Dissection of the role of paeoniflorin in the traditional Chinese medicinal formula Si-Ni-San against contact dermatitis in mice

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Keywords:
Traditional Chinese medicine
Si-Ni-San
Paeoniflorin
Selective deletion
Immunosaffinity column

Introduction

Traditional Chinese medicine (TCM) is one of the most ancient and greatest traditions, with sound philosophical basis and a wide range of practices. In TCM, Chinese multi-herb formulas are the most commonly employed therapeutic method assisting Chinese people in dealing with diseases. Scientific evidence for TCM is generally achieved through rigorous experimental design. For instance, various methods including activity-guided isolation (Cakir et al., 2003; Schleich et al., 2006), serum pharmacology (Cao et al., 2003), computer predicting (Wang et al., 2005), herbal code database (Yi and Chang, 2004), and pharmacogenomics (Efferth et al., 2008) have been used in the research of TCM. However, these are not sufficient to understand how these formulas work, since the integrated effect of the formula is not just the simple addition of the efficacy of each active ingredient. Research in TCM has been dominated by the search for its biological basis, identifying active substances, and investigating mechanisms of action. In contrast, research in the role and interaction of active ingredients in the formulas is still scarce, thus hampering the understanding of the rationality of formula design in TCM.

Aims: The roles of specific active ingredients in Chinese medicinal formulas have not been clearly elucidated. In this study, we selectively deleted and replenished paeoniflorin from Si-Ni-San, a traditional Chinese prescription, and aimed to identify the molecular basis of how paeoniflorin exerted its effect in Si-Ni-San.

Main methods: Contact dermatitis was induced in mice with picryl chloride. Paeoniflorin was selectively deleted from Si-Ni-San by an immunosaffinity column. Quantitative real-time PCR, western blot, and enzyme-linked immunosorbent assay were used in this study.

Key findings: Both Si-Ni-San and paeoniflorin significantly reduced ear swelling in mice while the paeoniflorin-deleted Si-Ni-San (Si-Ni-SanPF) showed little ameliorative effect. In lipopolysaccharide-evoked macrophages, Si-Ni-San and paeoniflorin markedly inhibited tumor necrosis factor-α production, cyclooxygenase-2 activity, as well as extracellular signal-regulated kinase 1/2 phosphorylation while Si-Ni-SanPF exhibited no or slight inhibitory effect. Furthermore, the inhibitory effect on the production of tumor necrosis factor-α reappeared when different proportions of paeoniflorin were replenished in Si-Ni-SanPF. In addition, the expression of macrophage migration inhibitory factor in T cells, rather than macrophages, was significantly inhibited by Si-Ni-San, but not Si-Ni-SanPF. Our data indicate paeoniflorin is the principal component of Si-Ni-San, exerting negative regulation on the function of macrophages in contact dermatitis.

Significance: The present study suggests that dissecting the role of specific constituents in medicinal formulas through selective deletion and replenishment may be a useful strategy in recognizing and validating an active ingredient in traditional Chinese medicine.

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be part of the process of MIF-mediated COX-2 activation (Mitchell et al., 1999).

Si-Ni-San is one representative of TCM derived from *Treatise on Febrile Diseases* (Zhang and Gu, 1996), a medical classic written by Zhongjing of the 3rd century. Si-Ni-San is effective in clinical practice for treating various inflammatory diseases including hepatitis, gastritis, and colitis (Guo et al., 1999; Zhang and Zhang, 2000; Chen et al., 2004), closely correlating with DTH mechanisms. The main components of Si-Ni-San are Radix Bupleuri Chinensis (Chaihu), Radix Paeoniae Alba (Shaoyao), Fructus Citri Aurantii (Zhishi), and Radix Glycyrrhizae Uralensi (Gancao), with saikosaponin, paeoniflorin, naringin and glycyrrhizin as major active ingredients (Yamamoto et al., 1975; Takagi and Harada, 1969; Roussef et al., 1987; Nikitina, 1966). Among these ingredients, paeoniflorin has been widely investigated as an antioxidant, cognitive enhancer, endothelium-dependent vasodilator, and neuroprotective agent (Goto et al., 1996; Ryu et al., 2001; Tabata et al., 2001; Chen et al., 2006). However, the actual contribution of paeoniflorin in Si-Ni-San against contact dermatitis, a typical T cell-mediated DTH response, remains unclear. In this study, the roles and mechanisms of paeoniflorin in Si-Ni-San were examined through both analytical and synthetic research approaches.

