International Immunopharmacology 9 (2009) 1437–1443

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Si-Ni-San, a traditional Chinese prescription, and its active ingredient glycyrrhizin ameliorate experimental colitis through regulating cytokine balance

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ARTICLE INFO

Article history:
Received 30 June 2009
Accepted 26 August 2009

Keywords:
Inflammatory bowel disease
Crohn's disease
TNBS
Si-Ni-San
Glycyrrhizin

ABSTRACT

Si-Ni-San, a traditional Chinese medicinal formula, exerts an important function in the treatment of inflammatory bowel diseases based upon thousands of years of clinical practice, but the underlying mechanism is still unclear. In this study, we investigated the therapeutic potential of Si-Ni-San and its ingredient glycyrrhizin in trinitrobenzene sulfonic acid (TNBS)-induced experimental colitis in mice, a well-characterized murine model for Crohn's disease. Si-Ni-San and glycyrrhizin significantly ameliorated TNBS-induced colitis with reduced mortality and recovery of body weights. In addition, Si-Ni-San and glycyrrhizin dose-dependently decreased macroscopic inflammation scores, microscopic histoligical scores, and myeloperoxidase activity. Furthermore, Si-Ni-San and glycyrrhizin caused a decrease in pro-inflammatory cytokines including IFN-γ, IL-12, TNF-α and IL-17 and an increase in regulatory cytokine IL-10 in colon of the mice. It should be noticed the therapeutic effect of Si-Ni-San at 450 mg/kg was much better than that of its contained content of glycyrrhizin at 10 mg/kg. In conclusion, Si-Ni-San and glycyrrhizin significantly ameliorated TNBS-induced colitis in mice through regulating pro- and anti-inflammtory cytokine production.

1. Introduction

Imbalance of cytokine regulation is critically involved in the pathogenesis of human autoimmune diseases, such as multiple sclerosis, rheumatoid arthritis, lupus nephritis and inflammatory bowel diseases (IBD, including Crohn's disease and ulcerative colitis) [1–4]. Increasing evidence demonstrates that these autoimmune diseases are characteristically associated with the over-production of pro-inflammatory cytokines. For example, an increased production of IFN-γ, IL-12, TNF-α and IL-17 was usually found in Crohn's disease [5–7]. Against the pro-inflammatory cytokines, the anti-inflammatory cytokines including IL-10 and TGF-β play a protective effect to ameliorate the inflammation, but its function is usually defective in the process of above diseases [8–10]. Thus, reconstruction for the balance of pro- and anti-inflammatory cytokines may be a promising approach to the treatment of autoimmune diseases.

IBD is a kind of chronic inflammatory and relapsing diseases in alimentary tract. Patients with IBD do have an increased risk of colorectal cancers, and their quality of life can be limited by complications such as toxic megacolon, bowel perforation and surgical complications. Although the etiology of IBD is still unclear, it is believed that altered immunological function, resulting from a synergism between genetic susceptibility and certain environmental factors, contributes to the mucosal inflammation of the gastrointestinal tract and as well the abnormal T cell responses to commensal microflora [4]. Nowadays, the therapies of IBD usually include the biological therapy and chemical drug therapy. For example, Infliximab, a chimeric immunoglobulin G1 monoclonal antibody to TNF-α, dramatically improved symptoms of patients with Crohn's disease and ulcerative colitis. However, biological therapies always carried a significant medication expense as well as a definite safety risk, such as the induction of autoimmune phenomena, neurotoxicity and the development of an immune response to engineered proteins [11,12]. Besides, some chemicals are available for the therapy of IBD, medicament such as 5-aminosalicylic acid, sulfasalazine and glucocorticoids, which can inhibit related inflammatory mediators through different mechanisms engaged in the regulation of the immune and inflammatory responses of IBD. However, their adverse reactions during prolonged treatment and the high relapse rate unavoidably limit their clinical application [13,14]. Therefore, there is urgent need to seek for alternative remedies for Crohn's diseases.

