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

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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)− 1α, IL-1β, 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)), IκB-α, and nuclear factor (NF)-κB (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, IκB-α, and NF-κB (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 Lebwohl, 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

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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-1α and IL-1β) 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-IκB-α (P-IκB-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 μL 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 × 250 mm, 5 μm; USA). The mobile phase was composed of solvent A (acetonitrile) and solvent B (12 mM ammonium acetate, 0.1% acetic acid). The linear gradient lution 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 goup): 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 × 2 cm. After 24 h, we treated the skin with 100 μL of 7% (w/v) DNCB, which had been dissolved in a vehicle containing acetone and olive oil (4:1). After 5 days, 5 μL of 0.5% (w/v) DNCB dissolved in the same vehicle was used to treat both sides of the right