



Anti-inflammatory and immune response regulation of Si-Ni-San in 2,4-dinitrochlorobenzene-induced atopic dermatitis-like skin dysfunction

Hui-Jie Fan^{a,b}, Ze-Ping Xie^a, Zi-Wen Lu^a, Zhang-Bin Tan^{a,b}, Yi-Ming Bi^{a,b}, Ling-Peng Xie^{a,b}, Yu-Ting Wu^{a,b}, Wen-Tong Zhang^{a,b}, Kevin Liu-Kot^c, Bin Liu^{a,d,*}, Ying-Chun Zhou^{a,b,*}

^a School of Traditional Chinese Medicine, Southern Medical University, Guangzhou, Guangdong 510515, China

^b Department of Traditional Chinese Medicine, Nanfang Hospital, Southern Medical University, Guangzhou, Guangdong 510515, China

^c Department of Biology, Boston University, Boston, MA, 02215, USA

^d Department of Cardiology, The Second Affiliated Hospital of Guangzhou Medical University, Guangzhou, Guangdong 510260, China

ARTICLE INFO

Keywords:

Atopic dermatitis

Si-Ni-San

Inflammatory

Immune response

ABSTRACT

Ethnopharmacological relevance: Si-Ni-San (SNS) is a well-known decoction in traditional Chinese medicine. Although studies have indicated that the anti-inflammatory and anti-allergic properties of SNS and its components can account for their therapeutic effects, the role and mechanism of SNS in treating skin dysfunction remain unclear.

Aim of the study: Atopic dermatitis (AD), a disorder known for its prevalence in infants and adults, severely influences the quality of life of affected patients. In this study, we aimed to investigate the anti-inflammatory and immune response modulations of SNS in 2,4-dinitrochlorobenzene (DNCB)-induced AD-like skin dysfunction.

Materials and methods: Dermatitis was induced in Kunming mice by the topical application of DNCB. SNS or dexamethasone (positive control) was topically applied every day over the course of the 21-day study. The following were assessed: dermatitis severity scores; ear and dorsal skin haematoxylin and eosin staining; interleukin (IL)–1 α , IL-1 β , IL-2, IL-4, IL-6, and tumour necrosis factor (TNF)- α cytokine levels in the serum; spleen index; spleen CD4 + /CD8 + T lymphocyte ratio; and phosphorylation levels of mitogen-activated protein kinases (MAPKs- p38, extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinase (JNK)), I κ B- α , and nuclear factor (NF)- κ B (p65) in skin lesions.

Results: SNS significantly alleviated the symptoms of AD-like lesions induced by DNCB, decreased the infiltration of inflammatory cells in the ear and dorsal tissues, suppressed the increased cytokine levels in the serum, reduced the CD4 + /CD8 + T lymphocyte ratio in the spleen, and downregulated the activation of MAPKs, I κ B- α , and NF- κ B (p65) in the dorsal skin. The effects were similar to those of dexamethasone.

Conclusions: SNS alleviated the DNCB-induced AD-like skin dysfunction in mice through anti-inflammatory and immune system modulation, indicating that SNS shows potential for AD treatment in clinical settings.

1. Introduction

Atopic dermatitis (AD) is a chronic, relapsing inflammatory disease characterized by erythematous dry skin lesions, intensive pruritus, xerosis, and lichenification (Bak et al., 2012). AD occurs equally in men and women and appears during infancy and adulthood. It affects approximately 18% of children and 4% of adults worldwide (Kim et al., 2014). AD occurs more frequently in children and young adults than in the elderly. Symptom outbreak appears in 85% of children within their first 5 years of life (Rudikoff and Lebowitz, 1998). Because of their

evident clinical presentation, prominent erythematous, itchy skin lesions are a common reason for patients or their caregivers to seek treatment for AD. A common residual effect of these patches is the lichenification of the skin, which can lead to social stigma and mental health problems. Patients with AD can suffer from lack of sleep, emotional anxiety, stress, and social difficulties. This persistent disease decreases the overall quality of life of patients and their caregivers (Kiebert et al., 2002). At the cellular level, a common characteristic of patients with AD is epidermal barrier dysfunction due to the absence of certain structural proteins, such as filaggrin (Bak et al., 2012). This

* Correspondence to: School of Traditional Chinese Medicine, Southern Medical University, 1063 Shatai Road, Guangzhou, Guangdong 510515, China.

E-mail addresses: 15521107492@163.com (H.-J. Fan), 326301458@qq.com (Z.-P. Xie), 1148131331@qq.com (Z.-W. Lu), 249880441@qq.com (Z.-B. Tan), 451645244@qq.com (Y.-M. Bi), 845639520@qq.com (L.-P. Xie), 1603406288@qq.com (Y.-T. Wu), zhangwentong.com@qq.com (W.-T. Zhang), kliukot@bu.edu (K. Liu-Kot), xmhoolv@163.com (B. Liu), zhychun@126.com (Y.-C. Zhou).

<https://doi.org/10.1016/j.jep.2018.04.032>

Received 6 December 2017; Received in revised form 19 April 2018; Accepted 21 April 2018

Available online 24 April 2018

0378-8741/ © 2018 Elsevier B.V. All rights reserved.

condition makes AD patients prone to environmental allergens, which can abnormally activate the inflammatory cascade due to cutaneous hyperactivity (Brown and Reynolds, 2006).

A previous study compared the cross sections of the dorsal epidermis of AD and nonatopic mouse models and found that the thickness of the epidermis was different between the two models; specifically, 2,4-dinitrochlorobenzene (DNCB)-induced skin lesions were thicker in the AD mice than in the normal mice (Lee et al., 2016). In patients with AD, the stratum corneum is damaged, thereby exposing the inner layer to allergens and infections and promoting moisture loss from the skin (Schwartz and Friedman, 2016). Subsequent behavioural itching responses exacerbate the condition with further damage to the epidermal barrier. Thus, epidermal barrier dysfunction exposes the skin to environmental allergens and promotes inflammatory reactions and pruritic sensations, creating a vicious cycle (Catherine Mack Correa and Nebus, 2012).

CD4 + T helper (Th) and CD8 + cytotoxic T cells are common T lymphocytes studied in AD pathogenesis (Komatsu et al., 2016; Leonardi et al., 2007). In patients with AD, the CD4 + /CD8 + T lymphocyte ratio increases in response to inflammation (Bak et al., 2012; Kagi et al., 1994; Prinz et al., 1999). In terms of immune responses, antigen-presenting cells migrate to lymph nodes and present allergens to naive T cells (Siracusa et al., 2011). In AD pathogenesis, a naive T cell binds to a substrate pocket and differentiates into a Th2 cell in the presence of free-floating cytokines, such as interleukin (IL) – 4, IL-5, IL-10, and IL-13. Primed Th2 cells release IL-4, which activates B cells to produce immunoglobulin E (IgE) that is specific to the activating allergen. IL-4 is accompanied by IL-5, which promotes eosinophil production and proliferation (Brandt and Sivaprasad, 2011). Th2 cells are also involved in the induction of the acute phase of AD, whereas Th1, Th2, and non-Th cells are involved in the persistence of AD symptoms (Chen et al., 2004; Christensen et al., 2011; Novak et al., 2003). Specifically, Th1-associated cytokines, such as IL-2 and interferon (IFN)- γ , are increased, along with the inflammatory cytokine tumour necrosis factor (TNF)- α , after allergen exposure (Brandt and Sivaprasad, 2011). Concurrently, other Th2 cytokines (IL-3 and IL-10) and non-Th proinflammatory cytokines (IL-1 α and IL-1 β) contribute to AD persistence (Chen et al., 2004). Thus, Th1, Th2, and non-Th cells share a network, which plays an important role in the inflammatory progress of AD.

Si-Ni-San (SNS) (containing root of *Bupleurum chinense* DC. [Radix Bupleuri], root of *Paeonia lactiflora* Pall. [Radix Paeoniae Alba], rhizome of *Glycyrrhizauralensis* Fisch. [Radix Glycyrrhizae, honeyed], and fruit of *Citrus aurantium* L. [Fructus Aurantii Immaturus], at a ratio of 1:1:1:1) is a well-known decoction in traditional Chinese medicine. Thousands of years ago, SNS was first reported in Shang Han Lun, which was written by a Chinese doctor named Zhongjing Zhang. SNS can alleviate depression (Tanaka et al., 2013), improve sleep (Li et al., 2013), prevent liver damage (Jiang et al., 2003), prevent acute gastric mucosal lesions (Ohta et al., 2006), ease irritable bowel syndrome (Yu et al., 2005), and reduce contact sensitivity (Zhang et al., 2006). *B. chinense* DC. (Radix Bupleuri) can attenuate inflammation in hypertrophied 3T3-L1 adipocytes (Fu et al., 2016), whereas *P. lactiflora* Pall. (Radix Paeoniae Alba) can inhibit mast cell activation in a dose-dependent manner (Jeon et al., 2016). *G. uralensis* Fisch. (Radix Glycyrrhizae) has been shown to attenuate dextran sulphate sodium-induced ulcerative colitis, which involves inflammatory responses (Han et al., 2016). Although studies have indicated that the anti-inflammatory and anti-allergic properties of SNS and its components can account for their therapeutic effects, the role and mechanism of SNS in treating skin dysfunction remain unclear. Therefore, in this study, we aimed to investigate the anti-inflammatory and immune-related contributions of SNS to treat DNCB-induced skin dysfunction in a mouse model.