Traditional Chinese medicine has a long history of treating various diseases by using various prescriptions that were formulated according to the symptoms of patients and the characteristic theories. Some of the prescriptions have been proven to give an evidence for their efficacy in pharmacological and biological levels [15,16]. One example is Si-Ni-San, a traditional Chinese medicinal formula derived from *Treatise on Febrile Diseases* [17], a medical classic written by Zhongjing Zhang in the 3rd century. Si-Ni-San is considered to the effective in clinical practice for treating various inflammatory diseases including gastritis, colitis and hepatitis [18–20]. The main components of Si-Ni-San are Radix Bupleuri Chinensis (Chaihu), Radix Paeoniae Alba

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doi:10.1016/j.intimp.2009.08.017

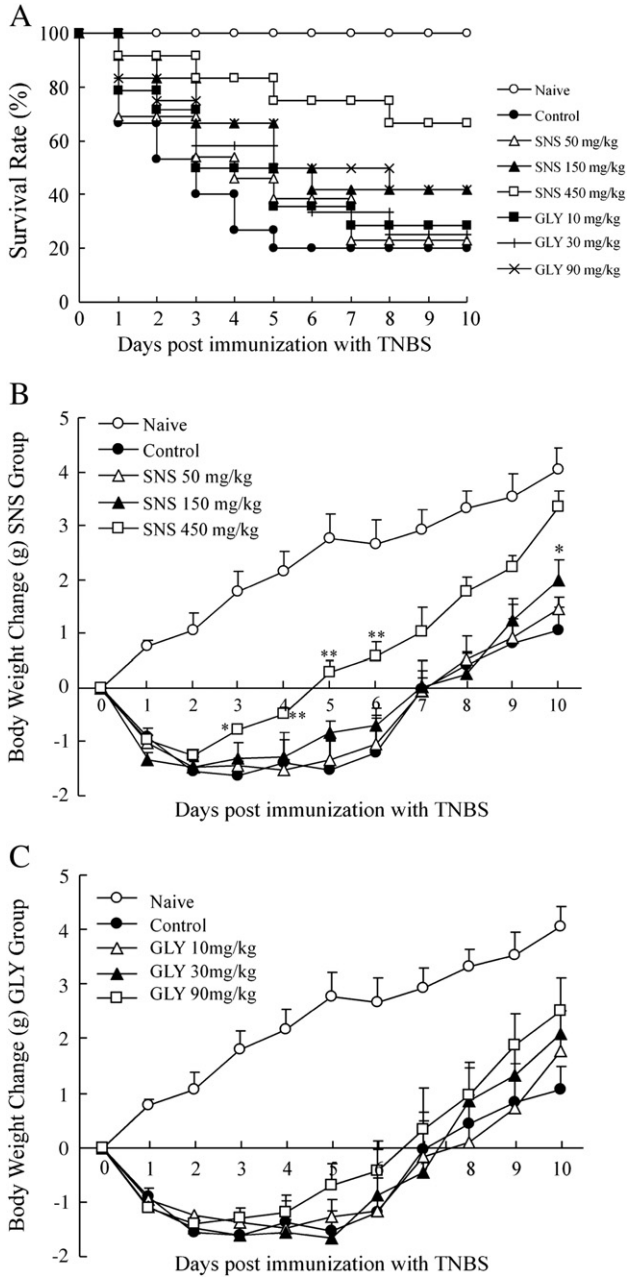


Fig. 1. Effects of Si-Ni-San and glycyrrhizin on the survival rate (A) and body weights (B, C) in mice with TNBS-induced colitis. The colitis was induced by intrarectally injecting TNBS. Si-Ni-San and glycyrrhizin were given intragastricly once a day. Body weight increase or decrease of each mouse, which are compared to the initial time, have been measured every day, as well as the animal survival rate. Naive: mice only receive 100 μL 50% ethanol vehicle; Control: mice receive 0.5 mg TNBS in 50% ethanol; SNS: Si-Ni-San; GLY: glycyrrhizin. Data are presented as mean ± SEM. $n = 12–15$. $^{*}P < 0.05$, $^{*}P < 0.01$ vs control.

(Shaoyao), Fructus Citri Aurantii (Zhishi), and Radix Glycyrrhizae Uralensi (Gancao), with saikosaponin, paeoniflorin, naringin and glycyrrhizin as major active ingredients respectively [21–24]. Among these ingredients, the salt diammonium of glycyrrhizin, diammonium glycyrrhizinate, has been reported to exert a protective effect on a rat model of ulcerative colitis [25]. Previously, we have demonstrated that Si-Ni-San significantly ameliorated experimental hepatitis and contact dermatitis in mice [26–28]. In this study, therefore, we examined the therapeutic potential of Si-Ni-San in TNBS-induced colitis in mice, a well-characterized murine model for Crohn's disease [29] and compared the effect of the prescription with its active ingredient glycyrrhizin.