**Materials and methods**

**Animals**

Eight-to-ten-week-old female BALB/c and ICR mice were purchased from Experimental Animal Center of Nanjing Medical University (Nanjing, China). Male New Zealand white strain rabbits weighing 2–3 kg were purchased from Jiangsu Academy of Agricultural Sciences (Nanjing, China). Animal welfare and experimental procedures were carried out strictly in accordance with the *Guide for the Care and Use of Laboratory Animals* (The Ministry of Science and Technology of China, 2006) and the related ethical regulations of our university. All efforts were made to minimize animals’ suffering and to reduce the number of animals used.

**Drugs and reagents**

Radix Bupleuri Chinensis (Chaihu), Radix Paeoniae Alba (Shaoyao), Fructus Citri Aurantii (Zhishi) and Radix Glycyrrhizae Uralensi (Gancao) were purchased from Nanjing Medicinal Material Co. (Nanjing, China) and identified by Dr. Boyang Yu (Department of Chinese Medicinal Prescription, China Pharmaceutical University) as *Bupleurum chinense* DC., *Citus aurantium* L., *Paonia albiflora* Pall., and *Glycyrrhiza uralensis* Fisch., respectively. They were mixed in an equal ratio (25 g of each drug in total 100 g) to make up Si-Ni-San. The ethanol extract of Si-Ni-San was lyophilized to obtain a powder with 21.0% yield. The content of paeoniflorin (Fig. 1) in Si-Ni-San was determined by high-performance liquid chromatography (HPLC) as 1.5%. The powders were dissolved in normal saline for in vivo assay by oral administration to mice and in medium for in vitro assay. The reagents used in this study were purchased as follows. Paeoniflorin (purity >98%, Wako, Japan); Sephadex G-25 (Beijing Bairder Biotechnology Co., Ltd, Beijing, China); cellulose DE-32 (Beijing Dingguo Biotechnology Co., Ltd, Beijing, China); CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech, Piscataway, NJ); picryl chloride (Nacalai tesque Inc, Kyoto, Japan); injection dexamethasone sodium phosphate (Dex, Nanjing 3rd pharmaceutical factory, Nanjing, China); RPMI 1640, Dulbecco’s modified Eagle medium (DMEM) and fetal bovine serum (Gibco/Invitrogen, Carlsbad, CA); ELISA kit for TNF-α (R&D Systems, Minneapolis, MN); 3-(4, 5-dimethyl-2-thiazyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT), bovine serum albumin, ovalbumin, nimesulide, arachidonic acid, hemat in porcine, 2',7'-dichlorofluorescin diacetate, 2,4,6-trinitrobenzenesulfonic acid hydrate, lipopolysaccharide (LPS) and concanavalin A (Con A) (Sigma Chemical Co., St. Louis, MO); anti-α Tubulin, anti-Acin and MIF (Santa Cruz Biotechnology, Santa Cruz, CA); anti-p38, anti-phospho-p38, anti-ERK1/2, anti-phospho-ERK1/2, anti-JNK and anti-phospho-JNK antibody (Cell Signaling Technology, Beverly, MA).

**Generation of the immunoaffinity column**

Paeoniflorin with carrier protein conjugates was synthesized by a modified synthetic procedure (Lu et al., 2003). Paeoniflorin–bovine serum albumin conjugate was used to elicit an immune response and applied in rabbits to get anti-paeoniflorin polyclonal antibodies, while paeoniflorin–ovalbumin conjugate was used as the coating antigen of enzyme-linked immunosorbent assay (ELISA) for antisemum titer. The purification of antibody was carried out on a Gel-HPLC system and the purity was more than 95%. The anti-paeoniflorin polyclonal antibody was coupled to CNBr-Sepharose 4B to generate the immunoaffinity column as we described previously (Zhang et al., 2005).