2. Materials and methods

2.1. Chemicals and reagents

Compound dexamethasone acetate (DEX) containing 0.075% dexamethasone was provided by Sanjiu Medical&Pharmaceutical Co., Ltd. (Shenzhen, China). DNCB was purchased from Shanghai Chemical Reagent Company (Shanghai, China), and acetone was provided by Guanghua Chemical Reagent Company (Guangzhou, Guang Dong, China). The primary antibodies, including those to detect glyceraldehyde 3-phosphate dehydrogenase (GAPDH), extracellular signal-regulated kinase (ERK), phospho-ERK (P-ERK), p38, phospho-p38 (P-p38), c-Jun N-terminal kinase (JNK), phospho-JNK (P-JNK), phospho-I κ B- α (P-I κ B- α), p65, phospho-p65 (P-p65), and anti-rabbit secondary antibody, were purchased from Cell Signalling Technology (Danvers, MA, USA). The Quantibody[®] Mouse Interleukin Array Kit was purchased from RayBiotech (Norcross, GA, USA). The Mouse T Lymphocyte Subset Antibody Cocktail for flow cytometry was purchased from BD Biosciences (Franklin Lakes, NJ, USA).

2.2. Preparation of SNS and high-performance liquid chromatography (HPLC) for SNS quality control

Radix Bupleuri, Radix Paeoniae Alba, Radix Glycyrrhizae, and Fructus Aurantii Immaturus used in SNS were purchased from Zisun Herbal Pharmaceutical Co., Ltd. (Guangzhou, Guang Dong, China). First, 10 g of each herb was soaked in 500 mL water for 30 min, and then boiled for 30 min. The extract was collected and the residue was boiled in water two more times, as above. Then, the extracts were pooled, concentrated to 200 mL, and kept at 4 °C. Fresh SNS was made every 3 days. SNS HPLC was conducted using an Agilent Technologies 1260-HPLC System (Palo Alto, CA, USA). Briefly, 1 μ L SNS was injected into the apparatus with an auto sampler. Chromatographic separation was achieved at a flow rate of 1.0 mL/min with an Agilent Eclipse Plus C18 column (4.6 $\times$ 250 mm, 5 μ m; USA). The mobile phase was composed of solvent A (acetonitrile) and solvent B (12 mM ammonium acetate, 0.1% acetic acid). The linear gradient elution was performed from 3% to 5% solvent A for 0–2 min, 5–15% solvent A for 2–8 min, 15–20% solvent A for 8–10 min, 20–45% solvent A for 10–15 min, 45–55% solvent A for 15–20 min, 55–75% solvent A for 20–23 min, 75–95% solvent A for 23–27 min, and 95–5% solvent A for 27–30 min. The separation temperature was 35 °C, with a detection wave length of 240 nm.

2.3. Animals

According to the guidelines of the Institute of Animal Care and Use Committee of Southern Medical University, 50 eight-week-old male Kunming (KM) mice with body weights ranging from 20 to 24 g were housed in a controlled environment, with the temperature between 22 and 26 °C, humidity of 40–60%, and a 12-h light/dark cycle. All mice were provided access to tap water and standard chow and were allowed to acclimatize to the new environment for 1 week before initiation of the experiment.

2.4. Experimental design

We divided the mice randomly into 5 groups ($n = 10$ per group): control, DNCB, positive control (DEX-0.1 mg/day), SNS-low dose (SNS-40 mg/day), and SNS-high dose (SNS-80 mg/day) groups. Except for the control group, skin dysfunction was induced by DNCB treatment as described previously. First, we shaved the dorsal hair in an area of approximately 2 cm $\times$ 2 cm. After 24 h, we treated the skin with 100 μ L of 7% (w/v) DNCB, which had been dissolved in a vehicle containing acetone and olive oil (4:1). After 5 days, 5 μ L of 0.5% (w/v) DNCB dissolved in the same vehicle was used to treat both sides of the right

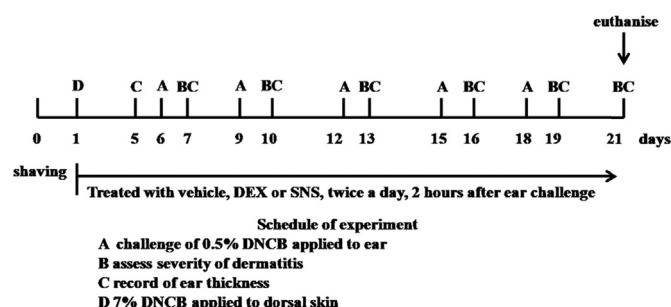


Fig. 1. Schedule of the experiment. Skin dysfunction was evoked by administering 7% DNCB in a vehicle made of acetone and olive oil (4:1) on the dorsal skin on day1. Additional applications of 0.5% DNCB were administered on days 6, 9, 12, 15, and 18. Dermatitis scores and ear thicknesses were also assessed. DNCB, 2,4-dinitrochlorobenzene; DEX, dexamethasone; SNS, Si-Ni-San.

ear of each mouse every 3 days to promote skin dysfunction. On the dorsal skin and both sides of the right ear, the control and DNCB groups were treated with 200 μ L water, whereas the positive control and SNS groups were treated with 200 μ L of DEX (containing 0.025% dexamethasone in water, equal to 0.1 mg dexamethasone per day) or SNS (SNS-40 mg/day, 100 μ L of SNS diluted with 100 μ L water; SNS-80 mg/day, 200 μ L of SNS) twice a day, respectively. After 21 days, all mice were euthanised. The schedule of this experiment is shown in Fig. 1. Prior to euthanasia, blood was collected retro-orbitally from the experimental mice, and the resulting samples were centrifuged at 3000 rpm for 15 min. The serum was kept at -80°C .

2.5. Evaluation of dermatitis severity

We evaluated the dermatitis severity according to erythema, oedema, and scratches present on the skin lesions, which were recorded every 3 days, by using the criteria listed in Table 1. In this experiment, we calculated the dermatitis severity score six times. Moreover, we measured the right ear thickness of each mouse seven times, once the day before the initial DNCB treatment, and then once after each of the six DNCB treatments.

2.6. Haematoxylin and eosin staining

After the mice were euthanised, their dorsal skins and right ears were removed. Then, the skins and ears were fixed in 10% formalin for 24 h, embedded in paraffin blocks, and sectioned into slices with a thickness of 5 μ m. The sections were stained with haematoxylin and eosin (H&E) to detect the corneum, epidermis, dermis, lymphocytes, neutrophils, and eosinophilic dermal infiltrates.

2.7. Western blot analysis

Protein samples were obtained from dorsal back skin samples of the

Table 1
Criteria of dermatitis severity.

Item	Severity index	score
Erythema	none	0
	mild	1
	moderate	2
	severe erythema and mild scar	3
	severe erythema and scar	4
Oedema	none	0
	mild	1
	moderate	2
	severe	3
Scratching	no	0
	yes	1

mice. First, the skin tissue was homogenized with RIPA buffer containing protease inhibitors (Beyotime Biotechnology, Shanghai, China), and the protein concentration of each sample was measured using an Enhanced BCA Protein Assay Kit (Beyotime Biotechnology). Then, 20 μ g of protein was denatured in sodium dodecylsulphate (SDS) buffer. The proteins were separated by electrophoresis on 10% SDS polyacrylamide gels and were then transferred to polyvinylidene fluoride (PVDF) membranes. After blocking the membranes with 5% nonfat milk dissolved in Tris-buffered saline with 0.1% Tween 20[®] (TBST) at 25 $^{\circ}\text{C}$ for 1 h, the membranes were incubated with different primary antibodies (GAPDH [1:3000 dilution in 5% bovine serum albumin (BSA)], P-ERK, ERK, P-p38, p38, P-JNK, JNK, P-I κ B- α , p65, and P-p65 [1:1000 dilution in 5% BSA]) at 4 $^{\circ}\text{C}$ overnight. Following incubation, the membranes were washed thrice with TBST and then incubated with anti-rabbit horseradish peroxidase conjugated secondary antibody (1:5000 dilution in 5% nonfat milk) at 25 $^{\circ}\text{C}$ for 1 h. The western blot signals were detected using a KODAK Image Station 4000MM Digital Imaging System. The intensity of each band was quantified with Image J software and normalised to the intensity of the corresponding loading control.

2.8. Inflammatory cytokine array

Inflammatory cytokines in the serum were detected using the Quantibody[®] Mouse Interleukin Array Kit. All procedures were in accordance with the manufacturer's instructions. The signals were detected with an InnoScan 300 microarray scanner (Innopsys, Carbonne, France).

2.9. Spleen index and flow cytometry

Each spleen was weighed after the mice were euthanized and was normalised to the body weight to calculate the spleen index. Then, to analyse the CD4 + and CD8 + lymphocytes, we cut the spleens into small pieces and placed them in cold phosphate-buffered saline (PBS). The pieces were subsequently homogenized in Roswell Park Memorial Institute (RPMI) 1640 medium containing 1% foetal bovine serum (FBS). All steps were followed according to the instructions provided with the Mouse T Lymphocyte Subset Antibody Cocktail (BD Biosciences).