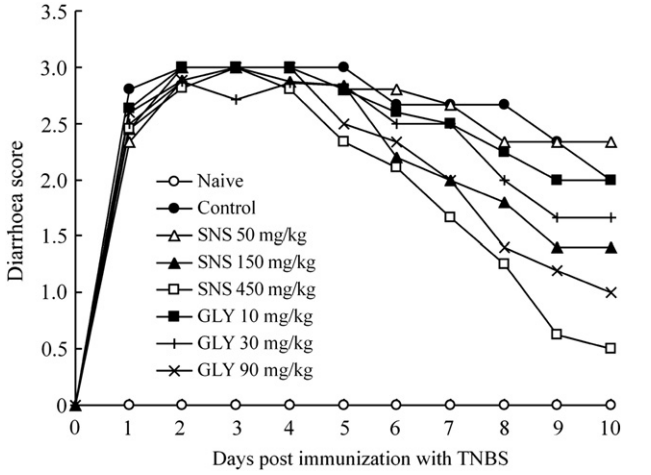


Fig. 2. Effects of Si-Ni-San and glycyrrhizin on the diarrhea score of TNBS-induced colitis in mice. The colitis was induced by intrarectally injecting TNBS. Si-Ni-San and glycyrrhizin were given intragastricly once a day. The diarrhea score was observed as described in methods. Naive: mice only receive 100 μL 50% ethanol vehicle; Control: mice receive 0.5 mg TNBS in 50% ethanol; SNS: Si-Ni-San; GLY: glycyrrhizin. Data are presented as mean of the diarrhea score.

2. Materials and methods

2.1. Mice

Specific pathogen-free, eight- to ten-week-old female C57BL/6 mice were purchased from Experimental Animal Centre of Yangzhou University (Yangzhou, China). Animal welfare and experimental procedures were carried out strictly in accordance with the Guide for the Care and Use of Laboratory Animals (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.

2.2. Preparation of Si-Ni-San and quantitative analysis of glycyrrhizin

The crude drugs used in this study are purchased from Nanjing Medicinal Material Co. (Nangjing, China) and identified as Bupleurum Chinese DC (Radix Chinensis, Chaihu), Paeonia albiflora Pall (Radix Paeoniae Alba, Shaoyao), Citrus aurantium L. (Fructus Citri Aurantii, Zhshi) and Glycyrrhiza uralensis Fisch (Radix Glycyrrhizae Uralensis, Gancao). They are mixed in an equal ratio (25 g of each drug in total 100 g) to make up Si-Ni-San, a mixed powder of material crude drugs. These materials are used for making 70% ethanol extracts. Briefly, the materials (100 g) are extracted twice with 5-fold volumes of 70% ethanol (500 mL) at 70 °C for 1 h each time. Then the supernatant, after centrifuging at 2000 g, is pooled and lyophilized to make a powder with 23.2% yields for Si-Ni-San. The dosages of these extracts were indicated as the powders. The contents of main components in the 70% ethanol extracts of Si-Ni-San are determined by high-performance liquid chromatography (HPLC) as 1.2% of saikosaponin a, 1.4% of paeoniflorin, 7.9% of naringin and 2.1% of glycyrrhizin.

2.3. Reagents

Trinitro-benzene-sulfonic acid (TNBS) and dexamethasone were purchased from Sigma Chemical Co. (St. Louis, MO). MPO kit was purchased from Jiancheng (Nanjing, China). ELISA kits for interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-12 (IL-12), IL-10, IL-17 and TGF-β were purchased from R&D Systems

