Full Length Research Paper

Effect and mechanism of Shao-Yao Gan-Cao Tang on adjuvant-induced arthritis in rats

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Shaoyaogancao-tang (SGT) is a widely used traditional medical formula to relieve different types of pain in China. Here, we investigated the suppressive effect of SGT on rat adjuvant-induced arthritis (AA) and the underlying mechanism. Paw swelling and histological score were calculated during or after SGT treatment. Levels of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and prostaglandin E₂ (PGE₂) in serum were analyzed by enzyme-linked immunosorbent assay, and nitric oxide (NO) in serum were analyzed by Griess reaction method. Cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) proteins expression in synovial tissues were detected by Western blot assay. SGT markedly relieved the paw swelling and lowered the histological score. SGT also notably decreased the levels of TNF-α, IL-1β, PGE₂ and NO in serum. In addition, SGT significantly suppressed the expression of COX-2 and iNOS proteins in synovial tissues. These findings suggest that SGT have pronounced anti-arthritis effect on AA in rats through inhibiting the production of inflammatory mediators, and indicate that SGT would be a promising candidate as a novel anti-rheumatic drug for further investigation.

Key words: Shaoyaogancao-tang, adjuvant-induced arthritis, rheumatoid arthritis, traditional Chinese medicine, inflammation.

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation of the synovial membranes in the joints, followed by erosion of cartilage and bone, ultimately leading to joint destruction and deformity. Inflammatory mediators, such as TNF-α, IL-1β, PGE₂ and NO, produced by the invading tumor-like synovium are primary factors of joint inflammation and articular destruction in RA (Schett, 2008). In inflammatory conditions, PGE₂ is mainly catalyzed by COX-2 and NO is mainly catalyzed by iNOS (Yao et al., 2005). Inhibition of inflammatory cytokine action has proved to be an effective target for new methods of therapeutic modalities, as evidenced by the use of TNF-α-neutralizing agents in the amelioration of joint inflammation in RA (Statkute and Ruderman, 2010). Currently the precise causes of RA remain unknown. Treatments are focused on the reduction of pain, inflammation, and joint damage. Traditional anti-rheumatic drugs used in clinic show severe adverse reactions and potential toxic effects (Amoroso et al., 2003). Although biologic agents are regarded as a promising method to improve RA (Ahn et al., 2010), severe adverse and toxic effects (Rosenblum and Amital, 2011), as well as expensive costs limit the clinical application (Katikireddi, 2010). Hence, natural herbal therapies have widely caused attention in recent years (de Sousa et al., 2012; Venkatesha et al., 2011). Traditional Chinese Medicine (TCM) has existed for thousands of years in China and traditional medical formula have been proven effective for RA patients in clinical practice. SGT is a generally used traditional medical formula which consists of Shaoya (Paeonia lactiflora Pall.) and Gancao (Glycyrrhiza uralensis Fisch.). In spite of such, the formula is chemically quite complex.

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and the exact chemical ingredients are not well known, certain bioactive chemicals have been identified, such as peoniflorin present in *P. lactiflora* Pall, and glycyrrhizin present in *G. uralensis* Fisch. (He et al., 2003). Though pharmacological studies of peoniflorin and glycyrrhizin have been reported (Asl and Hosseinizadeh, 2008; Yan et al., 2004), traditional medical formula is the most common form in TCM clinical experiences; therefore, exploring the pharmacological effect and mechanism of formulae hold great significance. SGT has been used for rheumatism in China for hundreds of years. However, the effect of SGT on inflammatory mediators production in RA has not yet been studied. Hence, we investigated the effect of SGT on the arthritis development and some key inflammatory mediators production in AA rats. We found here that SGT suppressed arthritis progress, accompanying the reduction of key inflammatory factors.

**MATERIALS AND METHODS**

**Plant material and extraction**

Shaoyao (*P. lactiflora* Pall.) and Gancao (*G. uralensis* Fisch.) were purchased from Chongqing Tongjungy Pharmacy (Chongqing, China) and were identified by Dr Jifen Zhang, College of Pharmaceutical Sciences, Southwest University (Chongqing, China). The two herb materials (1:1, w/w) were extracted twice with boiling water (1:10, v/v) for 2 h respectively. The solution was filtered and concentrated then made into freeze-dried powder.

