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***Salvia miltiorrhiza* (Dan Shen) Significantly Ameliorates Colon Inflammation in Dextran Sulfate Sodium Induced Colitis**

Xiao-Dong Wen^{1,2,3,*}, Chong-Zhi Wang^{2,3}, Chunhao Yu^{2,3}, Zhiyu Zhang^{2,3}, Tyler Calway^{2,3}, Yunwei Wang⁴, Ping Li¹, and Chun-Su Yuan^{2,3,5}

¹State Key Laboratory of Natural Medicines, China Pharmaceutical University, Nanjing 210009, China

²Tang Center for Herbal Medicine Research, University of Chicago, Chicago, IL 60637, U.S.A

³Department of Anesthesia and Critical Care, University of Chicago, Chicago, IL 60637, U.S.A

⁴Department of Medicine, University of Chicago, Chicago, IL 60637, U.S.A

⁵Committee on Clinical Pharmacology and Pharmacogenomics, University of Chicago, Chicago, IL 60637, U.S.A

Abstract

Inflammatory bowel disease increases the risks of human colorectal cancer. In this study, the effects of *Salvia miltiorrhiza* extract (SME) on chemically-induced colitis in a mouse model were evaluated. Chemical composition of SME was determined by HPLC analysis. A/J mice received a single injection of AOM 7.5 mg/kg. After one week, these mice received 2.5% DSS for 8 days, or DSS plus SME (25 or 50 mg/kg). DSS-induced colitis was scored with the disease activity index (DAI). Body weight and colon length were also measured. The severity of inflammatory lesions was further evaluated by colon tissue histological assessment. HPLC assay showed that the major constituents in the tested SME were danshensu, protocatechuic aldehyde, salvianolic acid D and salvianolic acid B. In the model group, the DAI score reached its highest level on Day 8, while the SME group on both doses showed a significantly reduced DAI score (both $P < 0.01$). As an objective index of the severity of inflammation, colon length was reduced significantly from the vehicle group to model group. Treatment with 25 and 50 mg/kg of SME inhibited the reduction of colon in a dose-related manner ($P < 0.05$ and $P < 0.01$, respectively). SME groups also significantly reduced weight reduction ($P < 0.05$). Colitis histological data supported the pharmacological observations. Thus, *Salvia miltiorrhiza* could be a promising candidate in preventing and treating colitis and in reducing the risks of inflammation-associated colorectal cancer.

Keywords

Salvia miltiorrhiza; Dan Shen; Traditional Chinese Medicine; Dextran Sodium Sulfate; DSS; Azoxymethane; AOM; Colitis; Carcinogenesis; Colorectal Cancer; Chemoprevention

Introduction

Colorectal cancer (CRC) is the fourth most common form of cancer in the United States and the second leading cause of cancer-related deaths in the West (NCI, 2012b). In fact, 1 in 18

*Corresponding author: Xiao-Dong Wen, Ph.D., State Key Laboratory of Natural Medicines, China Pharmaceutical University, 24 Tongjia Lane, Nanjing 210009, P.R. China. Tel: +86-25-8327-1382; endeavour@yahoo.cn.

American men and 1 in 20 women are at risk for developing invasive CRC in their lifetime (NCI, 2012a). It has been estimated that the economic impact of CRC is up to \$8.4 billion or over 10% of all cancer treatment expenditures. In Chinese patients undergoing screening colonoscopy, large and proximal serrated polyps were detected in 2.3% and 7.2%, respectively, and the individuals with these polyps have a higher risk of synchronous advanced neoplasia (Leung *et al.*, 2012). Therefore, it is a particularly relevant public health task worldwide to develop more effective evidence-based botanical intervention for patients diagnosed with CRC (Randhawa and Alghamdi, 2011; Wang *et al.*, 2012a).