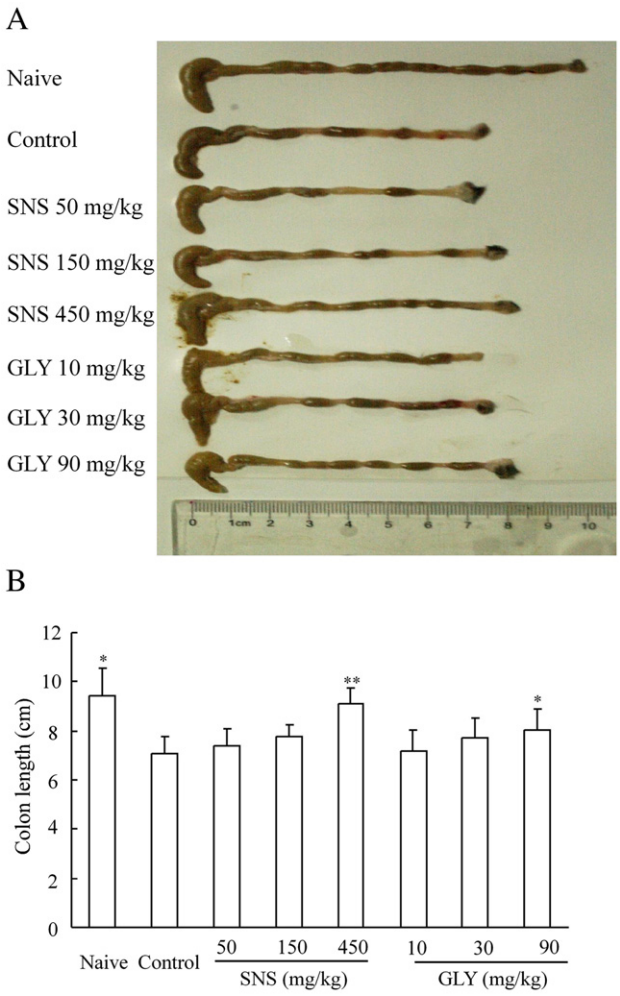


Fig. 3. Effects of Si-Ni-San and glycyrrhizin on the macroscopic appearance changes of colons in mice with TNBS-induced colitis. (A) Macroscopic changes of colons in TNBS-induced colitis in mice. (B) The colon length from TNBS-colitis mice. The colitis was induced by intrarectally injecting TNBS. Si-Ni-San and glycyrrhizin were given intragastricly once a day. After 10 days colons were taken out and observed the macroscopic change as described in methods. Naive: mice only receive 100 μL 50% ethanol vehicle; Control: mice receive 0.5 mg TNBS in 50% ethanol; SNS: Si-Ni-San; GLY: glycyrrhizin. Data are presented as mean ± SEM. $^{*}P<0.05$, $^{**}P<0.01$ vs control.

(Minneapolis, MN). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

2.4. TNBS-induced colitis in mice

Colitis was induced by intrarectal injection of TNBS as previously described [30]. Briefly, mice were fasted for 24 h with free access to drinking water. They were anesthetized by sodium pentobarbital (50 mg/kg, i.p.). Next, 100 μL of a 10 mg TNBS in 2 mL of 50% ethanol solution was injected intrarectally through a flexible catheter of 3.5 cm length. After TNBS injection, mice were held upside down in a 45° position for 1 min to prevent leakage of the TNBS solution and were replaced in their cages with free access to food and water afterward. Ten days after the TNBS injection, mice were sacrificed, and the colon is collected and evaluated for colon length. We also collect the sample of colon for protein extraction and histological assay. The samples were either stored in −70 °C or fixed in 10% formalin. Body weights, diarrhea score and survival rate of animals are monitored every day. Diarrhea is scored as follow criteria: 0, normal; 2, loose previous termstools and 4, diarrhea.

2.5. Microscopic histoligical scores

Colonic segments were fixed in 10% formaldehyde, embedded in paraffin, and cross sections of 5 μm were stained with hematoxylin and eosin. The histological scores were assessed according to criteria as below: 0: no signs of inflammation; 0.5: very slight inflammation; 1: low level of leucocyte infiltration; 2: moderate level of leucocyte infiltration; 3: high level of leucocyte infiltration, high vascular density, thickening of bowel wall; 4: transmural infiltrations, loss of goblet cells, high vascular density, critical bowel wall thickening. Final data are the average scores of each animal in the same group, and the higher score means more serious inflammation.

2.6. MPO activity measurement

Colonic tissues from mice of each group were stored in −70 °C until assayed. All experiments were performed within 1 week of collection of tissue. Myeloperoxidase (MPO) activity was measured according to the method described by user's guide of kit. Tissue was homogenized in hexadecyltrimethylammonium bromide in 50 mM potassium phosphate buffer. Aliquots were then added to O-dianisidine hydrochloride solution. Absorbance was read at 450 nm using a microplate reader. MPO was expressed in units per milligram of tissue, where 1 unit corresponds to the activity required to degrade 1 mmol of hydrogen peroxide in 1 min at 24 °C.

2.7. Cytokine assay

Cytokines (IFN-γ, TNF-α, IL-12, IL-17, IL-10, TGF-β) were determined using ELISA kits from R&D systems (Minneapolis, MN).

2.8. Statistical analysis

All results shown represent means ± SEM. Statistical analyses were performed using an unpaired, two-tailed Student's *t*-test. The significance of difference is indicated as $P<0.05$.

