Salvia miltiorrhiza increases the expression of transcription factor Foxp3 in experimental murine colitis

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Abstract. *Salvia miltiorrhiza* (SM) is mainly used for the treatment of coronary heart disease in China, but previous studies demonstrated that it also shows anti-inflammatory effects and the underlying mechanisms of these effects are not well understood. The present study aimed to investigate the effect of an injection of SM powder on the expression of transcription factor Foxp3 (Foxp3) in experimental colitis in mice. Mice were grouped and treated with SM powder for injection at the time of colonic instillation of trinitrobenzene sulfonic acid. Expression studies were performed by quantitative polymerase chain reaction and western blot analysis and histological studies were performed by hematoxylin and eosin staining. Myeloperoxidase activity was also tested for the evaluation of colitis. In the treated groups, the expression of Foxp3 mRNA and protein in the spleen were increased, the inflamed colonic lesions were relieved and the myeloperoxidase activity in the colon decreased significantly. Thus, it was demonstrated that SM exhibited its anti-inflammatory by promoting Foxp3 expression. SM may be effective for the treatment of inflammatory disease, particularly for inflammatory bowel disease.

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Abbreviations: SM, *Salvia miltiorrhiza*; Foxp3, transcription factor Foxp3; TNBS, trinitrobenzene sulfonic acid; PCR, polymerase chain reaction; H&E, hematoxylin and eosin stain; IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; TNF-α, tumor necrosis factor-α; Tr, regulatory T cells; IFN-γ, interferon-γ; IL, interleukin; OR, odds ratio

Key words: Salvia miltiorrhiza, inflammatory bowel disease, Foxp3, colitis, mice

Introduction

Inflammatory bowel disease (IBD) is a chronic inflammatory condition that may affect almost any part of the gastrointestinal tract. It is hypothesized that IBD results from the interactions between genetic predisposition, bacterial microflora, environmental influences and immune system disorders (1-4). It has been demonstrated that cytokines are crucially involved in the pathogenesis of IBD. Corticosteroids, 5-aminosalicylates, azathio-prine (6-mercaptopurine), methotrexate, thalidomide and monoclonal antibodies against TNF-α (Infliximab) are used for the treatment of IBD (5). However, there is concern regarding the safety of the drugs since patients with IBD usually undergo treatment with prolonged immunosuppressive therapies. It was previously reported that the use of corticosteroids, azathioprine/6-mercaptopurine and infliximab were individually associated with significantly increased risk of opportunistic infection. The use of any one of these drugs yielded an odds ratio (OR) of 2.9, whereas the use of 2 or 3 of these drugs yielded an OR of 14.5 for opportunistic infection. Immunosuppressive medications, particularly when used in combination, and at an older age are associated with an increased risk of opportunistic infections (6), thus efforts to improve the immunization status among patients with IBD are required (7). For this reason, there is a requirement of the identification of efficient and safe drugs for the treatment of IBD. *Salvia miltiorrhiza* (SM) is effective in the management of coronary heart disease (8). SM has previously been shown to exhibit anti-inflammatory bioactivity; however, the underlying mechanism remains unknown. It was demonstrated that SM can suppress the production of TNF-α, interleukin-1β (IL-1β), IL-12, interferon-γ (IFNγ) and nuclear factor-κB (9-11). A previous study demonstrated that SM decoction can increase the expression of transcription factor Foxp3 (Foxp3) in cultured lymphocytes (12). While Foxp3 is a master regulatory gene for the development and function of $CD4^+CD25^+$ regulatory T cells (Tr) which are hypothesized to exhibit a significant role in the self-tolerance and downregulation of inflammation in the intestine. The present study aimed to investigate the effect of injection with SM powder on the expression of Foxp3 in murine colitis induced by trinitrobenzene sulfonic acid (TNBS).

