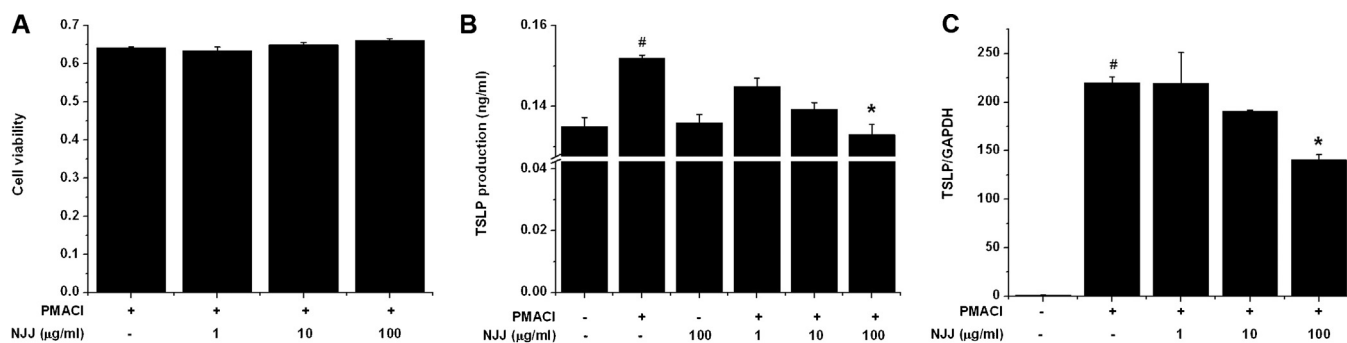


Fig. 6. NJJ suppresses the level of TSLP in the activated HMC-1 cells. (A) Cell viability was analyzed with an MTT assay. (B) HMC-1 cells (4×10^5) were pretreated with NJJ (1, 10, and 100 $\mu\text{g/ml}$) for 2 h and stimulated with PMACI for 7 h. The production of TSLP was analyzed with the ELISA. (C) HMC-1 cells (1×10^6) were pretreated with NJJ (1, 10, and 100 $\mu\text{g/ml}$) for 2 h and stimulated with PMACI for 5 h. mRNA expression of TSLP was analyzed with the real time-PCR analysis. # $p < 0.05$, significantly different from inactivated cells. * $p < 0.05$, significantly different from PMACI-activated cells.

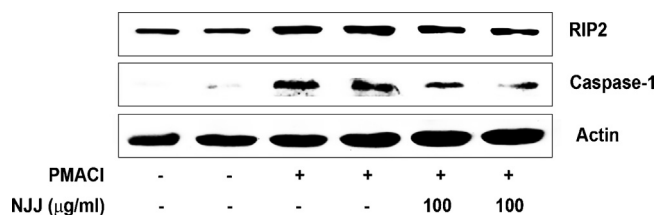


Fig. 7. NJJ suppresses the expressions of RIP2 and caspase-1 in activated HMC-1 cells. HMC-1 cells (5×10^6) were pretreated with NJJ (100 $\mu\text{g/ml}$) for 2 h and stimulated with PMACI for 1 h. The levels of RIP2 and caspase-1 were analyzed with Western blot analysis.

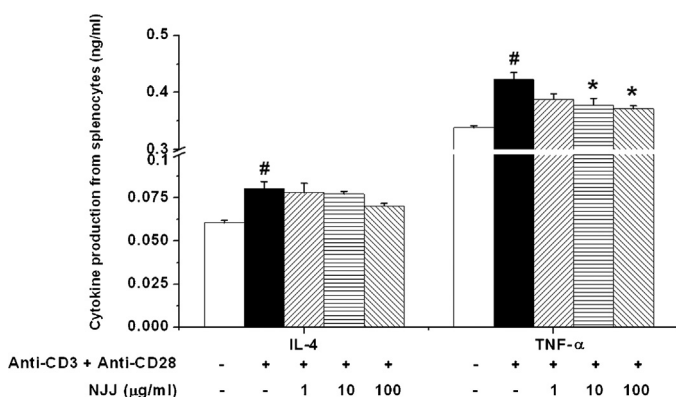


Fig. 8. NJJ suppresses the production of TNF- α from the stimulated splenocytes. Splenocytes ($2.5 \times 10^6/\text{ml}$) were stimulated with immobilized anti-CD3 and soluble anti-CD28 antibodies and treated with NJJ (1, 10, and 100 $\mu\text{g/ml}$). The productions of IL-4 and TNF- α were analyzed with the ELISA. # $p < 0.05$, significantly different from unstimulated cells. * $p < 0.05$, significantly different from anti-CD3 and anti-CD28-stimulated cells.

caspase-1; whereas NJJ suppressed the expressions of RIP2 and caspase-1 increased in the activated HMC-1 cells (Fig. 7).

NJJ suppresses the production of TNF- α from the stimulated splenocytes

Finally, we studied whether NJJ would regulate the productions of inflammatory cytokines from the anti-CD3 and anti-CD 28 antibodies-stimulated splenocytes. As shown in Fig. 8, the stimulated splenocytes increased the production of TNF- α ; whereas NJJ significantly suppressed the production of TNF- α from the stimulated splenocytes ($p < 0.05$). However, NJJ did not significantly

suppress the production of IL-4 from the stimulated splenocytes (Fig. 8).