Salvia miltiorrhiza Bunge (Dan Shen or 丹参 in Chinese) or *S. miltiorrhiza* is a perennial herbal plant of the Labiatae family. This herb can be found throughout most of China, and it is mainly produced in Jiangsu, Anhui, Hebei, Shanxi and Sichuan provinces. Based on traditional Chinese medicine (TCM) descriptions, *S. miltiorrhiza* root is bitter in flavor, slightly cold in nature, and mainly manifests its therapeutic actions in the heart, pericardium and liver meridians (Chen and Chen, 2004; Zhou *et al.*, 2005). The herb activates and nourishes blood, removes blood stasis, regulates menstruation and calms the spirit, and is used for the treatment of coronary heart disease and other cardiovascular disorders, blood circulation diseases, menstrual disorders including menostasis, and hepatitis (Chan *et al.*, 2012; Wu and Wang, 2012).

Recent pharmacological studies reported that *S. miltiorrhiza* reduced small intestine inflammation and pancreatitis (Xiping *et al.*, 2010). The lipid-soluble extract of the herb showed significant inhibitory effects on drug-induced inflammatory mediators, and these effects were supported by *in vivo* observation (Li *et al.*, 2012). *S. miltiorrhiza* and its constituents also attenuated the inflammatory response and apoptosis after rat spinal cord injury (Yin *et al.*, 2012).

The inflammatory bowel disease (IBD), including ulcerative colitis and Crohn's disease, is associated with colonic mucosal ulceration, shortening of the colon, diarrhea, blood in the feces, and weight loss (Fiocchi, 1998; Kim *et al.*, 2011; Kohn and Meddi, 2012). It is well known that chronic inflammation in the colon can lead to cancer in humans (Hanauer, 1996; Geier *et al.*, 2007). However, to date, the effects of *S. miltiorrhiza* on acute experimental colitis have not been reported. In addition, only limited studies used its water-soluble extract, which represents a different group of chemical constituents. In this study, we used a water-soluble extract of *S. miltiorrhiza*, and evaluated its effects on colon inflammation induced by dextran sulfate sodium (DSS).

Materials and Methods

Chemicals and Reagents

HPLC grade acetonitrile was obtained from Merck (Darmstadt, Germany). Formic acid (purity no less than 96%) was obtained from Tedia (Fairfield, OH). Milli Q water was supplied by a water purification system (US Filter, Palm Desert, CA). Azoxymethane (AOM) was obtained from the NCI Chemical Carcinogen Reference Standard Repository, Midwest Research (Kansas City, MO). Dextran sodium sulfate (DSS, molecular weight of 36,000–50,000 Da) was obtained from MP Biomedicals (Solon, OH). Hemocult Sensa test strips were obtained from Beckman Coulter, Inc. (Brea, CA).

Herbal Extract and Standards

S. miltiorrhiza extract (SME), lyophilized powder for injection (lot number 110546, permission code of Chinese State Food and Drug Administration: Z10970093), was purchased from the Harbin Pharm Group Co. Ltd. (Harbin, Heilongjiang, China). Chemical

standards of danshensu, protocatechuic aldehyde, salvianolic acid D and salvianolic acid B (Fig. 1B) were purchased from Shanghai Tauto Biotechnology Co., Ltd. The purities of the standards were determined to be higher than 98% by high performance liquid chromatography.

High Performance Liquid Chromatography (HPLC) Analysis

The HPLC system was an Agilent 1100 instrument with a quaternary pump, an automatic injector, and a photodiode array detector. The separation was carried out on a Zobax Extend C-18 column (5 μ , 4.6 mm $\times$ 250 mm). For the HPLC analysis, a 10- μ L sample was injected into the column and eluted at room temperature with a constant flow rate of 1.0 mL/min. For the mobile phase, 0.2% formic acid in water (solvent A) and acetonitrile (solvent B) were used. The gradient program was optimized as the following: 0–10 min, 5–18% B and 95–82% A; 10–20 min, 18–20% B; 20–40 min, 20–25% B; 40–50 min, 25–35% B; 50–60 min, 35–70% B; and 60–70 min, 70–100% B. The detection wavelength was set to 280 nm. The SME stock solution (6 mg/ml) was prepared by dissolving SME powder in water and was stored at -70°C . Before analysis, the stock solution was diluted to 0.6 mg/ml with water, filtered through a 0.22 μm membrane filter and then centrifuged at 13,000 g at 4°C for 10 min. The supernatant of the SME solution was injected into the HPLC system for analysis.