Materials and methods

Experimental animals. Specific pathogen free, Balb/c female mice (age, 6-8 weeks) were provided by the Laboratory Animal Center of China Medical University (Shenyang, China). The mice were housed under standard conditions (25˚C and 12-h light-dark cycle, 5 mice per 80 cm^2 cage) for at least one week prior to the start of the experiments. Throughout, the mice were fed with standard pellet diet *ad libitum* with the exception of when they were fasted for 24 h with free access to drinking for the colitis induction. The experimental settings involving mice were approved by the local authority for Animal Care and Use. The present study was performed in compliance with the animal welfare legislations of China Medical University (Shenyang, China). All efforts were made to minimize animals' suffering and to reduce the number of animals used.

Drugs and reagents. SM powder for injection (Lot no. 20090612; permission code of State Food and Drug Administration: Z10970093) was purchased from the Second Chinese Medicine Factory of Harbin Pharm Group Co. Ltd., (Harbin, China). It was extracted from 1,500 g dried root of SM with boiled distilled water and ethanol. Filtration and lyophilization were also used to obtain 40 g brownish powder. The dried powder contained 8% sodium Danshensu ($C_9H_9O_5Na$) and 16% protocatechuic aldehyde ($C_7H_6O_3$) determined by colorimetric methods (13). In total, 5% TNBS was purchased from Sigma-Aldrich Trading Co., Ltd. (Shanghai, China). A myeloperoxidase activity test kit was purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Rabbit anti-Foxp3 antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). UltraSensitve™ S-P kit was purchased from Maixin-Bio. Co. Ltd. (Fuzhou, China). RNAsimple Total RNA kit, TIANScript RT kit and SYBR-Green mastermix were purchased from Tiangen Biotech. Co. Ltd. (Beijing, China).

Colitis induction. Colitis was induced by intrarectal injection of TNBS as described previously (14,15). Briefly, subsequent to adaptively being housed for seven days, the mice were fasted for 24 h with free access to water. The mice were administered intrarectally with a single dose of TNBS 100 mg/kg (1% TNBS in 30% ethanol solution) subsequent to being anesthetized by 5% chloral hydrate [3 ml/kg, intraperitoneal (i.p.)]. The mice were held in a vertical position for 30 sec to ensure that TNBS was distributed evenly within the entire colon and cecum.

Groups and treatments. Mice with colitis were divided into five groups (n=5 per group) randomly. The drugs were administered by i.p injection daily following the induction of colitis. The drugs used for each group were as follows: Group A, none; group B, sterile normal saline 10 ml/kg; group C, 2% of 10 ml/kg SM normal saline (as 25 times for human); group D, 4% SM normal saline 10 ml/kg (as 50 times for human); and group E, 6% SM normal saline 10 ml/kg (as 75 times for human). The normal mice were also studied as group N when required.

Colon sampling for histological and myeloperoxidase activity analysis. The mice were sacrificed by 10% chloral hydrate (3 ml/kg; i.p.) seven days later following a colonic instillation of TNBS. The entire colon was dissected and the colon content was removed by gently rinsing with cold phosphate-buffered saline. Colon sections of ~0.5 cm were obtained from the distal, transversal and proximal segments of the colon. The colon specimens were fixed in 4% paraformaldehyde for 24 h and 5 μM paraffin-embedded sections were stained with hematoxylin and eosin for routine histological examination. The colon tissues for myeloperoxidase activity analysis were enclosed in sterile tubes and immediately frozen into liquid nitrogen. The specimens were stored at -80˚C until the testing was performed. The myeloperoxidase activity assay was performed according to the manufacturer's instructions of the test kit, as described in a previous study (16). Briefly, the colon tissue homogenate was mixed with reagents and incubated for the reactions. The absorbance of reaction products was measured at 460 nm (1 cm optical path). Myeloperoxidase activity was expressed in units/g of tissue. One unit corresponded to the activity required to degrade 1 μmol of hydrogen peroxide at 37˚C.