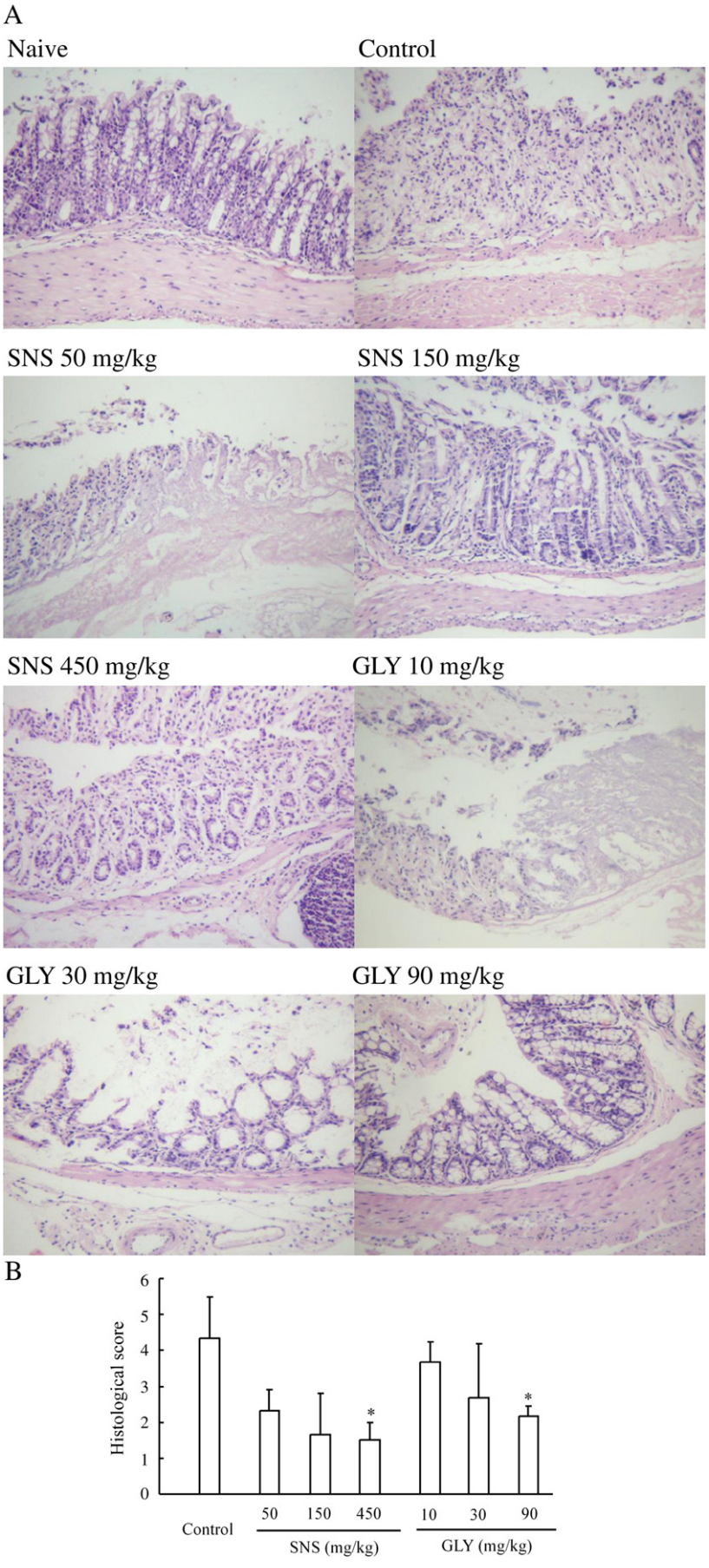
3. Results

3.1. Effects of Si-Ni-San and glycyrrhizin on the survival rate and body weight in TNBS-induced colitis

As illustrated in Fig. 1, when intragastricly administered daily from day 1 postimmunization onwards, Si-Ni-San showed a significant prolongation of the survival rate and recovery from the decrease in body weights in mice with colitis. Similarly, glycyrrhizin also prolonged the survival rate and increased body weights of mice, though the effect was less than that of Si-Ni-San (Fig. 1A and C). In this case the group of 450 mg/kg of Si-Ni-San, which contains about 10 mg/kg of glycyrrhizin, Si-Ni-San showed much better potency than each group of glycyrrhizin from 10 to 90 mg/kg. Mice treated with 90 mg/kg of glycyrrhizin returned to the initial weight at about 6th day, while mice treated with 450 mg/kg of Si-Ni-San reached the same effect much earlier, at about 4th day. Moreover, at the terminated time of experiment (10th day), the body weights in 450 mg/kg of Si-Ni-San-treated group almost reached the level in vehicle control group (Fig. 1B).