2.10. Statistical analysis

SPSS 21.0 software was used for statistical analysis. Data are presented as means $\pm$ standard errors of the means (SEMs) or means $\pm$ standard deviations (SDs), and were analysed by one-way analysis of variance, followed by least significant difference post-hoc tests. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. HPLC chromatograms of SNS

The compounds, paeoniflorin, liquiritin, naringin, hesperidin, and glycyrrhizic acid, which are known to be present in SNS, were analysed by HPLC. We identified the peak of each compound in the SNS extract by comparison with the standards shown in Fig. 2A. Fig. 2B illustrates that the SNS used in our experiment contains these five major components.

3.2. SNS attenuates DNCB-induced AD-like symptoms

To investigate the preventive effects of SNS on the DNCB-induced AD-like symptoms in the KM mice, we assessed the dermatitis severity of their dorsal skin. Representative images of the dorsal skin from each treatment group are shown in Fig. 3A. The images captured on day14

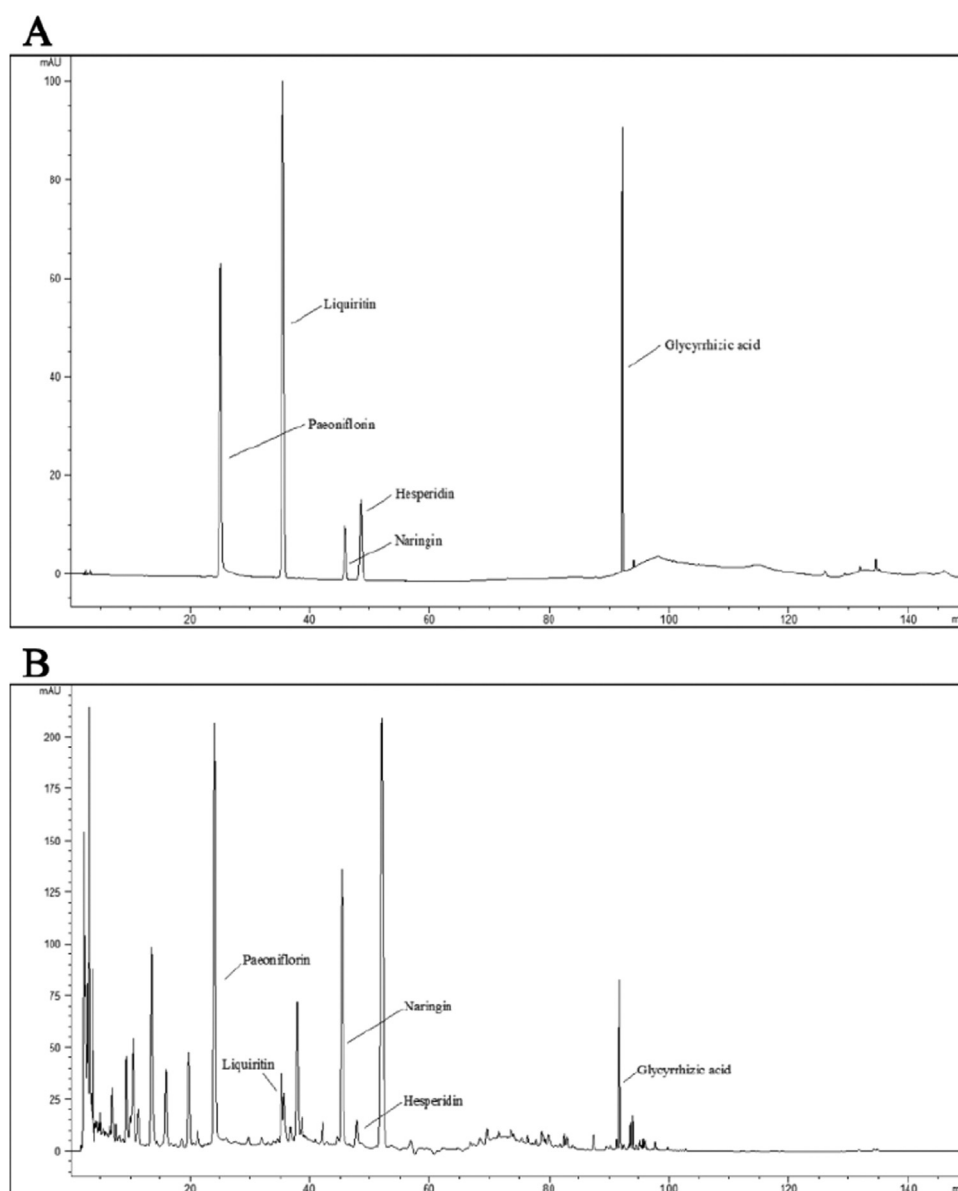


Fig. 2. HPLC chromatograms of SNS. Chromatographic separation was achieved at a flow rate of 1.0 mL/min using an Agilent Eclipse Plus C18 column (4.6 × 250 mm, 5 μm; USA). A) Chromatogram of purified standards known to be present in SNS; peaks from left to right are paeoniflorin, liquiritin, naringin, hesperidin, and glycyrrhizic acid. B) Chromatogram of the SNS extract used in this study. SNS, Si-Ni-San.

show that the AD severity in the DEX-0.1 mg/day, SNS-40 mg/day, and SNS-80 mg/day groups was noticeably relieved. In addition, erythema, oedema, and excoriations (scratching) of the skin area in the experimental mice were recorded on days 7, 10, 13, 16, 19, and 21 according to the criteria outlined in Table 1. The dermatitis scores of the control, DNCB, DEX-0.1 mg/day, SNS-40 mg/day, and SNS-80 mg/day groups were calculated and are plotted in Fig. 3B; the final scores on day 21 were 0.2 ± 0.05 , 6.2 ± 1.1 , 2.5 ± 1.6 , 4.3 ± 0.6 , and 3.5 ± 0.4 , respectively. The results indicate that DNCB can remarkably induce skin dysfunction over time, whereas DEX-0.1 mg/day ($P < 0.01$), SNS-40 mg/day ($P < 0.05$), and SNS-80 mg/day ($P < 0.05$ or $P < 0.01$) can significantly alleviate these symptoms.

3.3. SNS reduces inflammatory reactions on the ear

The AD-like mouse model was sensitized on the dorsal skin and challenged on the ear by using DNCB (Karuppagounder et al., 2014). To observe the severity of ear inflammation, we performed H&E staining

and measured the ear thickness; we found that the ear thickness of the DNCB-induced mice was increased. In addition, DNCB-induced inflammation in the ear tissue and the resulting phenotypic alterations were clearly evident. SNS elicited remarkable therapeutic effects by suppressing hyperkeratosis and inflammatory cell infiltration, as apparent in the histopathology of the ear tissue (Fig. 4A). Moreover, DEX-0.1 mg/day, SNS-40 mg/day, and SNS-80 mg/day decreased the ear swelling compared with that of the DNCB group (0.421 ± 0.02 , 0.488 ± 0.04 , 0.464 ± 0.06 , and 0.625 ± 0.03 mm, respectively; $P < 0.01$; Fig. 4B).

3.4. SNS reduces inflammatory reactions and inhibits epidermal hyperplasia in dorsal skin dose-dependently

To further investigate the therapeutic effects of SNS on AD-like dorsal lesions, we examined the H&E stained sections under higher magnification to evaluate epidermal hyperplasia and infiltration of inflammatory cells in the dorsal skin tissue (Fig. 5A). After 21 days of