Spleen sampling for quantitative polymerase chain reaction (qPCR). The spleen specimens were packaged in RNase-free tubes and immediately placed in liquid nitrogen. The specimens were stored at -80˚C for the analysis of Foxp3 mRNA by qPCR. The frozen spleen tissue was divided into three sections and the RNAsimple Total RNA kit was used for the extraction of the total RNA. All the procedures were conducted according to the instructions of the RNAsimple Total RNA kit. The RNA concentration was determined by ultraviolet spectroscopy and the integrity was assessed by denaturing agarose gel electrophoresis. The TIANScript RT kit was used for the reverse transcription of total RNA, 1.5 μg total RNA was transcribed into cDNA in a 14.5 μl reaction, containing 1 μl oligo $(dT)_{15}$, 1 μl random primers, 2 μl dNTP (2.5 mM each) and ddH_2O was added to make the volume up to 14.5 μl, and were incubated at 70˚C for 5 min and 0˚C for 2 min. The product was mixed with 4 μl 5X First-Strand Buffer (Tiangen Biotech. Co. Ltd.), 0.5 μl RNasin (Tiangen Biotech. Co. Ltd.) and 1 μl (200 U) TIANScript M-MLV (Tiangen Biotech. Co. Ltd.) and then incubated at 42˚C for 50 min and 95˚C for 5 min to give 20 μl cDNA. The product of 1 μl cDNA mixed with 0.5 μl forward and 0.5 μl reverse primers, 10 μl SYBR-Green mastermix and 8 μl ddH_2O were used for the real-time fluorescent qPCR in the Exicycler™96 (Bioneer Corporation, Daejeon, Korea). An initial denaturation/activation step at 95˚C for 10 min was followed by 40 cycles at 95˚C for 10 sec, 60˚C for 20 sec and 72˚C for 30 sec and finally held at 4˚C for 5 min. The primers used are shown in Table I.

Triplicates were run for each sample. The specificity of the amplification products was controlled by a melting curve analysis. The mean expression of Foxp3 mRNA was normalized with housekeeping gene β-actin in the same samples. The relative quantification was performed using the comparative threshold cycle ($2^{-\Delta\Delta ct}$) method (relative gene expression). The expression of Foxp3 mRNA measured in the normal mice was considered the unit value, and the results obtained were reported as the relative levels with respect to the unit value.

Spleen sampling for western blot analysis. Spleen specimens were packaged in sterile tubes and immediately frozen in liquid

Table I. Primers used for PCR.

Gene	Sequence, 5'-3'	Length, bp	Tm, ˚C	Size, bp
Foxp3	F: AGCAGGAGAAAGCGGATACC	20	58.60	177
	R: TCTGTGAGGACTACCGAGCC	20	57.30	
β-actin	F: ACGTTGACATCCGTAAAGAC	20	50.18	200
	R: GAAGGTGGACAGTGAGGC	18	51.61	

PCR, polymerase chain reaction; bp, base pairs; F, forward; R, reverse.