3.2. Effects of Si-Ni-San and glycyrrhizin on the diarrhea score and colon length in mice with TNBS-induced colitis

Diarrhea was the main symptom of colitis in mice, we monitored diarrhea of each mouse every day. As showed in Fig. 2, both Si-Ni-San and glycyrrhizin could significantly relieve the diarrhea symptom, and it is worth noting that 450 mg/kg of Si-Ni-San almost recovered from the diarrhea at the end of experiment. Likewise, Si-Ni-San showed


A
Naive
Control
SNS 50 mg/kg
SNS 150 mg/kg
SNS 450 mg/kg
GLY 10 mg/kg
GLY 30 mg/kg
GLY 90 mg/kg
B
Histological score
0
1
2
3
4
5
6
*
*
Control
50
150
450
10
30
90
SNS (mg/kg)
GLY (mg/kg)

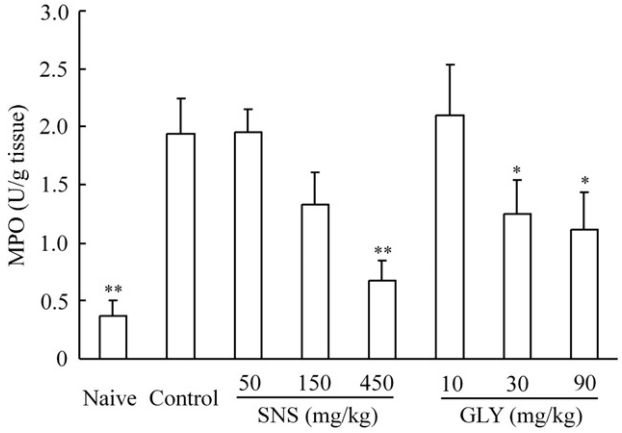


Fig. 5. Effects of Si-Ni-San and glycyrrhizin on the myeloperoxidase (MPO) activity in TNBS-induced colitis in mice. The colitis was induced by intrarectally injecting TNBS. Si-Ni-San and glycyrrhizin were given intragastricly once a day. Colons were taken out 3 days postimmunization and subjected to MPO activity assay. Naive: mice only receive 100 μL 50% ethanol vehicle; Control: mice receive 0.5 mg TNBS in 50% ethanol; SNS: Si-Ni-San; GLY: glycyrrhizin. Data are presented as mean ± SEM. $^{*}P<0.05$, $^{**}P<0.01$ vs control.

better therapeutic effect than glycyrrhizin did. Colitis always resulted in the edema and incrassation of pathogenic intestinal tract, which finally shorten the whole length of colon. The length of colon from the appendix to the anus was measured, and both Si-Ni-San and glycyrrhizin dose-dependently recovered the colon length from the shortening (Fig. 3A and B).

3.3. Effects of Si-Ni-San and glycyrrhizin on the microscopic changes in mice with TNBS-induced colitis

Colons from control group showed evidence of mucosal congestion, erosion, loss of goblet cells, the thickening of the colon wall and high level of polymorphocyte infiltration. Against these changes, Si-Ni-San and glycyrrhizin significantly improved the above parameters. Fig. 4A was a representative photo of H&E staining for colon tissues. The histological score was showed in Fig. 4B.

3.4. Effects of Si-Ni-San and glycyrrhizin on the myeloperoxidase activity in mice with TNBS-induced colitis

Mice treated with TNBS exhibited a significant increase in MPO activity. Si-Ni-San and glycyrrhizin significantly reduced the increase in MPO activity in a dose-dependent manner (Fig. 5).

3.5. Effects of Si-Ni-San and glycyrrhizin on the pro- and anti-inflammtory cytokine production in mice with TNBS-induced colitis

As shown in Fig. 6, Si-Ni-San and glycyrrhizin significantly inhibited the production of pro-inflammtory cytokines including IFN-γ, IL-12, TNF-α and IL-17. However, they significantly enhanced the production of anti-inflammtory cytokine IL-10 but showed no influence on TGF-β.

4. Discussion

Traditional Chinese medicine that has been practiced for thousands of years in clinic often offers some unique advantages and provides a vast source of pharmaceutical material for the development of effective drugs. The substantial obstacle, however, is that the scientific evidence how it takes effect is largely unclear. Si-Ni-San, a famous traditional Chinese prescription, is widely used as a medication recipe to treat gastrointestinal diseases including colitis in clinic in China for thousands of years [19]. Therefore, it is worth trying to find scientific evidence for the effect of this traditional prescription. The aim of the present study is to investigate the therapeutic potential and underlying mechanisms of Si-Ni-San on experimental colitis in mice. In this study we provide evidence that Si-Ni-San and its active ingredient glycyrrhizin have a beneficial effect on TNBS-induced colitis in mice. This effect is mediated by an immunological pathway involving Th1, Th17, and Treg cells.