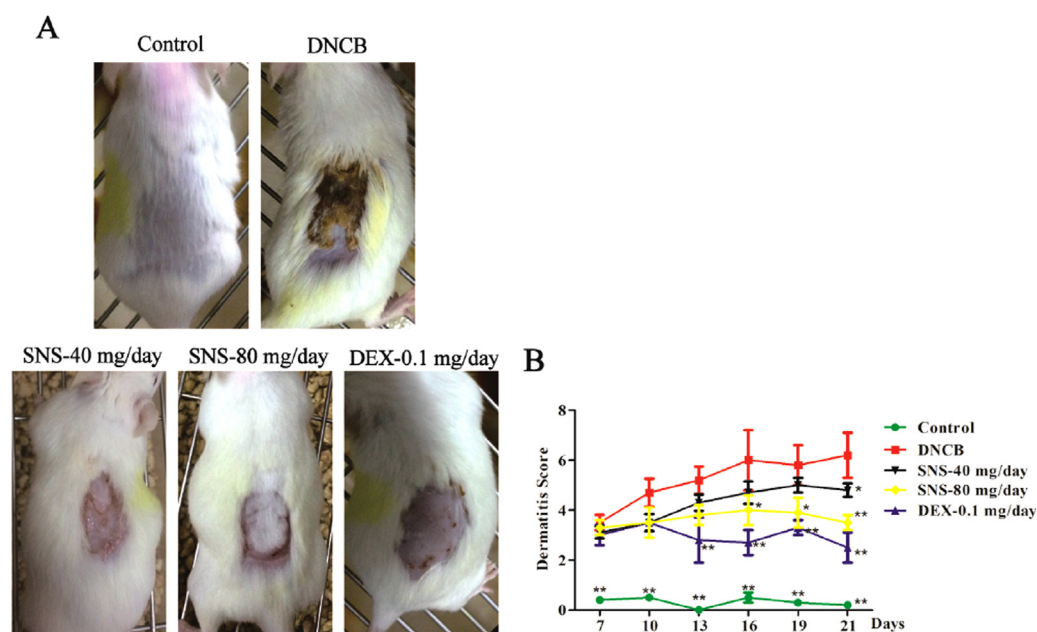


Fig. 3. SNS attenuates DNCB-induced AD-like skin lesions and reduces dermatitis scores in a dose-dependent manner. (A) Representative dorsal skin pictures of the control group (no induction or treatment), and the DNCB-induced groups with (DEX-0.1 mg/day positive control, SNS-40 mg/day, or SNS-80 mg/day) or without (DNCB group) treatment were captured on day 14. (B) Severity of the dorsal skin was evaluated based on the criteria in Table 1. The dermatitis score was summed every 3 days according to the erythema, oedema, and scratches present on the lesions. Data are presented as means $\pm$ SEM ($n = 10$). * $P < 0.05$ vs DNCB, ** $P < 0.01$ vs DNCB. DNCB, 2,4-dinitrochlorobenzene; DEX, dexamethasone; SNS, Si-Ni-San; AD, atopic dermatitis.

treatment, the DEX-0.1 mg/day, SNS-40 mg/day, and SNS-80 mg/day groups (89.14 ± 14.2 , 138.78 ± 11.1 , and $79.22 \pm 10.1 \mu\text{m}$, respectively) had significantly less epidermal thickening than the DNCB group did ($229.70 \pm 25.26 \mu\text{m}$, $P < 0.01$; Fig. 5B). These results indicate that DEX and SNS have significant attenuating effects on AD.

3.5. SNS attenuates the increase in serum inflammatory cytokines induced by DNCB in a dose-responsive manner

To evaluate the effects of SNS on serum inflammatory cytokine levels, we collected serum when the mice were euthanised and a cytokine protein array was performed. According to the results shown in Fig. 6, topical DNCB treatment increased the serum levels of IL-1 α , IL-1 β , IL-2, IL-4, IL-6, and TNF- α compared with the corresponding levels in the

control group ($P < 0.01$). Moreover, these cytokine levels were suppressed significantly in the SNS-40 mg/day and SNS-80 mg/day groups ($P < 0.01$) in comparison with those of the DNCB group. This result indicates that SNS has a similar anti-inflammatory activity as that of DEX ($P < 0.01$ versus the DNCB group).

3.6. SNS suppresses the enhanced CD4 + /CD8 + ratio in the spleen and spleen index

The CD4 + /CD8 + T lymphocyte ratio in the spleen and spleen index were increased significantly in the DNCB group (2.79 ± 0.2 and 44.2 ± 3.2 , respectively) compared with those of the control group (2.01 ± 0.1 and 28.9 ± 1.7 , respectively, $P < 0.05$). Moreover, the positive control DEX group had an over-inhibited immune response,

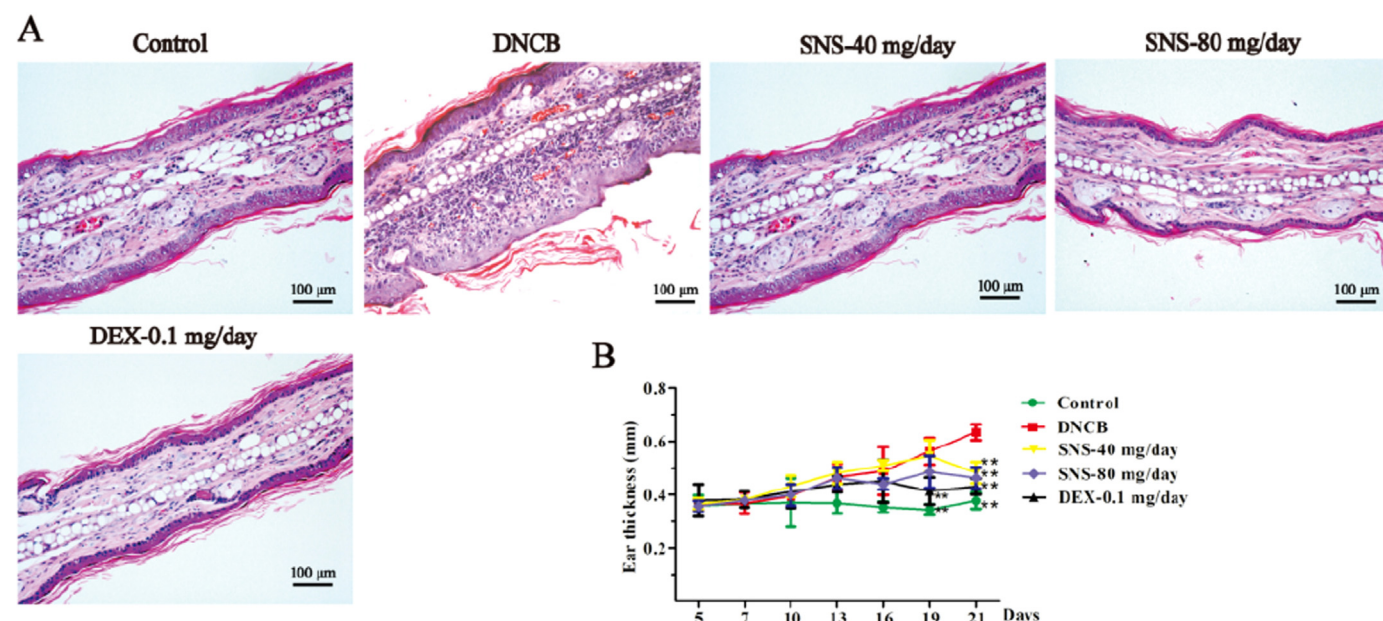


Fig. 4. SNS suppresses hyperkeratosis and inflammatory cell infiltration in the ear, as revealed by H&E staining and ear thickness. The right ears of the mice in each group were removed after euthanasia on the last day of the experiment. (A) Fixed ear sections were subjected to H&E staining (magnification: $200\times$, images are representative of six independent samples). (B) Ear thickness was measured with a thickness gauge on days 5, 7, 10, 13, 16, 19, and 21. Data are presented as means $\pm$ SEM ($n = 6$). ** $P < 0.01$ vs DNCB. DNCB, 2,4-dinitrochlorobenzene; DEX, dexamethasone; SNS, Si-Ni-San; H&E, haematoxylin and eosin.

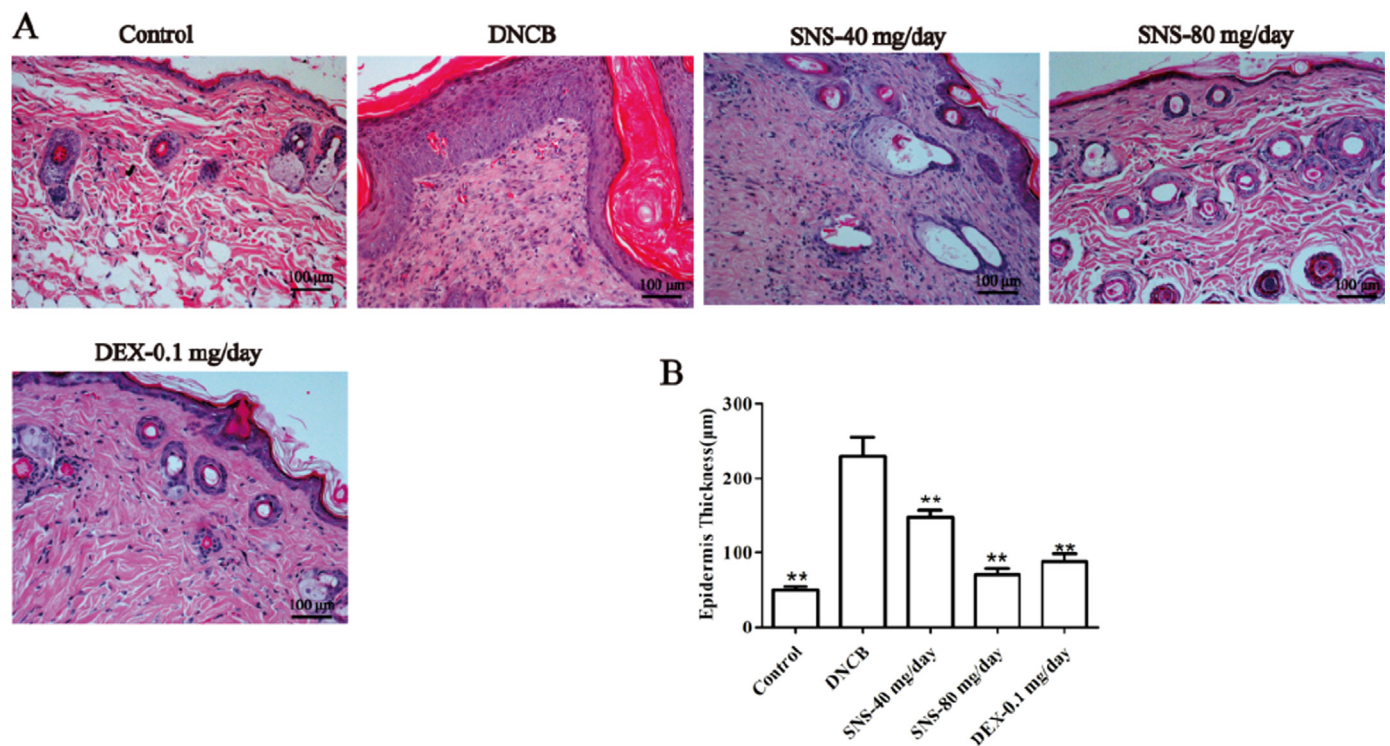


Fig. 5. SNS inhibits epidermal hyperplasia of dorsal skin dose-dependently, as revealed by H&E staining. The histology of the dorsal skin samples was examined by H&E staining after the final drug administration. (A) Representative H&E-stained dorsal skin sections are shown (magnification: 200 ×). (B) Epidermal thickness (μm) in the H&E-stained sections was measured under a microscope. Data are plotted as means ± SEM ($n = 6$). ** $P < 0.01$ vs DNCB. H&E, haematoxylin and eosin; DNCB, 2,4-dinitrochlorobenzene.