133 g SGT freeze-dried extract powder was obtained from 600 g raw material (the yield was 22.17%). The freeze-dried powder was stored at -20°C until use. The content of peoniflorin in SGT freeze-dried powder was quantitatively analyzed by high performance liquid chromatography (HPLC) method (Chen et al., 1999). In this study, SGT was found to contain 9.17 mg peoniflorin per g freeze-dried powders (Figure 1).

**Experimental animals and induction of arthritis**

Male SD rats weighing 160 to 180 g were purchased from Chongqing Medical University (Chongqing, China). All rats were housed in a temperature-controlled room (23 ± 2°C) under a light/dark cycle with lights on from 7:00 am to 7:00 pm. They were housed in a temperature-controlled room (23 ± 2°C) under a light/dark cycle with lights on from 7:00 am to 7:00 pm. They were allowed food and water *ad libitum*. The animals adapted to experiment environment for 1 week before experiments were carried out. All animal procedures were approved by the Ethical Committee in Animal Research of Chongqing Technology and Business University. Freund’s complete adjuvant was prepared by suspending heat-killed BCG (Shanghai biochemical factory, China) in sterile mineral oil (10 mg/ml). Arthritis was induced by a single injection of 100 μl of Freund’s complete adjuvant intradermally in the left hind footpad of the rat.

**Drug treatment**

Rats were randomly divided into six groups with 10 rats in each group: (1) normal group, (2) model group, (3) 0.1 mg/kg methotrexate (MTX, Shanghai Xinyi pharmaceutical factory, China), (4) 4 g/kg SGT group, (5) 13.33 g/kg SGT group, (6) 40 g/kg SGT group. Arthritic rats of groups 3, 4, 5 and 6 were treated with MTX or SGT by ig, Qd. For the normal and model groups, rats were treated with an equal volume of physiological saline. All the rats were received treatment from day 14 to day 27 after immunization.

**Clinical and histological assessments**

The left hind foot volume was measured by PBC7140 plethysmometer (Ugo Basile, Italy). All rats were sacrificed on day 27. The left ankle joints were surgically removed, fixed in 4% paraformaldehyde for 24 h, decalified in 10% EDTA for 14 days at 4°C, then embedded in paraffin. Serial paraffin sections (5 μm) were stained with hematoxylin and eosin (H&E). Histopathological changes in joints were scored (histological score) using the following parameters (Ono et al., 2004): 0: normal, 1: infiltration of inflammatory cells, 2: synovial hyperplasia, 3: pannus formation, 4: bone erosion, 5: bone destruction.

**Inflammatory mediators levels analysis**

TNF-α, IL-1β and PGE2 levels in serum were determined using commercial enzyme-linked immunosorbent (ELISA) kits (Pierce, USA). NO production in serum was detected with a commercial NO test kit based on the Griess reaction method (Nanjing Jiancheng bioengineering institute, China). Assays were performed according to the manufacturer’s instructions.

**Western blot analysis**

Frozen synovial tissue collected from knee joints of rats was homogenized in RIPA lysis buffer (50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) (Wei et al., 2012) and EDTA-free protease inhibitor cocktail (Roche, Switzerland). Equal amounts of protein samples were separated by SDS-PAGE electrophoresis and transferred onto PVDF membranes. After blocked with 5% nonfat milk in TBST buffer (10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.1% Tween 20) for 2 h at room temperature, PVDF membranes were incubated with anti-iNOS antibody, anti-COX-2 antibody or anti-β-actin antibody at 4°C overnight. Then the membranes were incubated for 1 h at room temperature with appropriate HRP-conjugated secondary antibody. The protein bands were detected with enhanced chemiluminescence reagents (Milipore, USA). Chemiluminescent signals were detected and analyzed using the ChemiDoc XRS imaging system (Bio-Rad, USA).

**Statistical analysis**

All data were presented as means ± standard error (S.E.M.). Statistical comparisons were evaluated by ANOVA test using the SPSS 16 software. Results were considered significant at *P* < 0.05.