**Cell lines**

Murine macrophage RAW264.7 cells were cultured in DMEM medium supplemented with 100 U/mL of penicillin, 100 µg/mL of streptomycin and 10% fetal bovine serum, under a humidified 5% (v/v) CO₂ atmosphere at 37 °C.

**Preparation of mouse peritoneal macrophages**

BALB/c mice were injected with 0.5 mL of sodium carboxymethylcellulose (3%) into the peritoneum. After 72 h, the mice were sacrificed and the peritoneum was injected with 10 mL DMEM medium with 5% fetal bovine serum. The peritoneal lavage was removed and the cells were centrifuged (200 g, 10 min, 4 °C) and resuspended in DMEM with 10% fetal bovine serum supplemented with l-glutamine and penicillin/streptomycin. Non-adherent cells were removed by rinsing with PBS.

**Picryl chloride-induced ear contact dermatitis**

ICR mice were sensitized by painting 0.1 mL of 1% picryl chloride in ethanol onto the shaved skin of their abdomens. Five days after sensitization, they were challenged on the right ear with 30 µL of 1% picryl chloride in olive oil. Ear swelling was evaluated by the difference in thickness between the right and left ears measured with an engineer’s micrometer (0.001 mm; Mitutoyo Co, Tokyo, Japan) 18 h after challenge. The negative control animals were normally sensitized and painted with olive oil alone when challenged. The positive control animals with contact dermatitis were given normal saline instead of drugs.

**Histology analysis**

Ear tissues were fixed in 10% formalin, embedded in paraffin, cut into 5-µm sections, deparaffinized in xylene, and serially dehydrated in decreasing concentrations of ethanol. Sections were stained with...
hematoxylin and eosin and examined under light microscopy to detect lymphocyte infiltration and to evaluate tissue structure.

**RT-PCR and real-time PCR analysis**

RNA was extracted from liver tissue using Trizol reagent (Invitrogen, Carlsbad, CA). One microgram of RNA was reverse transcribed to cDNA. The primer sequences used in polymerase chain reaction (PCR) were as follows: GAPDH, 5′-AAGCACCCCTCTTGGAC and 3′-CAGACTCATACAAGC-CACTT; IFN-γ, 5′-CTTCACGACACGACGAAAGAAGC and 3′-ACTAAAGC-CCCAACATGACAAC; TNF-α, 5′-ATCTTCTCTAATAATTTGAGTACA and 3′-TCCAAAATGGAAAGATGAGGGT; IL-1β, 5′-GCCATCTCTGGTAGTACCT and 3′-CTTGGTAGTCGTCGTGTCG; COX-2, 5′-AATCTACTAAGTTGTGAAGTGC-ATT and 3′-GTTAATTTGGGATGATGTATTAG; iNOS, 5′-ACAACGTGAGAAAACCGGTG and 3′-CCAGAAGCTACGGGCCTCGCA; MIF, 5′-GCAAGCAGCAGACTAACT and 3′-TACTGCGAGGTGTCACAC. The PCR cycle conditions were: 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s for 28 cycles. After the amplification, PCR products were separated by electrophoresis on 1.5% agarose gels and visualized using ethidium bromide. For quantitative real-time PCR analysis, amplification was carried out for 40 cycles of the same PCR conditions mentioned above and product was detected using SYBR Green I dye (Molecular Probes, Eugene, OR). Reactions were run in triplicate using GAPDH as the internal RNA control on an ABI 7000 Thermocycler (Applied Biosystems Inc., Foster City, CA).

**Enzyme-linked immunosorbent assay of TNF-α**

The level of TNF-α in cell culture supernatant was determined using ELISA kit from R&D systems (Minneapolis, MN).