Animal Treatment

The experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Chicago. All experiments were carried out in male A/J mice, 6–8 weeks old, weighing between 18 and 22 g, obtained from Jackson Laboratories (Bar Harbor, ME). Mice were caged under a controlled room temperature, humidity and light (12/12 h light/dark cycle) and allowed unrestricted access to standard mouse chow and tap water. The mice were allowed to acclimate to these conditions for at least 7 days before inclusion in the experiments.

The experimental protocol is shown in Figure 1A. There were 4 experimental groups ($n = 8$ per group). Group 1 is the vehicle control. Animals in Group 2 (model), Group 3 (low-dose), and Group 4 (high dose) initially received a single intraperitoneal injection of AOM (7.5 mg/kg). One week after the AOM injection (set as Day 1), these animals started to receive 2.5% DSS in drinking water for consecutive 8 days. Animals in Group 3 (low-dose group) and Group 4 (high-dose group) also received SME 25 mg/kg and 50 mg/kg intraperitoneal injection every other day, respectively. During treatment, animal body weight was measured daily. Animals were sacrificed on Day 12, entire colon (cecum to anus) was removed and colon length was measured from the ileocecal junction to the anus. Colon tissue samples were collected for additional observations.

Disease Activity Index

DSS induced colitis was scored as the Disease Activity Index (DAI) as described previously (Cooper *et al.*, 1993). The DAI score was obtained based on weight loss, stool consistency change, and bleeding in stool (Table 1). The minimal score was 0 and the maximal score was 4. The animals were scored for the DAI at the same time of each day, blind to the treatment received.

Histological Assessment

Paraffin-embedded tissue samples were serially sectioned, and one section from each mouse was stained with hematoxylin and eosin (H&E). The stained sections were subsequently examined for histopathological changes by a gastrointestinal pathologist.

Histology score was determined by multiplying the percent involvement for each of the three following histologic features by the percent area of involvement (Jin *et al.*, 2010): inflammation severity (from 0, none to 3, severe), inflammation extent (from 0, to 3, transmural), crypt damage (from 0, none to 4, crypts and surface epithelium lost), and percent area involvement (from 0, 0% to 4, 76–100%). The minimal score was 0 and the maximal score was 40.

Statistical Analysis

Data were presented as mean $\pm$ standard error (S.E.). Data were analyzed using analysis of variance (ANOVA) for repeated measures and Student's *t*-test. The level of statistical significance was set at $P < 0.05$.

Results

HPLC Analysis of SME

The HPLC-UV fingerprint of SME is shown in Figure 1A. The constituents in SME were well separated under the established HPLC condition. From the chromatogram of SME, four main peaks were observed. After comparing their retention times and UV spectra with standards, they were identified as danshensu, protocatechuic aldehyde, salvianolic acid D and salvianolic acid B (Fig. 1B). Based on their peak area, the major constituents in SME are the former two compounds.

SME Ameliorates DSS-Induced Colitis

After DSS treatments, animals showed apparent diarrhea and rectal bleeding starting from Day 5 in the model group. As the treatment continued, presence and development of inflammation were clearly demonstrated. The disease severity, scored by disease activity index (DAI) reached highest level on Day 8 (Fig. 2). Figure 2(B) shows the effects of SME on the reduction of the DAI score in a dose-related manner ($P < 0.01$). This suppression of the colitis was not only evident during DSS treatment, but also obvious several days after the cessation of its administration, suggesting that SME significantly promoted the recovery from the colitis.