Discussion

The various drugs for AD patients have been developed steadily. However, AD prevalence has considerably increased worldwide in recent years. Unfortunately, synthetic corticosteroid, a common and often efficacious drug for a wide variety of cutaneous conditions, could induce various cutaneous adverse effects (Kozower et al. 1974). Dyes in clothing can cause a rash or eruption in people who are allergic or sensitive to synthetic dyes. Natural products have provided a primary source of drugs for hundreds of years and continue to do so even today (Newman et al. 2000). As a natural dye, NJJ has been used in various clothing. In addition, NJJ has been used to treat skin diseases in TKM. Thus, we hypothesized that NJJ would have potential for treating skin diseases, especially AD. As a result, NJJ suppressed the development of AD in the mimic AD murine model. And NJJ suppressed the production and mRNA expression of TSLP through the blockade of RIP2/caspase-1 signal pathway in activated HMC-1 cells. NJJ suppressed the production of TNF- α from the stimulated splenocytes.

AD is one of the most common and severe skin diseases, which is characterized by eczematous lesional skin and the high production of IgE (Kang et al. 2006). The release of cytokines in skin may be of major importance in the development of many inflammatory skin disorders. Allergen-induced IgE synthesis can trigger eosinophils, basophils, and mast cells to release cytokines for the differentiation of Th cells into Th2 cells to secrete IL-4, IL-5, and IL-13 (Wong et al. 2001). Recently, TSLP has been known to act as a critical switch factor in the allergic reactions based on its capacity to differentiate proinflammatory Th2 cells from naive CD4 T cell precursors in human subjects (Hulse et al. 2010). The level of IL-4 mRNA in inflammatory infiltrating cells was increased in lesional skin of AD patients (Tazawa et al. 2004). The levels of IL-4 and IgE were significantly increased in serum from AD pediatric patients (Yeşilova et al. 2012). In the lesional skin of patients with AD, the level of IL-6 was increased compared to healthy individuals (Fedenko et al. 2011). The percentage of TNF- α ⁺ mast cells was higher in lesional skin than in healthy-looking skin of AD patients (Ackermann and Harvima 1998). TARC was produced highly in the lesional skin of patients with AD and attracted Th2 cells into the skin (Morita et al. 2010). In this study, the mRNA levels of TSLP, IL-4, IFN- γ , IL-6, TNF- α , and TARC were suppressed by oral administration of NJJ in AD-like lesional skin. The protein levels of TSLP, IL-4, and IL-6 were also suppressed by oral administration of NJJ in AD-like lesional skin. And serum IgE and IL-4 were suppressed

by oral administration of NJJ in AD murine model. Thus, we imply that NJJ would have an anti-AD effect by inhibiting the expression of inflammatory cytokines and chemokine.

Caspase-1 is a crucial regulator of nociceptive sensitization and inflammation (Liang et al. 2010). The protein kinase RIP2 is a member of the CARD protein family (caspase activation and recruitment domain) and has been shown to be an activator of NF- κ B (Yin et al. 2010). Caspase-1 transgenic mice overexpressing caspase-1 spontaneously developed chronic dermatitis and had a significant increase in the serum levels of histamine and IgE (Murakami et al. 2006). Macrophages from RIP2-deficient mice showed impaired NF- κ B activation and decreased cytokine production in response to lipopolysaccharide stimulation (Chin et al. 2002). Furthermore, TSLP was up-regulated by RIP2/caspase-1 signal pathway in mast cells (Moon and Kim 2011). In this study, the histologic analysis showed that the expression of mast cells-derived caspase-1 in AD-like lesional skin was suppressed by oral administration of NJJ. NJJ also suppressed the activation of caspase-1 in the activated HMC-1 cells. Thus, we imply that the inhibitory effect of NJJ on AD might be induced through the regulation of caspase-1 signaling pathways.

T cell receptor complex consisted of CD3 and T cell receptor (TCR) generates an activation signal in T cells. CD28 is one of the costimulatory molecules expressed on T cells, which are required for T cell activation. Naive T cells from spleen were stimulated with anti-CD3 and anti-CD28 antibodies (Ci et al. 2010). CD28-mediated costimulation together with CD3-mediated TCR signaling induced the production of primary and secondary cytokines (Iyoda et al. 2010). In this study, NJJ significantly suppressed the production of TNF- α from anti-CD3 and anti-CD28-stimulated splenocytes. Thus, we imply that the anti-AD effect of NJJ might be regulated at least partially through a suppressive action on T cells.

As a medicinal plant, NJJ has been known to have various bioactive compounds and biological activities (Iwaki et al. 2011). Kaempferol, tryptanthrin, and indirubin are natural constituents from the NJJ (Kataoka et al. 2001; Kim et al. 2010a,b). Kaempferol has been reported to inhibit rheumatoid arthritis (Yoon et al. 2013) and atherosclerosis (Kong et al. 2013). Tryptanthrin has been reported to have therapeutic effects on colitis (Micallef et al. 2002) and *Escherichia coli* infection (Bandeekar et al. 2010). Novel indirubin derivative had an anti-atherosclerotic property (Kim et al. 2010a,b). Indirubin inhibited inflammatory reactions in delayed-type hypersensitivity (Kunikata et al. 2000).

In conclusion, we determined scientifically the anti-AD effect of NJJ which has been used to treat skin disease in TKM. However, further study is necessary to clarify the specific concepts for external use of NJJ.

Conflicts of interest

None to declare.

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