**Western blot**

Proteins from cells and liver tissues were extracted in lysis buffer (30 mmol/L Tris, pH 7.5, 150 mmol/L sodium chloride, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 1% Nonidet P-40, 10% glycerol, and phosphatase and protease inhibitors). The proteins were then separated by SDS-PAGE and electrophoretically transferred onto polyvinylidene fluoride membranes. The membranes were probed with antibodies overnight at 4 °C, and then incubated with a HRP-coupled secondary antibody. Detection membranes were probed with antibodies overnight at 4 °C, and then incubated with a HRP-coupled secondary antibody. Detection was performed using a LumiGLO chemiluminescent substrate system (KPL, Guildford, UK).

**COX-2 enzyme activity assay**

COX-2 enzyme activity assay was performed as previously described (Tyson-Capper and Europe-Finner, 2006) with some modifications. Briefly, macrophages were collected in PBS with 2 mM CaCl₂, lysed ultrasonically for 30 s, times, and centrifuged (10,000 g at 4 °C for 15 min) to get protein lysate. To three tubes labeled A, B and C, protein lysate sample 60 µL, 1 M Tris–HCl 60 µL, 50 µL 2′,7′-dichlorofluorescein diacetate 60 µL, and 20 µM hematin 60 µL were added, then in tube C, 2.5 µM nimesulide 60 µL was added. Then being allowed to react at 30 °C for 10 min, 60 µL of 9.0 µM arachidonic acid was added to tubes B and C, and the tubes were cultured further for 20 min at 30 °C to complete the enzyme reaction. Finally, 2 mL cold PBS was added to all tubes to stop the reactions. Fluorescence was evaluated within 2 h (excitation wavelength 450 nm, emission wavelength 550 nm, aperture 5 nm), COX-2 activity is judged by the fluorescence intensity. Total COX activity refers to B–A, activity of COX-1 refers to C–A while activity of COX-2 comes to B–C.

**Statistical analysis**

Quantitative data are expressed as mean±SD. Statistical significance was determined by one-way analysis of variance followed by the Bonferroni post-hoc test for multiple comparisons or the two-tailed Student’s t-test. A P value of less than 0.05 was considered statistically significant.

**Results**

**Generation of the immunoaffinity column and deletion of paeoniflorin from Si-Ni-San**

The purified anti-paeoniflorin polyclonal antibodies were coupled to CNBr-Sepharose 4B to generate the immunoaffinity column. By detecting the uncoupled antibody in the effluent of the column using HPLC, we found 21 mg of antibody coupled to 1 g CNBr-Sepharose 4B. The breakthrough volume was tested to determine the mass of paeoniflorin that the immunoaffinity column could catch. The capacity of the immunoaffinity column was 1.1 µg. For the content of paeoniflorin in Si-Ni-San and the breakthrough volume detected above, the extract of Si-Ni-San was dissolved in 0.01 M PBS to a concentration of 12.5 µg/mL and 5.5 µL solution was injected to the column. The effluent was collected and lyophilized. The sample, from which paeoniflorin was deleted (Si-Ni-SanP¹⁰), was obtained. The immunoaffinity column was eluted by 10 mL of 40% ethanol and eluted by 10 mL of 0.01 M PBS to give regeneration. After passing through the column, the compound was almost undetectable and its content decreased approximately 97% (Fig. 2).

**The deletion of paeoniflorin significantly weakened the alleviative effect of Si-Ni-San on ear swelling and inflammatory cytokine expression of picryl chloride-induced contact dermatitis in mice**

When administered once a day intragastrically for 6 days following sensitization, 200 mg/kg of Si-Ni-San and 20 mg/kg of paeoniflorin

![Fig. 2. Selective depletion of paeoniflorin from Si-Ni-San.](http://example.com/fig2.png)
sensitized and painted with olive oil alone when challenged. The positive control mice with contact dermatitis were given normal saline instead of drugs. Values are means±SD of 10 mice. The negative control mice were normally given intragastrically and dexamethasone (10 mg/kg) was given intramuscularly for 6 days following sensitization.