Effect of SME on DSS-Induced Colon Length Change

Colon length, as an objective measure of the severity of inflammation, was measured (Fig. 3). The control group animal had an average colon length of 8.6 ± 0.5 cm. The DSS treatment led to a very significant reduction of colon length, and the length in the model group animals was reduced to 5.5 ± 0.6 cm ($P < 0.01$ compared to control). Treatment with a low-dose and high-dose of SME inhibited the reduction of the colon in a dose-related manner (6.7 ± 0.6 and 7.8 ± 0.5 cm, both $P < 0.01$ compared to the model group).

Effect of SME on Body Weight Changes

Figure 4 showed body weight changes in different experiment groups. Compared to control with a slow gain of the weight, the model group had significant weight reduction starting from Day 6. This reduced weight continued up to Day 9, one day after cessation of the DSS. However, both the low-dose and high-dose SME groups significantly reduced the body weight reduction (both $P < 0.05$ compared to the model group).

Effect of SME on Colon Histopathology Changes

From H&E staining of histological sections, colon tissue from model animals showed increasingly severe inflammatory lesions extensively throughout: significant and complete loss of crypts; surface erosion with exuberant inflammatory exudate; patchy re-

epithelization; lamina propria fibrosis with acute and chronic inflammatory infiltrate; and submucosa edema and mixed inflammatory cell infiltration. For mice treated with SME, there was a significant reduction in colon inflammation. This reduction is particularly evident in the high-dose group: serosa colon tissue had mild inflammation, mucosa had tightly packed glands with a normal amount of goblet cells; lamina propria showed patchy neutrophilic infiltrate which extended into the submucosa; and normal serosa observed. Figure 5 shows representative H&E staining histological sections (A), and overall histology scores are provided (B).

Discussion

Traditional Chinese medicine (TCM) is under the guidance of its unique theory – a theory that is deeply influenced by both ancient philosophy and accumulated clinical experience. Chinese herbal medicine is a major component of TCM, and has played a critical role in the promotion of health, prevention of disease, and treatment of illnesses for Chinese people for several thousand years (Wang *et al.*, 2011; Xu *et al.*, 2011). *S. miltiorrhiza* is a very commonly used Chinese herbal medicine, and it has a strategic position in TCM. The herb is mostly used for cardiovascular illness since it promotes circulation, removes blood stasis, nourishes and tranquilizes the blood (Bensky *et al.*, 2004; Chen and Chen, 2004; Liu *et al.*, 2011). Modern biomedical studies have shown that constituents of *S. miltiorrhiza* could be beneficial in intervening against cardiovascular disorders (Chan *et al.*, 2011; Jiang *et al.*, 2011).

Compared to the cardiovascular studies of *S. miltiorrhiza*, an understating of its role in inflammation conditions is limited, while gastrointestinal inflammation is largely unexplored. Interestingly, only one study reported that the water-soluble extract of the herb, not other constituents, possessed anti-inflammatory properties *in vitro* and investigated its mechanism of action related to lipopolysaccharide, a bacterial product with a high content in the intestinal lumen. An *in vitro* kinase assay showed that the water-soluble extract directly inhibited lipopolysaccharide-induced I κ B kinase activity in IEC-18 cells. The extract blocked the lipopolysaccharide-induced NF- κ B signaling pathway by targeting the IKK complex in intestinal epithelial cells (Kim *et al.*, 2005). Modulation of bacterial product-mediated NF- κ B signaling by the water-soluble extract could represent a strategy towards the prevention and treatment of intestinal inflammation. In this study, we used a water-soluble extract of *S. miltiorrhiza* to observe its effects on colon inflammation in a DSS-induced colitis mouse model.