with the spleen CD4 + /CD8 + T lymphocyte ratio and spleen index (1.38 ± 0.2 and 22.0 ± 1.4 , respectively, $P < 0.01$) being even lower than those of the control group. Meanwhile, the ratio and spleen index in the SNS-40 mg/day (2.27 ± 0.1 and 35.4 ± 1.4 , respectively, $P < 0.01$) and SNS-80 mg/day (2.05 ± 0.1 and 33.0 ± 2.0 , respectively, $P < 0.01$) groups were lower than those in the DNCB group (Fig. 7). These results indicate that the DEX treatment may excessively suppress the immune response in AD. Moreover, SNS partly inhibited the immune response induced by DNCB in a dose-dependent manner.

3.7. Effects of SNS on the phosphorylation of mitogen-activated protein kinases (MAPKs), IκB-α, and nuclear factor (NF)-κB (p65)

In previous studies, the phosphorylation of MAPKs (p38, ERK, and JNK), IκB-α, and NF-κB (p65) was shown to be activated in DNCB-induced AD-like skin lesions (Chan et al., 2013; Lee et al., 2016). In our present study, we further tested the changes in signalling that were induced by SNS. As shown in Fig. 8, P-ERK, P-JNK, P-p38, P-IκB-α, and P-p65 levels in the DNCB group were increased ($240.6 \pm 34.4\%$, $478.7 \pm 56.4\%$, $427.3 \pm 44.2\%$, $235.6 \pm 38.2\%$, and $435.2 \pm 31.1\%$, respectively; $P < 0.01$) compared with those of the control group. Meanwhile, SNS dose-dependently abolished the increase in P-ERK, P-JNK, P-p38, P-IκB-α, and P-p65 induced by DNCB ($P < 0.01$ versus the DNCB group).

4. Discussion

AD, which is closely associated with inflammatory dysfunction, is a common skin disease with multifactorial causes. AD is accompanied by erythema, pruritus, and xerosis (Kim et al., 2015; Peng and Novak, 2015), and it affects many aspects of patients' lives, including their educational well-being, socialising, and emotional health. This reduces their overall quality of life. AD can be a considerable burden on national health care costs, and it can negatively affect the work

performance of many individuals. At present, topical corticosteroids are the most common treatments for AD. However, the use of topical corticosteroid results in many side effects, such as downregulating the activity of the immune system, thinning of the skin, and loss of skin pigment (Dhossche et al., 2017; Saraswat, 2010). Therefore, alternative methods should be developed to treat AD without side effects, while retaining the same therapeutic advantages.

SNS can treat diseases related to inflammation and immune dysfunction, which have similar mechanisms to those of AD, (e.g., liver damage, hepatitis, colitis, and delayed-type hypersensitivity (Jiang et al., 2003; Ohta et al., 2006; Sun et al., 2009; Tanaka et al., 2013)). However, evidence that definitively shows whether SNS affects AD is still lacking. Therefore, we conducted studies to illuminate the influence of SNS on AD and the mechanism involved. To our knowledge, our study is the first to demonstrate that topical use of SNS can effectively alleviate the symptoms of DNCB-induced AD-like disease in mice. According to our results, SNS dose-dependently alleviates eczematous symptoms, diminishes ear swelling, suppresses inflammatory cytokine production, and regulates the immune response. These results indicate that SNS significantly improves AD-like symptoms.

According to some previous studies, lymphocyte proliferation and proinflammatory cytokine secretion were closely related to AD (Bak et al., 2012; Leung, 2000). In AD, the production of inflammatory cytokines, such as IL-1β, IL-2, IL-4, IL-6, and TNF-α, was excessively increased. Therefore, the reduction in proinflammatory cytokine secretion is crucial to improve AD symptoms. These previous results showed a similar change in inflammatory cytokines as we observed in our mouse model. Moreover, the results of our study indicated that SNS dose-dependently alleviated inflammation by decreasing the serum levels of IL-1α, IL-1β, IL-2, IL-4, IL-6, and TNF-α compared with those in the DNCB group. These findings were similar to those of the previous reports, which indicated that SNS downregulates inflammatory cytokines, such as TNF-α, in Crohn's disease (Ohta et al., 2006), and that SNS reduces IL-1β and TNF-α in LPS-activated Raw264.7 macrophages

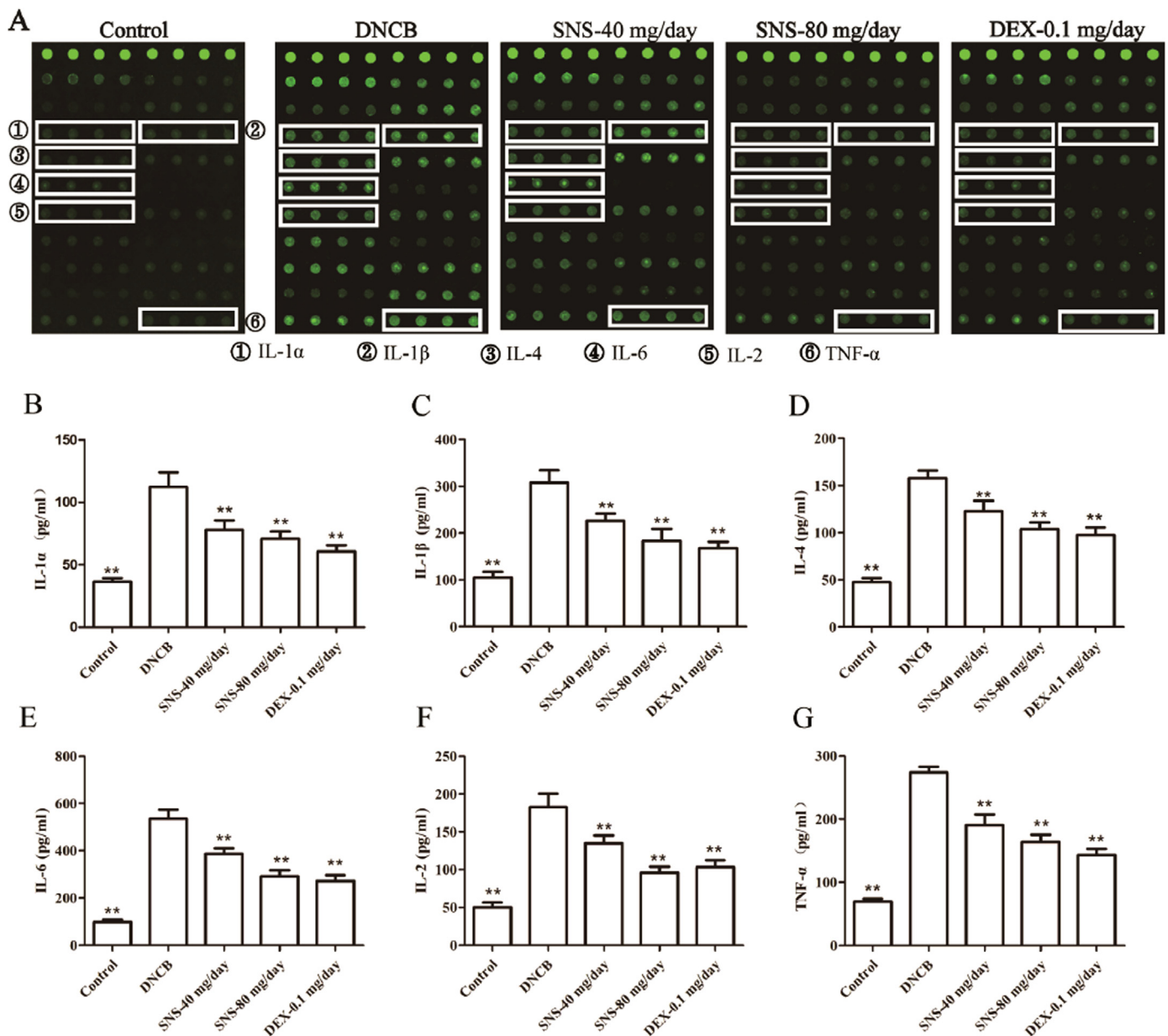


Fig. 6. SNS dose-dependently reduces IL-1 α , IL-1 β , IL-2, IL-4, IL-6, and TNF- α levels in the serum. Serum was collected from the experimental mice retro-orbitally on the last day of the experiment. The inflammatory cytokines in the serum were detected using the Quantibody[®] Mouse Interleukin Array Kit. (A) Images of IL-1 α , IL-1 β , IL-4, IL-6, IL-2, and TNF- α signals in the protein array. (B–G) Quantitative data for IL-1 α , IL-1 β , IL-4, IL-6, IL-2, and TNF- α , respectively, were obtained from the arrays. Data are presented as means $\pm$ SEM ($n = 10$). ** $P < 0.01$ vs DNCB. DNCB, 2,4-dinitrochlorobenzene; DEX, dexamethasone; SNS, Si-Ni-San; IL, interleukin; TNF, tumour necrosis factor.