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RAW264.7 cells and primary peritoneal macrophages were pre-treated with drugs for 1 h, then stimulated with 100 ng/mL LPS for 24 h. Si-Ni-San significantly inhibited TNF-α production from RAW264.7 cells while Si-Ni-SanPF− showed little inhibitory effect (Fig. 4A). The similar result was also observed in primary peritoneal macrophages (Fig. 4B). The positive control dexamethasone also noticeably inhibited TNF-α production in both RAW264.7 cells and primary peritoneal macrophages. To further confirm the role of paeoniflorin in the whole formula, different proportions of paeoniflorin were then replenished to Si-Ni-SanPF−. The supplementation of paeoniflorin to Si-Ni-SanPF− at the proportions of 0.01–1:1 dose-dependently recovered the inhibition on TNF-α production (Fig. 4C). The similar tendency was also observed in the macrophage phagocytosis assay (Supporting information Fig. S1) and nitric oxide secretion assay (Supporting information Fig. S2).

The deletion of paeoniflorin reduced the down-regulatory effect of Si-Ni-San− on the phosphorylation of ERK1/2 MAPK and the activity of COX-2 in LPS-stimulated RAW264.7 cells

As shown in Fig. 5A, pretreatment with Si-Ni-San and paeoniflorin markedly downregulated LPS-evoked phosphorylation of ERK1/2, but not p38 or JNK MAPKs. Also, Si-Ni-SanPF− no longer showed suppression of the downregulation on ERK1/2 phosphorylation. None of the three groups of drugs influenced the total cellular p38, ERK1/2, or JNK expression. Next, we examined the effect of drugs on COX-2, which was one of the important molecules downstream of ERK1/2 MAPK signaling (Kim et al., 2008). Although Si-Ni-San, paeoniflorin, and Si-Ni-SanPF− all downregulated the COX-2 enzyme

significantly inhibited ear swelling by 50.9% and 33.5%, respectively. Such alleviative effect was significantly blocked when paeoniflorin was selectively deleted from the whole formula. (The inhibitory rate of Si-Ni-SanPF− was only 21.7%). Dexamethasone as a positive control also inhibited ear swelling (Fig. 3A). The histopathologic changes in the ear were mainly observed in the dermis, including severe inflammatory infiltration, vascular congestion, and moderate edema in the control group. Against the positive control, the mice treated with Si-Ni-San only showed a mild cellular infiltration and vasodilation without edema, while the mice treated with Si-Ni-SanPF− revealed a moderate cellular infiltration and mild edema (Fig. 3B). Furthermore, the elevated expressions of inflammatory mediators including IFN-γ, TNF-α, IL-1β, COX-2, and iNOS in ear tissue from mice with picryl chloride-induced contact dermatitis were obviously inhibited by Si-Ni-San, but not Si-Ni-SanPF− (Fig. 3C). We also examined the expressions of other inflammatory mediators by RT-PCR on the macrophage cell line RAW264.7 cells in vitro. Si-Ni-San and paeoniflorin significantly inhibited the expression of TNF-α, COX-2, and IL-1β in the LPS-activated RAW264.7 macrophages, while Si-Ni-SanPF− had almost no inhibitory effect (Fig. 3D).

The deletion of paeoniflorin blocked the inhibitory effect of Si-Ni-San− on the production of TNF−α in LPS-activated macrophages
activity. Si-Ni-San$^{3\text{R}}$ appeared to have a much lower inhibitory effect than the other two (Fig. 5B). Furthermore, the addition of paeoniflorin back to Si-Ni-San$^{3\text{R}}$ at the proportion of 0.1:1 significantly enhanced the inhibitory effect. The positive control dexamethasone also significantly inhibited COX-2 enzyme activity. It was notable that the viability of both untreated cells, as well as cells treated with drugs of various doses, was more than 95%, which was measured by trypan blue exclusion (data not shown).

The deletion of paeoniflorin reversed the inhibitory effect of Si-Ni-San on macrophage migration inhibitory factor (MIF) secreted by T lymphocytes other than macrophages.