**RESULTS**

**Effect of SGT on paw swelling of AA rats**

Arthritis was developed in the immunized rats 3 days after challenge. Joint swelling and erythema were observed as clear signs of the evolution of arthritis. The incidence of arthritis reached to 100% at the beginning of drug administration and did not vary among Model, MTX and SGT groups. Paw swelling was more serious in
Figure 1. HPLC chromatograms of Peoniflorin (A) and SGT (B).

Model group rats than Normal group (P < 0.01, Table 1). 40 g/kg SGT significantly suppressed the increase of paw swelling on day 24 (P < 0.05) and on day 27 (P < 0.01, Table 1). However, 13.3 g/kg SGT significantly
suppressed the increase of paw swelling only on day 27 (P < 0.05, Table 1). As a positive controlled drug, 0.1 mg/kg MTX remarkably suppressed the increase of paw swelling as early as day 21 (on day 21 P < 0.05, on day 24 and 27 both P < 0.01, Table 1).

Effect of SGT on joint inflammation and destruction of AA rats

No synovitis, pannus formation, focal cartilage or bone erosion was seen in ankle joints of Normal group rats. While in ankle joints of Model group rats massive inflammatory cell infiltrate, synovium hyperplasia, periartthritis and focal bone erosion were clearly observed. 13.33 and 40 g/kg SGT markedly ameliorated the severity of arthritis, although inflammatory cell infiltrate, synovium hyperplasia and focal bone erosion were seen in ankle joints. Histological score represent the degree of severity of articular inflammation and destruction. 13.33 and 40 g/kg SGT significantly decreased the histological score relative to Model group (P < 0.05 or P < 0.01, Table 1). 0.1 mg/kg MTX also profoundly ameliorated the pathological characters of arthritis and decreased the histological score (P < 0.01, Table 1).

Effect of SGT on inflammatory mediators levels in serum of AA rats

The serum levels of TNF-α, IL-1β, PGE2 and NO were significantly increased in Model group rats than in Normal group rats on day 27 (P < 0.01, Table 2). 13.33 and 40 g/kg SGT markedly decreased the serum levels of all the inflammatory mediators compared with Model group, and the reduction of PGE2 was the most obvious (P < 0.05 or P < 0.01, Table 2). 0.1 mg/kg MTX also significantly decreased the serum levels of TNF-α, IL-1β, PGE2 and NO (all P < 0.01, Table 2).

Effect of SGT on iNOS and COX-2 expression in synovial tissues of AA rats

The COX-2 and iNOS proteins could not be detected in synovial tissues of Normal group rats, but both were considerably increased in the Model group rats on day 27. SGT decreased iNOS protein expression in a dose-dependent manner, corresponding to about 12% inhibition at 13.33 g/kg, 42% inhibition at 40 g/kg (P < 0.05 or P < 0.01, Table 3). SGT also reduced COX-2 protein expression in a dose-dependent manner, corresponding to about 33% inhibition at 13.33 g/kg, 52% inhibition at 40

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Table 1. Effects of SGT on paw swelling and histological score of AA rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose</th>
<th>Paw swelling (ml)</th>
<th>Histological score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 14</td>
<td>Day 18</td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td>0.23 ± 0.01</td>
<td>0.25 ± 0.01</td>
</tr>
<tr>
<td>Model</td>
<td></td>
<td>1.01 ± 0.07*</td>
<td>1.17 ± 0.10*</td>
</tr>
<tr>
<td>MTX</td>
<td>0.1 mg/kg</td>
<td>1.01 ± 0.09</td>
<td>1.16 ± 0.05</td>
</tr>
<tr>
<td>SGT</td>
<td>4 g/kg</td>
<td>1.04 ± 0.07</td>
<td>1.14 ± 0.10</td>
</tr>
<tr>
<td>SGT</td>
<td>13.3 g/kg</td>
<td>1.03 ± 0.10</td>
<td>1.15 ± 0.07</td>
</tr>
<tr>
<td>SGT</td>
<td>40 g/kg</td>
<td>1.03 ± 0.12</td>
<td>1.15 ± 0.07</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± S.E.M (n = 10). *P < 0.01 compared with Normal group; **P < 0.05, ***P < 0.01 compared with Model group.