(Sun et al., 2009).

A variety of cytokines are secreted by lymphocytes. Moreover, lymphocytes play crucial roles in the immune system. In chronic AD, CD4⁺ T lymphocytes primarily secrete Th1 cytokines, such as IFN- γ and IL-2, which are responsible for cell-mediated immunity. In acute AD, lymphocytes also release Th2 cytokines, such as IL-4, IL-5, IL-10, and IL-13, accompanied by non-Th cytokines, such as IL-1 α , IL-1 β , IL-6, and TNF- α . In AD development, Th1, Th2, and non-Th proinflammatory cytokines are closely associated with immune function. Moreover, Th1, Th2, and non-Th proinflammatory cytokines are abnormally produced due to sensitization (Kang et al., 2017; Luz Correa et al., 2014). On the basis of our results, we found that SNS inhibits the excessive production of IL-1 α , IL-1 β , IL-2, IL-4, IL-6, and TNF- α . This result suggests that SNS can regulate the immune response.

The CD4⁺ and CD8⁺ ratio is associated with inflammatory and

immune responses in the body (Prinz et al., 1999). When the body is infected with exogenous antigens, the concentration of CD4⁺ lymphocytes will increase to release proinflammatory cytokines that help fight infections (Chan et al., 2013). Consistently, our results revealed that DNCB can enhance the CD4⁺/CD8⁺ ratio, which is similar to that described in other studies (Bak et al., 2012; Luz Correa et al., 2014). Moreover, the CD4⁺/CD8⁺ ratio was decreased in the SNS group compared with the ratio in the DNCB group. This result suggests that SNS can downregulate the excessive immune response induced by DNCB. Moreover, our results showed a very low CD4⁺/CD8⁺ ratio in the positive control group, suggesting that long-term DEX treatment (21 days in our study) is associated with immune deficiency. This result is consistent with the side effects of DEX mentioned above. Thus, topical SNS may become an effective and novel treatment to improve AD without over suppression of immune function.

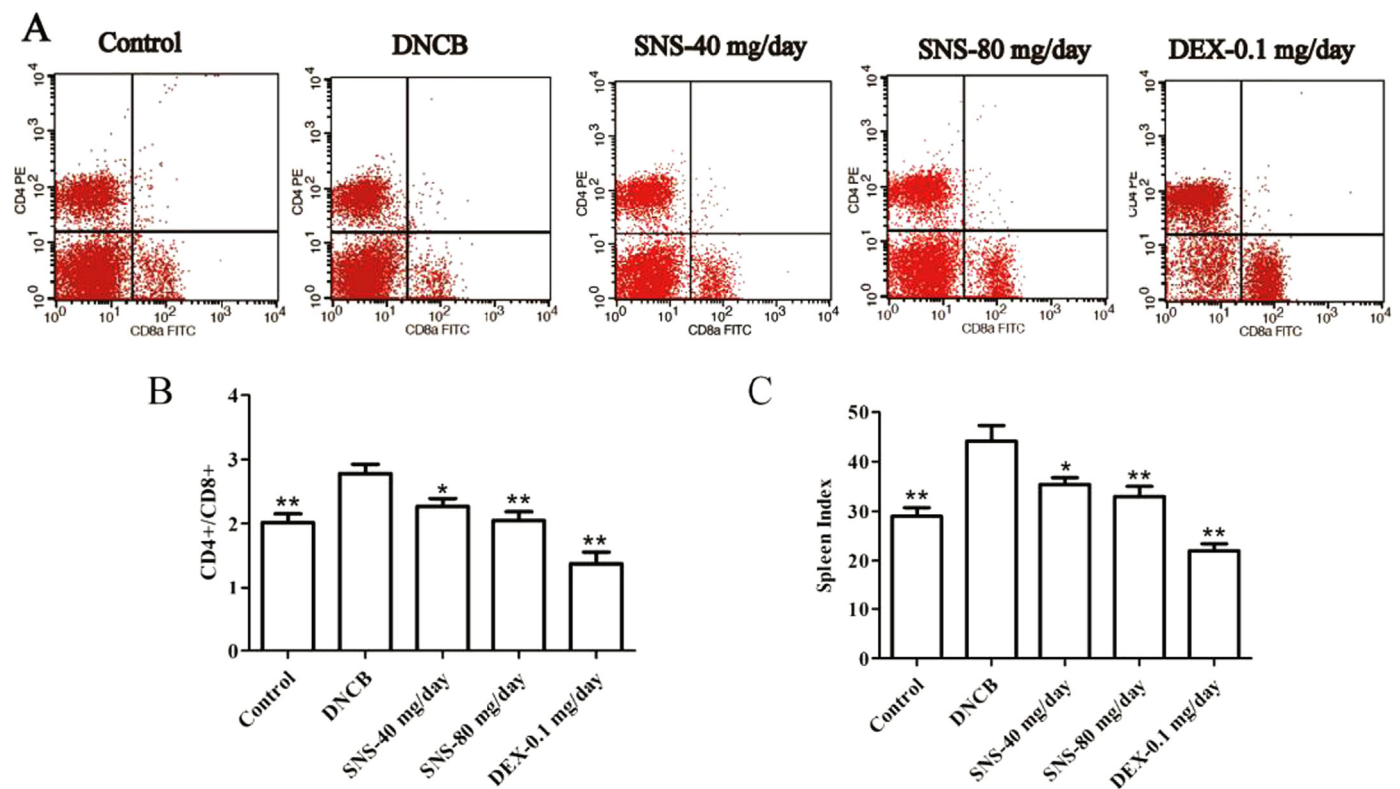


Fig. 7. SNS reduces the ratio of CD4 + /CD8 + in the spleen and decreases the spleen index. (A) CD4 + and CD8 + cells were extracted from the spleen immediately after euthanasia, according to the instructions supplied with the Mouse T Lymphocyte Subset Antibody Cocktail and were subjected to flow cytometry. Bivariate plots are shown that are representative of five independent assessments, which were quantified and plotted as the ratio of CD4 + /CD8 + in part B. (C) Spleen index = spleen weight (mg)/body weight (g) $\times 10$ ($n = 10$). Data are plotted as means $\pm$ SEM. * $P < 0.05$ vs DNCB, ** $P < 0.01$ vs DNCB. DNCB, 2,4-dinitrochlorobenzene; DEX, dexamethasone; SNS, Si-Ni-San; PE, phycoerythrin; FITC, fluorescein isothiocyanate.

To further understand the mechanism of the anti-inflammatory and immune response regulation of SNS, we studied its influence on NF- κ B (p65) and MAPK signalling. Previous studies have indicated that the activation of NF- κ B (p65) and MAPK signalling, leading to proinflammatory cytokine secretion, can contribute to the development of inflammation and immune regulation (Chan et al., 2013; Lawrence, 2009; Luz Correa et al., 2014). In the NF- κ B (p65) inflammatory response, extracellular signals bind to toll-like receptors, which activate I κ B kinase (IKK) in the cell cytoplasm. Afterward, the activated IKK complex phosphorylates two serine residues on I κ B- α , which is an NF- κ B inhibitory protein, promoting the ubiquitination, dissociation, and subsequent proteasomal degradation of I κ B- α (Wuertz et al., 2012). After I κ B- α is unbound from the NF- κ B p50/p65 hetero dimer, the latter transcription factor translocates to the nucleus, binds to specific sequences called response elements in the DNA, and activates gene transcription. This phenomenon results in the production of inflammatory mediators, including TNF- α , IL-1 β , monocyte chemoattractant protein 1 (MCP-1), and granulocyte-macrophage colony-stimulating factor (GM-CSF). Furthermore, inflammatory cytokines can activate NF- κ B (p65), thereby forming an amplifying loop called positive feedback activation (Chen et al., 2016; Swaroop et al., 2016). In our study, I κ B- α and NF- κ B (p65) phosphorylation levels were significantly increased by DNCB and decreased by SNS in a dose-dependent manner.