We next tested whether paeoniflorin could influence the proliferation, activation, and cytokine production of T lymphocytes and found that the deletion of paeoniflorin did not affect the regulative effect of Si-Ni-San on T lymphocytes (Supporting information Fig. S3). Conversely, Si-Ni-San dramatically inhibited MIF expression on T lymphocytes activated by Con A, and the deletion of paeoniflorin reversed the inhibitory effect of Si-Ni-San (Fig. 6A–C). In contrast, Si-Ni-San and paeoniflorin did not affect MIF expression in RAW264.7 macrophages activated by LPS (Fig. 6D–F).

**Discussion**

In order to explore the active principles of a Chinese herb and its blended formula, a common method is to isolate the compounds contained by a chemical purification or the activity-guided purification (Cakir et al., 2003; Schleich et al., 2006). Such assay is usually based on the activity of purified compounds, but it is difficult to identify the actual contribution of one single ingredient to the whole formula. It is important to design a method to examine the role of active ingredients in the formula. In this trial, a specific ingredient was deleted from the formula, using a gene knockout from a genome in active ingredients in the formula. In this trial, a specific ingredient was deleted from the formula, using a gene knockout from a genome in

![Fig. 4. The deletion of paeoniflorin blocked the inhibitory effect of Si-Ni-San on the production of TNF-α in LPS-activated macrophages.](image)

(A) Production of TNF-α on RAW264.7 macrophages stimulated by LPS. (B) Production of TNF-α on primary peritoneal macrophages stimulated by LPS. (C) Production of TNF-α on LPS-activated RAW264.7 macrophages when paeoniflorin was replenished to Si-Ni-San$^{3\text{R}}$. Si-Ni-San contained 1.5% of paeoniflorin, so the 0.01 proportion of Si-Ni-San$^{3\text{R}}$ with paeoniflorin represented $1\times10^{-5}$ g/mL+$1.5\times10^{-7}$ g/mL, 0.1 proportion of Si-Ni-San$^{3\text{R}}$ with paeoniflorin represented $1\times10^{-5}$ g/mL+$1.5\times10^{-5}$ g/mL, and 1:1 represented $1\times10^{-5}$ g/mL+$1.5\times10^{-5}$ g/mL. Each column represents the mean±SD of three independent experiments and each experiment includes triplicate sets. $^\text{*P}<0.05$, $^\text{**P}<0.01$ vs. cont, $^\text{Y}P<0.05$.

**Fig. 5.** The deletion of paeoniflorin reduced the down-regulation of Si-Ni-San on the phosphorylation of ERK1/2 MAPK and the activity of COX-2 of LPS-stimulated RAW264.7 macrophages. (A) Macrophages were treated with or without $1\times10^{-5}$ g/mL of Si-Ni-San, Si-Ni-San$^{3\text{R}}$, paeoniflorin and $5\times10^{-7}$ g/mL of dexamethasone in the presence of 100 ng/mL LPS for 24 h. Cytokine in supernatant was examined by ELISA. (B) Macrophages were treated with or without $1\times10^{-5}$ g/mL of Si-Ni-San, Si-Ni-San$^{3\text{R}}$, paeoniflorin and $5\times10^{-7}$ g/mL of dexamethasone in the presence of 100 ng/mL LPS for 24 h. COX-2 enzyme activity was then examined. The 1:0.1 proportion of Si-Ni-San$^{3\text{R}}$ with paeoniflorin represented $1\times10^{-5}$ g/mL+$1.5\times10^{-5}$ g/mL. Spont: cells without treatment. Cont: cells stimulated with LPS only. Each column represents the mean±SD of three independent experiments and each experiment includes triplicate sets. $^\text{*P}<0.05$, $^\text{**P}<0.01$ vs. cont, $^\text{Y}P<0.05$. **
We successfully made the antibody against paeoniflorin and prepared an immunoaffinity column to obtain the paeoniflorin-deleted sample (Si-Ni-SanPF−). Considering Si-Ni-San was effective for the treatment of picryl chloride-induced murine ear contact dermatitis, a typical T cell-mediated DTH response, we first compared the differences between Si-Ni-San and Si-Ni-SanPF− on this murine model. Both paeoniflorin and the whole-formula Si-Ni-San showed a significant inhibition on the ear swelling, lymphocyte infiltration, and pro-inflammatory mediator expression. However, such strong inhibitory effect was almost reversed when paeoniflorin was selectively deleted. This finding suggests that paeoniflorin is at least partially responsible for alleviating the ear inflammation.