Table 2. Effects of SGT on TNF-α, IL-1β, PGE2 and NO production in serum of AA rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose</th>
<th>TNF-α (ng/L)</th>
<th>IL-1β (ng/L)</th>
<th>PGE2 (ng/L)</th>
<th>NO (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>-</td>
<td>10.5 ± 3.3</td>
<td>42.6 ± 8.5</td>
<td>195.7 ± 21.9</td>
<td>33.2 ± 6.7</td>
</tr>
<tr>
<td>Model</td>
<td>-</td>
<td>34.9 ± 6.1*</td>
<td>93.1 ± 10.7*</td>
<td>690.4 ± 90.6*</td>
<td>79.8 ± 12.3*</td>
</tr>
<tr>
<td>MTX</td>
<td>0.1 mg/kg</td>
<td>13.9 ± 3.6##</td>
<td>62.3 ± 11.6##</td>
<td>373.7 ± 53.5##</td>
<td>50.9 ± 8.6##</td>
</tr>
<tr>
<td>SGT</td>
<td>4 g/kg</td>
<td>34.4 ± 9.2</td>
<td>90.8 ± 7.5</td>
<td>635.4 ± 52.5</td>
<td>79.1 ± 13.9</td>
</tr>
<tr>
<td>SGT</td>
<td>13.3 g/kg</td>
<td>29.7 ± 7.4#</td>
<td>85.6 ± 8.9#</td>
<td>565.7 ± 49.7##</td>
<td>70.3 ± 6.9#</td>
</tr>
<tr>
<td>SGT</td>
<td>40 g/kg</td>
<td>22.6 ± 8.2##</td>
<td>76.8 ± 8.9##</td>
<td>527.3 ± 41.6##</td>
<td>64.5 ± 10.7##</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± S.E.M. (n=10). *P < 0.01 compared with Normal group; **P < 0.05, ***P < 0.01 compared with Model group.
Table 3. Effects of SGT on COX-2 and iNOS protein expression in synovial tissues of AA rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose</th>
<th>$A_{\text{iNOS}}/A_{\beta-\text{actin}}$</th>
<th>$A_{\text{COX-2}}/A_{\beta-\text{actin}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Model</td>
<td>-</td>
<td>2.15 ± 0.10</td>
<td>1.09 ± 0.08</td>
</tr>
<tr>
<td>MTX</td>
<td>0.1 mg/kg</td>
<td>0.95 ± 0.06#</td>
<td>0.38 ± 0.05##</td>
</tr>
<tr>
<td>SGT</td>
<td>4 g/kg</td>
<td>2.14 ± 0.14</td>
<td>0.99 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>13.3 g/kg</td>
<td>1.89 ± 0.09#</td>
<td>0.73 ± 0.04#</td>
</tr>
<tr>
<td></td>
<td>40 g/kg</td>
<td>1.25 ± 0.27##</td>
<td>0.52 ± 0.09##</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± S. E. M (n = 4). *P < 0.05, **P < 0.01 compared with Model group.

Figure 2. Effects of SGT on iNOS (A) and COX-2 (B) protein expression in synovial tissues of AA rats. Four independent experiments performed in duplicate. Densitometric quantitative analysis of the bands in Figure 3 was performed in Table 3.

to about 33% inhibition at 13.3 g/kg, 52% inhibition at 40 g/kg (P < 0.05 or P < 0.01, Figure 2). 0.1 mg/kg MTX profoundly reduced the iNOS and COX-2 proteins expression by approximately 56 and 65%, respectively (both P < 0.01, Table 3).

DISCUSSION

In the present study, we evaluated the effect and mechanism of SGT on AA in rats. We found that SGT markedly relieved paw swelling of arthritic rats as early as 10 days of treatment. Histological score is the index of joint inflammation and destruction. SGT significantly reduced the histological score, indicating that SGT markedly ameliorated the pathological characters of arthritis. Since we certified the efficacy of SGT on rats AA, we further investigated the potential mechanism.