Intrarectal injection of TNBS is a well-characterized model of experimental colitis, resulting in a Th1 immune response comparable to the inflammatory processes present in Crohn's diseases [31]. We used clinical, histological, and biochemical parameters to quantify the inflammation in the colon. TNBS-induced colitis caused a significant increase in all inflammatory parameters tested. Treatment with Si-Ni-San significantly decreased the diarrhea score, microscopic colonic inflammation score, and MPO activity in mice with colitis in a dose-dependent manner. It was worth noting that the therapeutic potential of Si-Ni-San (450 mg/kg) was much better than that of its active ingredient glycyrrhizin (10–90 mg/kg), at which 450 mg/kg Si-Ni-San contained about 10 mg/kg glycyrrhizin. This result may be interpreted with the integrated effect of the ingredients contained in Si-Ni-San. Such synergistic efficacy of multiple constituents in Si-Ni-San was also found in the amelioration of experimental hepatitis and contact dermatitis in mice as reported previously by our laboratory [16,27,28].

Next we investigated the immunological mechanisms underlying their efficacy. There is compelling evidence that a dysfunctional mucosal immune response to commensal bacteria and imbalance between pro-inflammatory and anti-inflammatory cytokine responses are involved in the pathogenesis of IBD, especially Crohn's disease [4,32]. As representatives of pro-inflammatory cytokines, IFN-γ and IL-12 have been shown to play a major role in the pathophysiology of several intestinal inflammatory diseases, especially Crohn's disease [5,32]. Recently, increasing evidence reveals that IL-17 is critical for the development of inflammation in many inflammatory conditions [6,33,34]. In this study we showed that the induction of colitis by TNBS injection caused a clear Th1 response in the colon that was significantly suppressed after administration of Si-Ni-San or glycyrrhizin. Intragastric administration of Si-Ni-San or glycyrrhizin, on the other hand, significantly decreased IL-17 production in colon, indicating that Si-Ni-San and glycyrrhizin are able to influence the recently discovered proinflammatory Th17 pathway. Here we were able to show that the inhibitory effect on Th17 cells as well as Th1 cells were involved in the mechanisms of therapeutic effect of Si-Ni-San and glycyrrhizin.

It is well known that the TNBS-induced experimental colitis is a self-limited process which reaches near complete remission after 7–10 days. Some regulatory cytokines including IL-10 and TGF-β play a critical role during the shutdown process of excess immune responses and maintain proper immune homeostasis [35,36]. Interestingly, the regulatory action of pro-inflammatory and anti-inflammatory cytokine by Si-Ni-San and glycyrrhizin treatment was found to induce shift of effector Th1/Th17 cells to regulatory T cells as characterized by up-regulation of IL-10 and corresponding down-regulation of IFN-γ, IL-12, TNF-α and IL-17. However, another anti-inflammatory cytokine TGF-β associated with experimental colitis was not markedly altered by Si-Ni-San and glycyrrhizin. These results suggested that the attenuation of inflammation after treatment with Si-Ni-San and glycyrrhizin might be linked to IL-10 — but not TGF-β-producing regulatory cells. Taken together, our results indicate that Si-Ni-San

Fig. 4. Effects of Si-Ni-San and glycyrrhizin on the microscopic changes in mice with TNBS-induced colitis. (A) Histopathological sections were stained by H&E, original amplification (×100). (B) Microscopic scores of colon. The colitis was induced by intrarectally injecting TNBS. Si-Ni-San and glycyrrhizin were given intragastricly once a day. Colons were taken out at the end of the experiment and subjected to microscopic histological examination as described in methods. Naive: mice only receive 100 μL 50% ethanol vehicle; Control: mice receive 0.5 mg TNBS in 50% ethanol; SNS: Si-Ni-San; GLY: glycyrrhizin. Data are presented as mean ± SEM. $^{*}P<0.05$ vs control.