Given the crucial role of macrophages in the progress of contact dermatitis, we next examined the influence of Si-Ni-San, paeoniflorin, and Si-Ni-SanPF− on LPS-activated macrophages in vitro. Triggered by LPS, macrophages secrete various pro-inflammatory cytokines, such as TNF-α, COX-2, and IL-1β (Tsatsanis et al., 2007). Si-Ni-San and paeoniflorin significantly inhibited LPS-evoked TNF-α production while Si-Ni-SanPF− almost completely lost its inhibitory effect both in the RAW264.7 macrophage cell line and primary peritoneal macrophages. Interestingly, this inhibitory effect reappeared when different proportions of paeoniflorin were replenished to the Si-Ni-SanPF− sample. In addition, the similar trend was observed in macrophage phagocytosis and nitric oxide production induced by LPS. In our system, we attributed the main effect of Si-Ni-San on macrophage function to paeoniflorin. However, only decuple supplemental of paeoniflorin (the supplementation of paeoniflorin to Si-Ni-SanPF− at the proportion of 0.1:1, Fig. 4C) can regain the efficacy of the paeoniflorin-deleted sample of Si-Ni-San. This phenomenon indicates that the therapeutic effect of Si-Ni-San is due to considerable complex mechanisms rather than cumulative efficacies of active ingredients in this formula. Previously, Stermitz et al. discovered that 5′-methoxyhydnocarpin, a compound which can be extracted in trace amounts from several Berberis medicinal plants producing berberine, could increase the level of accumulation of berberine in the cells by significantly inhibiting the metabolism of berberine.
inhibiting the multidrug-resistant pumps of the human pathogen *Staphylococcus aureus*, while 5′-methoxyhydnocarpin had no antimicrobial activity alone (Sternitz et al., 2000). This is a clear example of synergy between components of a medicinal plant described at a molecular level. This work suggests that natural synergy mechanisms concealed in traditional medicine are worth seeking, uncovering, and using in the combination of drugs in future. In the present study, we surmise that this selective deletion may interrupt the interaction of paeoniflorin with other ingredients in the formula, and this method may contribute to finding natural synergy mechanisms of compounds in traditional medicine.

Additionally, multiple signaling molecules are activated in these inflammatory processes, some of which are involved in cytokine production, such as phosphatidylinositol 3-kinase, p38, ERK1/2, and c-Jun NH2-kinase (JNK) (Hsu and Wen, 2002; Su et al., 2006). The inhibitory effect of paeoniflorin on TNF-α and COX-2 suggests that paeoniflorin might modulate intracellular signaling events that coordinate the activity of pro-inflammatory cytokines. Signal transduction through ERK1/2, JNK, and p38 MAPK pathways is a critical step in macrophage activation leading to increased expressions of pro-inflammatory cytokines. To better understand the role of paeoniflorin in Si-Ni-San on the molecular mechanisms underlying the anti-inflammatory effect, we further examined the effect of paeoniflorin on LPS-induced activation of MAPK signaling pathways. Our results show that paeoniflorin, as well as Si-Ni-San, selectively inhibited LPS-evoked phosphorylation of ERK1/2, rather than p38 and JNK in LPS-activated RAW264.7 macrophages, while such effect was almost blocked when paeoniflorin was deleted from the formula. A downstream molecule of ERK1/2 MAPK signaling, COX-2, was also significantly inhibited by Si-Ni-San and paeoniflorin, but not Si-Ni-SanPF, at a concentration of 10 µg/mL. These results suggest that the inhibitory effect of paeoniflorin on the expression of inflammatory cytokines and mediators involves suppression of the ERK1/2 MAPK signaling pathway.