MAPKs, which are known for their abilities to regulate cell functions and stimulate cell responses, also play important roles in AD pathogenesis by triggering inflammatory responses. Moreover, down-regulation of MAPK signalling can inhibit the activation of NF- κ B (Kaminska, 2005). The key MAPK, ERK, p38, and JNK, can be activated by proinflammatory cytokines, such as TNF- α and IL-1 β (Peti and Page, 2013; Xia et al., 2014). Thus, p38 and JNK pathways generate

signalling cascades that can produce more inflammatory cytokines. Furthermore, other effects of MAPKs are related to immune responses, apoptosis regulation, and cell differentiation (Hajar et al., 2015). Therefore, MAPKs are crucial targets for the treatment of inflammatory diseases. SNS inhibited the phosphorylation of MAPKs in the dorsal skin samples, which may be a major contribution to its anti-inflammatory function in AD-like mice. Hence, the presence of SNS can dose-dependently prevent the activation of MAPKs, which are important agents in the proinflammatory process, which is closely linked to AD.

5. Conclusions

Topical SNS alleviates the development of AD-like symptoms in KM mice by reducing inflammatory cytokine levels in the serum, decreasing inflammatory cell infiltration in the tissues, lowering the CD4 + /CD8 + ratio, and inhibiting MAPK and NF- κ B signalling. These effects are dose-dependent and do not appear to lead to excessive immune suppression, which is associated with the side effects of topical corticosteroids.

Acknowledgments

This work was supported by grants from National Natural Science Foundation of China (81673805, 81373575, 8177410, and 81601779), Guangdong Natural Science Foundation (2014A030110210), Planned Science Technology Project of Guangzhou (201607010146), Traditional Chinese Medicine Bureau of Guangdong Province (20161260), Climbing Program Special Funds of Guangdong Province (pdjh2017a0094) and National Training Program of Innovation and Entrepreneurship for Undergraduates (201712121006).

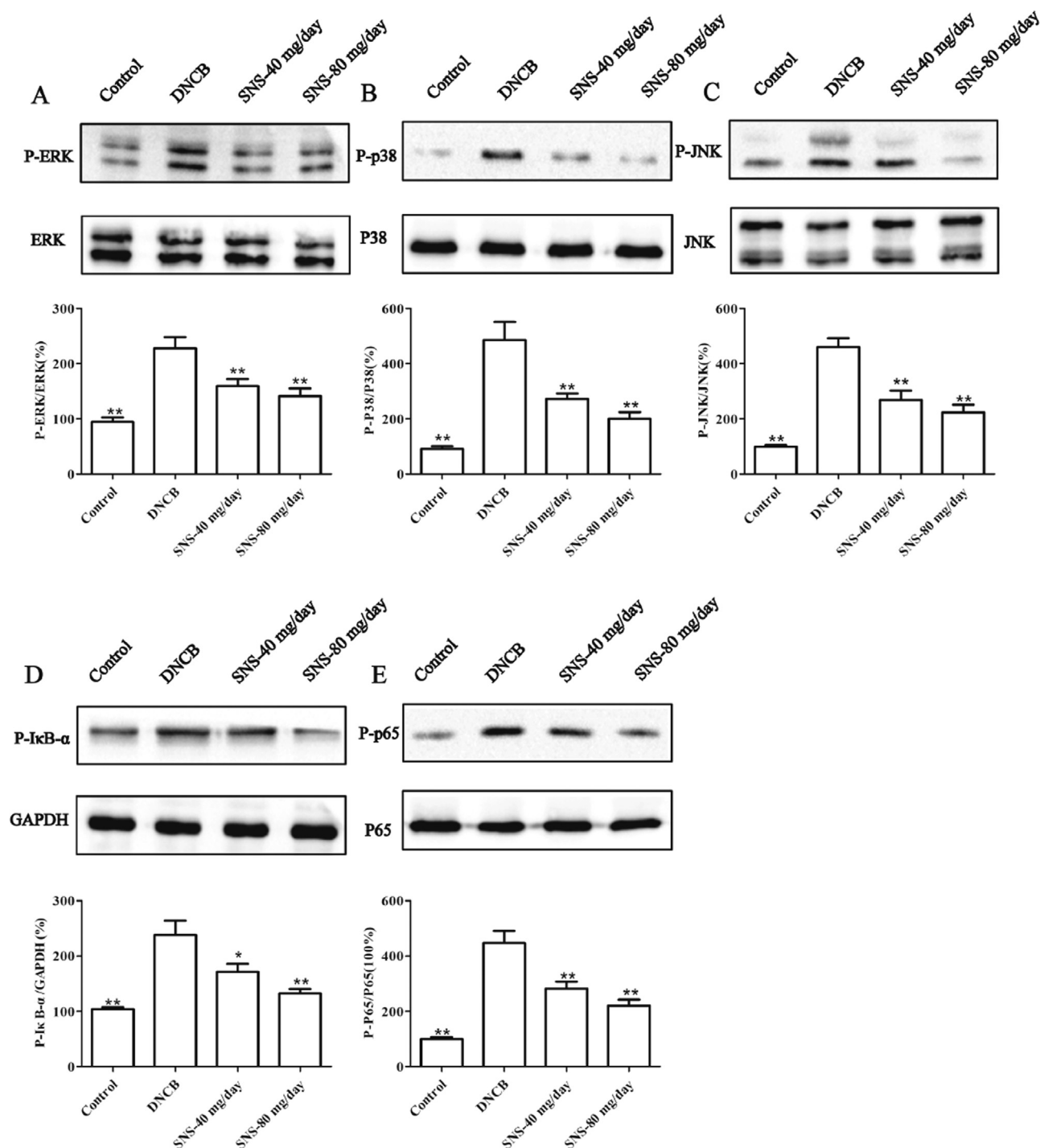


Fig. 8. SNS treatment dose-dependently suppresses the activation of MAPKs and NF- κ B signalling. The effects of SNS on ERK (A), p38 (B), JNK (C), I κ B- α (D), and NF- κ B (p65, E) phosphorylation in the dorsal skin of DNCB-stimulated KM mice treated with SNS-40 mg/day or SNS-80 mg/day. The levels of P-ERK, P-JNK, P-p38, and P-p65 were normalised to the levels of ERK, JNK, P38, and p65 proteins, respectively. Mouse GAPDH was used as the internal loading control for p-I κ B- α . Data are presented as means $\pm$ SD, $n = 6$. * $P < 0.05$ vs DNCB, ** $P < 0.01$ vs DNCB. DNCB, 2,4-dinitrochlorobenzene; SNS, Si-Ni-San.

Conflict of interest statement

The authors have no conflict of interest.

Author contributions

Designed the study: Huijie Fan, Yingchun Zhou, and Bin Liu.

Coordinated the study and finalized the manuscript: Huijie Fan, Bin Liu, and Zhangbin Tan.

Read and approved the manuscript: Yingchun Zhou, Bin Liu, and Kevin Liu Kot.

Performed the experiments: Huijie Fan, ZepingXie, Ziwen Lu, Yiming Bi, LingpengXie, and Yuting Wu.

Analyzed the data: Huijie Fan, Zhangbin Tan, Wentong Zhang and Kevin Liu Kot.