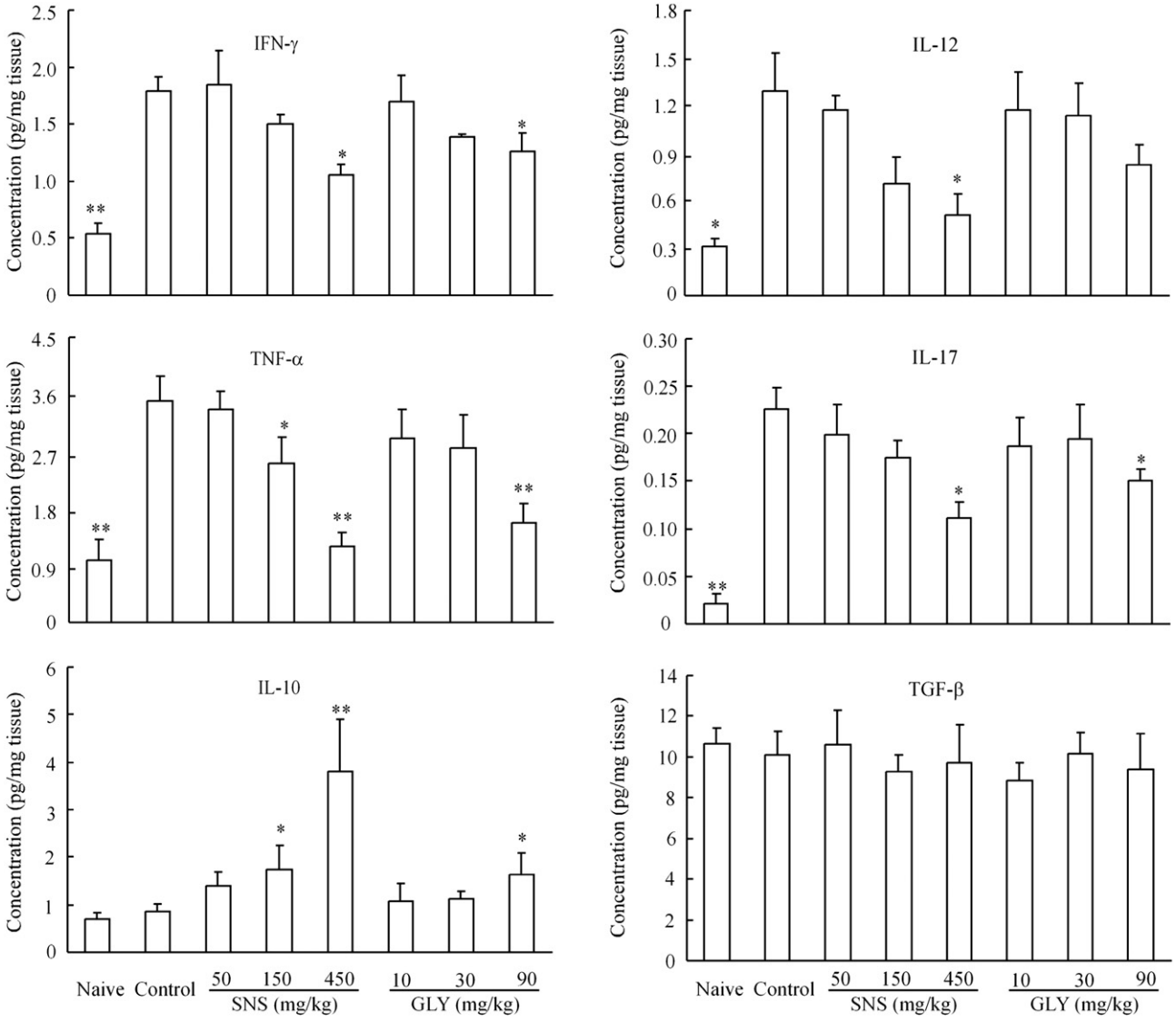


Fig. 6. Effects of Si-Ni-San and glycyrrhizin on the pro- and anti-inflammtory cytokine production in mice with TNBS-induced colitis. The colitis was induced by intrarectally injecting TNBS. Si-Ni-San and glycyrrhizin were given intragastricly once a day. Colons were taken out 3 days postimmunization, homogenated and examined the cytokine production by ELISA. Naive: mice only receive 100 μL 50% ethanol vehicle; Control: mice receive 0.5 mg TNBS in 50% ethanol; SNS: Si-Ni-San; GLY: glycyrrhizin. Data are presented as mean ± SEM. $^{*}P<0.05$, $^{**}P<0.01$ vs control.

and glycyrrhizin induce potent suppressive responses, including down-regulation of Th1 and Th17 cytokines and up-regulation of regulatory cytokine, establishing an environment that combats the pro-inflammatory response that causes the pathology observed in experimental colitis.

In conclusion, intrarectal injection of TNBS in mice caused severe colonic damage and inflammation. Treatment of mice with Si-Ni-San and glycyrrhizin significantly attenuated TNBS-induced inflammation of the murine colon. Our results suggest that the beneficial effect of Si-Ni-San and glycyrrhizin is linked to potentialization of regulatory T cells and suppression of proinflammatory T cells. Therefore, we conclude that treatment with Si-Ni-San and glycyrrhizin has therapeutic potential in IBD especially Crohn's disease.

Acknowledgement

This study was supported by Natural Science Foundation of China (Nos. 30672656, 30701095 and 30730107) and Natural Science Foundation of Jiangsu Province (No. BK2007716).

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