S. miltiorrhiza contains both hydrophobic and hydrophilic compounds. Previous phytochemical and biological studies were often focused on the hydrophobic compounds. More than 30 diterpene compounds have been isolated and identified from the hydrophobic fraction, such as tanshinones (including tanshinone I, IIA, IIB, cryptotanshinone) and related compounds. The antiproliferative potential of many natural tanshinones and their synthetic analogs on different cancer cell lines has been routinely evaluated with possible mechanisms of actions (Wang *et al.*, 2007; Dong *et al.*, 2011). Investigations on hydrophilic compounds, however, are very limited to date. The major constituents of hydrophilic extract include water-soluble phenolic acids and tanshinones. In this study, we evaluated the effects of the hydrophilic extract of *S. miltiorrhiza* on AOM/DSS-induced colitis in a mouse model. It would be interesting to compare pharmacological effects between hydrophobic and hydrophilic components in the same experimental setting. Nonetheless, the hydrophilic extract of *S. miltiorrhiza* showed satisfactory effect in our study.

In summary, we showed that, in a dose-related manner, SME inhibited DSS-induced colitis, resulting in an overall attenuation of inflammation DAI, including colon length and body

weight changes. The effects of the herb were further supported by histological characterization in mouse acute colitis. TCM has been practiced for thousands of years based on clinical experience. It is necessary to integrate existing TCM knowledge of diseases with modern biomedical technologies (Wang *et al.*, 2012b), and explore the mechanisms of action of the observed pharmacological effects. Future controlled clinical trials of *Salvia miltiorrhiza* on IBD symptoms are also warranted to evaluate the candidacy of the herb in the TCM-based CRC prevention and treatment.

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Figure 1.
HPLC analysis of *Salvia miltiorrhiza* extract (SME) and chemical structures of identified representative constituents in SME. (A) HPLC chromatogram of SME recorded at 280 nm. (B) Chemical structures of four identified compounds.

Figure 2.

Effects of *Salvia miltiorrhiza* extract (SME) on DSS-induced colitis. (A) Experimental protocol. (B) SME ameliorates the colitis, expressed as Disease Activity Index (DAI). Data from the control group are all zeros from Day 1 to Day 12 (no shown). The dose-related effects of SME are observed ($P < 0.05$).

Figure 3.

Mouse colon length changes in different groups. (A) Colon representative photographic images. (B) For model group, DSS treatment very significant reduces the colon length from control group (#, $P < 0.01$ compared to the control group). *Salvia miltiorrhiza* extract (SME) low-dose and high-dose treatment significantly decreased the colon length reduction (*, $P < 0.05$ and **, $P < 0.01$ compared to the model group, respectively). SME 25, SME 25 mg/kg; SME 50, SME 50 mg/kg.

Figure 4.

Effects of *Salvia miltiorrhiza* extract (SME) on mouse body weight changes. While the model group shows significant weight reduction from Day 6, SME low-dose (25 mg/kg) and high-dose (50 mg/kg) treatment significantly reduced the DSS-induced weight reduction (both $P < 0.05$ compared to the model group).

Figure 5. Effects of *Salvia miltiorrhiza* extract (SME) on the histological characterization in DSS-induced mouse colitis. (A) shows representative H&E staining histological sections of control, DSS only, DSS plus low-dose SME, and DSS plus high-dose SME. (B) shows the overall histology scores in these different groups (**, $P < 0.01$ compared to the model group). SME 25, SME 25 mg/kg; SME 50, SME 50 mg/kg.

Table 1

Scoring System for Disease Activity Index (DAI).

Score	Weight Loss	Stool Consistency	Blood in Stool
0	None	None	None
1	1–5%	---	Trace
2	5–10%	Loose stool	Hemoccul +
3	10–20%	---	Hemoccul ++
4	>20%	Diarrhea	Gross bleeding

The DAI was the combined scores of weight loss, stool consistency and bleeding divided by 3. Stool bleeding score: trace, scarcely blue; hemoccul +, blue easily detected; hemoccul ++, strongly blue. Acute clinical symptoms are diarrhea and/or grossly bloody stools.