RA is a chronic, systemic inflammatory disease characterized by chronic arthritis affecting several joints and accompanying synovial hyperplasia, ultimately leading to joint destruction, deformity and severe pain, which greatly reduce the quality of life. The proinflammatory mechanism of RA is considered to be closely associated with progressive joint destruction in the disease course (Voog et al., 2003). Since the exact cause of RA remains unknown, inhibiting the production and function of proinflammatory mediators are thought to be an effective method to treat RA. Many pro-inflammatory mediators are manifested to play key role in inflammatory development and bone erosion of RA, including TNF-α, IL-1β, PGE2 and NO (Karmakar et al., 2010).

Inflammatory cytokines appear to be centrally involved in the pathogenesis of RA. TNF-α was considered as a pivotal mediator in RA followed from the evidences of its potential degrading cartilage and bone in vitro (Zwerina et al., 2006). TNF-α is both an autocrine stimulator and a potent paracrine inducer of other inflammatory mediators, including IL-1β, PGE2 and NO (Feldmann and Maini, 2008). TNF-targeting biologic agents, named rituximab, abatacept, and tocilizumab, have been successfully approved in RA clinical treatment (Jin et al., 2010). IL-1β is confirmed to induce cytokines and PGs production (Chang et al., 2006). Importantly, IL-1β directly resulted in bone erosion in vitro by strong induction of matrix metalloproteinases generation and osteoclasts activation (Barksby et al., 2007). Hence, we explored the effect of SGT on TNF-α and IL-1β production in AA rats. Serum contents of TNF-α and IL-1β both were increased on day 27 after immunization in Model rats compared with Normal rats. However, TNF-α and IL-1β significantly decreased respectively by 35 and 17% after 40 g/kg SGT treatment compared with Model group. We also found that SGT showed less effective than 0.1 mg/kg MTX which decreased TNF-α by 60% and IL-1β by 33%, respectively.

At the same time, we investigated the NO and PGE2 changes after SGT treatment. NO concentration in serum was found proportional to RA disease activity or radiological progression (Emery et al., 2007). Various cell types are capable of producing NO in the inflammatory synovium, including fibroblasts, macrophages, neutrophils, osteoblasts, osteoclasts and endothelial cells (Nagy et al., 2010). In inflammatory conditions, iNOS is the key catalyst of NO generation. iNOS knockout mice were resistant to IL-1-induced bone erosion, suggesting that NO played a key role in the pathogenesis of articular damage in RA (van’t Hof et al., 2010 ). RA is often associated with severe pain thereby resulting in poor quality of life. PGE2, mainly synthesized by COX-2 in inflammatory conditions, is considered to be the critical mediator which contributes to arthritic pain and swelling in RA (Schaible et al., 2002). Non-steroidal anti-inflammatory drugs (NSAIDs), including nonselective NSAIDs and COX-2 selected inhibitors, lessen pain and stiffness through decreasing the production of PGE2 (Rao et al., 2008). Interestingly, PGE2 has been traditionally regarded as an immunosuppressant based on its inhibition of T cell activation and proinflammatory
mediators generation in vitro. However, recently researchers made extraordinary findings that PGE2 played a role of immunomodulator that acted on the EP4 receptor and facilitated Th1 differentiation and Th17 expansion, two Th subsets involved in immune inflammation (Yao et al., 2009). Here, we found that SGT markedly reduced PGE2 and NO production, and PGE2 reduction was more obvious than that of NO. In accordance with changes of PGE2 and NO levels, COX-2 and iNOS proteins expression in synovial tissues were significantly reduced by SGT treatment.

In conclusion, we have verified that SGT suppressed the joint inflammation and destruction in AA rats, and the potential mechanism may be inhibiting the production of key inflammatory mediators including TNF-α, IL-1β, PGE2 and NO. These data provide mechanistic evidence for anti-arthritic appliance of SGT in TCM and suggest that SGT is a promising candidate for novel therapeutic agents of RA.

Conclusion

The outcomes of this study demonstrated that SGT could significantly relieve arthritis symptom and improve the pathological changes. This was accomplished through reduction of the levels of inflammatory mediators in serum or in synovial tissues. Subsequent studies are necessary to examine the effects of SGT on NF-κB activation, the key transcription factor for the pro-inflammatory genes expression in inflammatory conditions, and further study the side effects of SGT.

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