A large body of evidence indicates that MIF is an integral component of the host antimicrobial alarm system and stress response that promotes the pro-inflammatory functions of immune cells. As a pivotal regulator of inflammation, it is rapidly released by immune cells that are exposed to microbial products or pro-inflammatory cytokines. In the process of inflammation, MIF that are secreted by activated T lymphocytes can promote the infiltration of macrophages in the inflammatory site, accompanying the release of TNF-α, IL-1β, and COX-2 (Calandra and Roger, 2003). Additionally, MIF can selectively induce rapid (within 30 min) and sustained (up to 24 h) phosphorylation of the ERK1/2 MAPK pathway (Mitchell et al., 1999). In the present study, we found that the deletion of paeoniflorin from the whole formula had no influence on the expression of CD69 in Con A-activated murine T lymphocytes, or the production of IFN-γ and TNF-α from the isolated lymphocytes sensitized with picryl chloride in vivo and challenged with 2,4,6-trinitrobenezensulfonic acid hydrate in vitro. These results clearly show that the inhibitory effects of Si-Ni-San on T lymphocyte proliferation, activation, and cytokine production are not obviously blocked or reversed when paeoniflorin is selectively deleted from Si-Ni-San. This phenomenon indicates that paeoniflorin may not make major contributions to the effect of Si-Ni-San in the downregulation of T lymphocytes. Intriguingly, we found that Si-Ni-San could dramatically suppress MIF expression in T lymphocytes, but not macrophages, and the deletion of paeoniflorin reversed the inhibitory effect of Si-Ni-San. These results suggest that MIF mediates the crosstalk of macrophages and T lymphocytes in the action of paeoniflorin, and that paeoniflorin in the formula might suppress the migration of macrophages through modulating this key molecule on T lymphocytes. Overall, paeoniflorin may act as an active constituent of Si-Ni-San in inhibiting contact dermatitis involving downregulation of macrophage functions. Its mechanisms may include inhibiting MIF secretion and ERK1/2 MAPK activation.

Previously, we demonstrated the mode of action of Si-Ni-San through drug-pairs (Sun et al., 2003), single herb (Jiang and Xu, 2004), and major active components (Zhang et al., 2006), while the actual role of active ingredients in the formula still remains unclear. As summarized in Fig. 7, in the present study, paeoniflorin exerted negative regulation on macrophage function including inhibiting MIF expression in T cells, down-regulating MIF-ERK1/2-COX-2 signaling, as well as LPS-triggered TNF-α and NO production in macrophages. In addition, we also found that saikosaponin a, another active ingredient of Si-Ni-San, mainly inhibited T lymphocyte activation and proliferation (unpublished data), while glycyrrhizin showed strong inhibition on cell adhesion to extracellular matrix and matrix metalloproteinase-2 and -9 activities of T lymphocyte. Collectively, these results support the notion that multiple components in the formula Si-Ni-San can hit multiple targets and exert synergistic anti-inflammatory efficacies. Arrows, positive effect; T-shaped arrows, negative effect; dashed arrows, speculative effect.

**Conclusion**

The attempt to specifically delete one or more components from a traditional Chinese formula and consecutively replenish the component to the deleted sample, as used in the present study, may be helpful for the identification of the role of active ingredients in Chinese medicinal formulas. A series of studies on Si-Ni-San in our group may be considered a useful pilot trial in exploring the mechanism of active ingredients in TCM and in helping to understand the rationality of Chinese multi-herb formulas.

**Competing interests**

The authors have declared that no competing interests exist.
Acknowledgments

The authors gratefully thank Dr. Xiao-Dong Liu (Shanghai University of Traditional Chinese Medicine, Shanghai) for valuable comments, Mrs. Ning Su (Southeast University School of Medicine, Nanjing) for technical assistance in the pathological experiment and BioMed Proofreading for careful language reviewing. This work was supported by funds from the National Natural Science Foundation of China (No. 30701095).

Appendix A. Supplementary data


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