Wrote the paper: Huijie Fan

References

- Bak, J.P., Kim, Y.M., Son, J., Kim, C.J., Kim, E.H., 2012. Application of concentrated deep sea water inhibits the development of atopic dermatitis-like skin lesions in NC/Nga mice. *BMC Complement. Altern. Med.* 12, 108.
- Brandt, E.B., Sivaprasad, U., 2011. Th2 cytokines and atopic dermatitis. *J. Clin. Cell. Immunol.* 2.
- Brown, S., Reynolds, N.J., 2006. Atopic and non-atopic eczema. *BMJ (Clinical research ed.)* 332, 584–588.
- Catherine Mack Correa, M., Nebus, J., 2012. Management of patients with atopic dermatitis: the role of emollient therapy. *Dermatol. Res. Pract.* 2012, 836931.
- Chan, C.C., Liou, C.J., Xu, P.Y., Shen, J.J., Kuo, M.L., Len, W.B., Chang, L.E., Huang, W.C., 2013. Effect of dehydroepiandrosterone on atopic dermatitis-like skin lesions induced by 1-chloro-2,4-dinitrobenzene in mouse. *J. Dermatol. Sci.* 72, 149–157.
- Chen, L., Martinez, O., Overbergh, L., Mathieu, C., Prabhakar, B.S., Chan, L.S., 2004. Early up-regulation of Th2 cytokines and late surge of Th1 cytokines in an atopic dermatitis model. *Clin. Exp. Immunol.* 138, 375–387.
- Chen, Y., Xian, Y., Lai, Z., Loo, S., Chan, W.Y., Lin, Z.X., 2016. Anti-inflammatory and anti-allergic effects and underlying mechanisms of Huang-Lian-Jie-Du extract: implication for atopic dermatitis treatment. *J. Ethnopharmacol.* 185, 41–52.
- Christensen, G.B., Hvid, M., Kvist, P.H., Deleuran, B., Deleuran, M., Vestergaard, C., Kemp, K., 2011. CD4⁺ T cell depletion changes the cytokine environment from a Th1/Th2 response to a Th17-like response in a murine model of atopic dermatitis. *Int. Immunopharmacol.* 11, 1285–1292.
- Dhossche, J., Simpson, E., Hajar, T., 2017. Topical corticosteroid withdrawal in a pediatric patient. *JAAD Case Rep.* 3, 420–421.
- Fu, H., Cheng, H., Cao, G., Zhang, X., Tu, J., Sun, M., Mou, X., Shou, Q., Ke, Y., 2016. The inhibition of mast cell activation of radix paeoniae alba extraction identified by TCPD based and conventional cell function assay systems. *PLoS One* 11, e0155930.
- Hajar, T., Leshem, Y.A., Hanifin, J.M., Nedorost, S.T., Lio, P.A., Paller, A.S., Block, J., Simpson, E.L., 2015. A systematic review of topical corticosteroid withdrawal ("steroid addiction") in patients with atopic dermatitis and other dermatoses. *J. Am. Acad. Dermatol.* 72 (541–549.e542).
- Han, H.M., Kim, S.J., Kim, J.S., Kim, B.H., Lee, H.W., Lee, Y.T., Kang, K.H., 2016. Ameliorative effects of *Artemisia argyi* Folium extract on 2,4-dinitrochlorobenzene-induced atopic dermatitis-like lesions in BALB/c mice. *Mol. Med. Rep.* 14, 3206–3214.
- Jeon, Y.D., Bang, K.S., Shin, M.K., Lee, J.H., Chang, Y.N., Jin, J.S., 2016. Regulatory effects of glycyrrhizae radix extract on DSS-induced ulcerative colitis. *BMC Complement. Altern. Med.* 16, 459.
- Jiang, J., Zhou, C., Xu, Q., 2003. Alleviating effects of si-ni-san, a traditional Chinese prescription, on experimental liver injury and its mechanisms. *Biol. Pharm. Bull.* 26, 1089–1094.
- Kagi, M.K., Wuthrich, B., Montano, E., Barandun, J., Blaser, K., Walker, C., 1994. Differential cytokine profiles in peripheral blood lymphocyte supernatants and skin biopsies from patients with different forms of atopic dermatitis, psoriasis and normal individuals. *Int. Arch. Allergy Immunol.* 103, 332–340.
- Kaminska, B., 2005. MAPK signalling pathways as molecular targets for anti-inflammatory therapy—from molecular mechanisms to therapeutic benefits. *Biochim. Et. Biophys. Acta* 1754, 253–262.
- Kang, H., Lee, C.H., Kim, J.R., Kwon, J.Y., Son, M.J., Kim, J.E., Lee, K.W., 2017. Theobroma cacao extract attenuates the development of dermatophagoides farinae-induced atopic dermatitis-like symptoms in NC/Nga mice. *Food Chem.* 216, 19–26.
- Karuppagounder, V., Arumugam, S., Thandavarayan, R.A., Pitchaimani, V., Sreedhar, R., Afrin, R., Harima, M., Suzuki, H., Nomoto, M., Miyashita, S., Suzuki, K., Watanabe, K., 2014. Resveratrol attenuates HMGB1 signaling and inflammation in house dust mite-induced atopic dermatitis in mice. *Int. Immunopharmacol.* 23, 617–623.
- Kiebert, G., Sorensen, S.V., Revicki, D., Fagan, S.C., Doyle, J.J., Cohen, J., Fivenson, D., 2002. Atopic dermatitis is associated with a decrement in health-related quality of life. *Int. J. Dermatol.* 41, 151–158.
- Kim, G.D., Kim, T.H., Park, Y.S., Ahn, H.J., Cho, J.J., Park, C.S., 2015. Immune response against 2,4-dinitrofluorobenzene-induced atopic dermatitis-like clinical manifestation is suppressed by spermidine in NC/Nga mice. *Scand. J. Immunol.* 81, 221–228.
- 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. Publ. Br. Ind. Biol. Res. Assoc.* 66, 341–349.
- Komatsu, K.I., Takanari, J., Maeda, T., Kitadate, K., Sato, T., Mihara, Y., Uehara, K., Wakame, K., 2016. Perilla leaf extract prevents atopic dermatitis induced by an extract of *Dermatophagoides farinae* in NC/Nga mice. *Asian Pac. J. Allergy Immunol.* 34, 272–277.
- Lawrence, T., 2009. The nuclear factor NF- κ B pathway in inflammation. *Cold Spring Harb. Perspect. Biol.* 1.
- Lee, H.J., Kim, M.H., Choi, Y.Y., Kim, E.H., Hong, J., Kim, K., Yang, W.M., 2016. Improvement of atopic dermatitis with topical application of *Spirodela polyrrhiza*. *J. Ethnopharmacol.* 180, 12–17.
- Leonardi, S., Rotolo, N., Vitaliti, G., Spicuzza, L., La Rosa, M., 2007. IgE values and T-lymphocyte subsets in children with atopic eczema/dermatitis syndrome. *Allergy Asthma Proc.* 28, 529–534.
- Leung, D.Y., 2000. Atopic dermatitis: new insights and opportunities for therapeutic intervention. *J. Allergy Clin. Immunol.* 105, 860–876.
- Li, Y., Liu, A., Wang, Y., Yan, X., 2013. The efficacy study on si ni san freeze-dried powder on sleep phase in insomniac and normal rats. *Evid.-Based Complement. Altern. Med.: eCAM* 2013, 947075.
- Luz Correa, B., Ornaghi, A.P., Cerutti Muller, G., Engroff, P., Pestana Lopes, R., Gomes da Silva Filho, I., Bosch, J.A., Bonorino, C., Bauer, M.E., 2014. The inverted CD4: CD8 ratio is associated with cytomegalovirus, poor cognitive and functional states in older adults. *Neuroimmunomodulation* 21, 206–212.
- Novak, N., Bieber, T., Leung, D.Y., 2003. Immune mechanisms leading to atopic dermatitis. *J. Allergy Clin. Immunol.* 112, S128–S139.
- Ohta, Y., Kobayashi, T., Hayashi, T., Inui, K., Yoshino, J., Nakazawa, S., 2006. Preventive effect of Shigyaku-san on progression of acute gastric mucosal lesions induced by compound 48/80, a mast cell degranulator, in rats. *Phytother. Res.: PTR* 20, 256–262.
- Peng, W., Novak, N., 2015. Pathogenesis of atopic dermatitis. *Clin. Exp. Allergy.: J. Br. Soc. Allergy. Clin. Immunol.* 45, 566–574.
- Peti, W., Page, R., 2013. Molecular basis of map kinase regulation. protein science: a publication of the protein. *Society* 22, 1698–1710.
- Prinz, B., Michelsen, S., Pfeiffer, C., Plewig, G., 1999. Long-term application of extracorporeal photochemotherapy in severe atopic dermatitis. *J. Am. Acad. Dermatol.* 40, 577–582.
- Rudikoff, D., Lebowitz, M., 1998. Atopic dermatitis. *Lancet (Lond., Engl.)* 351, 1715–1721.
- Saraswat, A., 2010. Topical corticosteroid use in children: adverse effects and how to minimize them. *Indian J. Dermatol., Venereol. Leprol.* 76, 225–228.
- Schwartz, J., Friedman, A.J., 2016. Exogenous Factors in Skin Barrier Repair. *J. Drugs Dermatol.: JDD* 15, 1289–1294.
- Siracusa, M.C., Comeau, M.R., Artis, D., 2011. New insights into basophil biology: initiators, regulators, and effectors of type 2 inflammation. *Ann. N. Y. Acad. Sci.* 1217, 166–177.
- Sun, Y., Dong, Y., Jiang, H.J., Cai, T.T., Chen, L., Zhou, X., Chen, T., Xu, Q., 2009. Dissection of the role of paeoniflorin in the traditional Chinese medicinal formula Si-Ni-San against contact dermatitis in mice. *Life Sci.* 84, 337–344.
- Swaroop, S., Sengupta, N., Suryawanshi, A.R., Adlakha, Y.K., Basu, A., 2016. HSP60 plays a regulatory role in IL-1 β -induced microglial inflammation via TLR4-p38 MAPK axis. *J. Neuroinflamm.* 13, 27.
- Tanaka, M., Satou, T., Koike, K., 2013. Anxiolytic-like effect of Shigyaku-san extract with low side effects in mice. *J. Nat. Med.* 67, 862–866.
- Wuertz, K., Vo, N., Kleetsas, D., Boos, N., 2012. Inflammatory and catabolic signalling in intervertebral discs: the roles of NF- κ B and map kinases. *Eur. Cells Mater.* 23, 103–119 (discussion 119–120).
- Xia, F., Wang, C., Jin, Y., Liu, Q., Meng, Q., Liu, K., Sun, H., 2014. Luteolin protects HUVECs from TNF- α -induced oxidative stress and inflammation via its effects on the Nox4/ROS-NF- κ B and MAPK pathways. *J. Atheroscler. Thromb.* 21, 768–783.
- Yu, S.P., Ye, H., Ha, N.L., Ding, S.Q., Chen, G., 2005. Effect of modified Sinisan on anorectal manometry of the constipation predominant type of irritable bowel syndrome. *Chin. J. Integr. Med.* 11, 27–30.
- Zhang, L., Dong, Y., Sun, Y., Chen, T., Xu, Q., 2006. Role of four major components in the effect of Si-Ni-San, a traditional Chinese prescription, against contact sensitivity in mice. *J. Pharm. Pharmacol.* 58, 1257–1264.