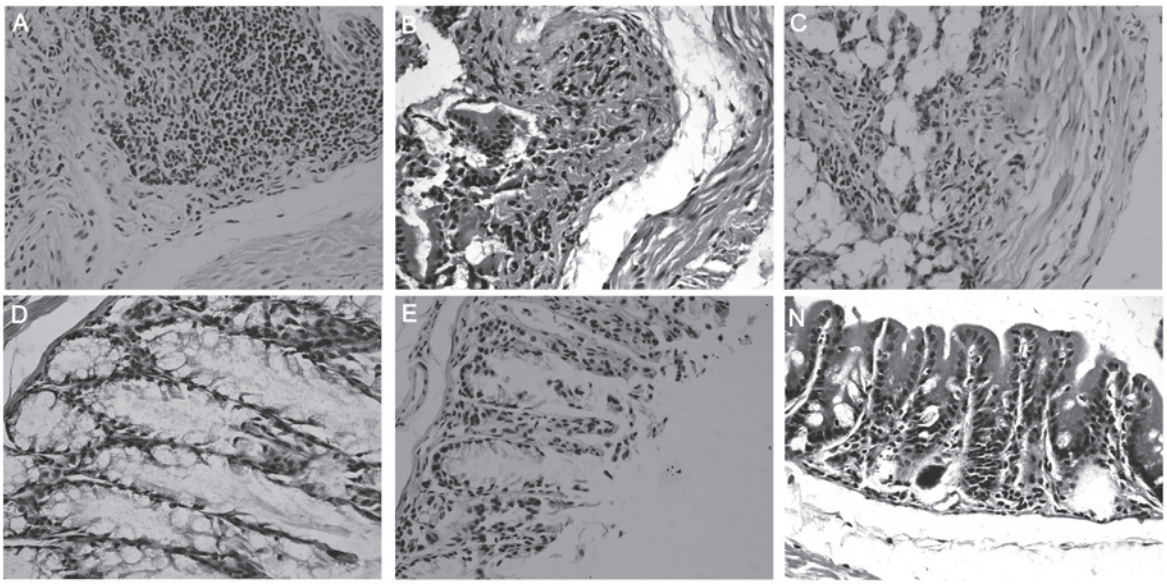

Figure 1. Hematoxylin and eosin staining of the colon. Balb/c mice were instilled with trinitrobenzene sulfonic acid (TNBS; 100 mg/kg) intracolonically and treated with daily injections of 2, 4 and 6% *Salvia miltiorrhiza* (SM; 10 ml/kg), normal saline (NS; 10 ml/kg) or none. The colon walls were thickened and granulomas with infiltration of inflammatory cells were observed in the colons of group A (TNBS), B (TNBS + NS) and C (TNBS + 2% SM). The colon lesions were significantly improved in the treated group D (TNBS+ 4% SM) and E (TNBS + 6% SM). N is the colon of a normal mouse. Original magnification, x400.

nitrogen. The specimens were stored at -80˚C for western blot analysis of Foxp3 protein. Briefly, the spleen tissue was lysed in ice-cold radio immunoprecipitation assay buffer [150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 50 mM Tris, (pH 8.0) and 1.0% henylmethylsulfonyl fluoride]. The quantity of protein was determined by a bicinchoninic acid assay (Boster Biological Technology Ltd., Wuhan, China) and 30 μg protein were loaded in each lane for electrophoresis. Following electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane. The primary antibody was rabbit anti-Foxp3 (1:500 dilution) and sheep anti-rabbit IgG conjugated to horseradish peroxidase (Boster Biological Technology Ltd.) was used as the secondary antibody. The reactions were developed with the electrochemiluminescence solution (Boster Biological Technology Ltd.). Blots were stripped and analyzed for β-actin, as an internal loading control, using a rabbit anti-β-actin (1:5,000 dilution). The optical density of the bands was measured by Image-Pro Plus 6.0 (Media Cybernatics Manufacturing, Warrendale, PA, USA), the value obtained was reported as the relative level with respect to β-actin in the same sample, and the value obtained from the normal mouse was considered the unit value.

Statistical analysis. All the data are presented as the mean ± standard deviation and the differences between groups were analyzed with a parametric test (t test). Software SPSS version 13.0 (SPSS, Inc., Chicago, IL, USA) was used for the analysis when appropriate and $P<0.05$ was considered to indicate a statistically significant difference.

Results

Histological evaluation of colon tissue. Mucosal erosion, ulceration, infiltration of inflammatory cells and granulomas were observed in the colon. The enteropathy of treated groups D and E was significantly relieved compared with groups A and B (Fig. 1).

Myeloperoxidase activity of colon tissue. Myeloperoxidase is an enzyme contained mainly in polymorphonuclear leucocytes, the myeloperoxidase activity is correlated with the number of inflammatory cells infiltrated in a given tissue (17). The myeloperoxidase activity in the treated groups C (3.16±0.32), D (2.65±0.24) and E (1.91±0.24 units/g) were significantly decreased compared with group A (3.80±0.39) and B (3.84±0.35 units/g). Furthermore, the activity in

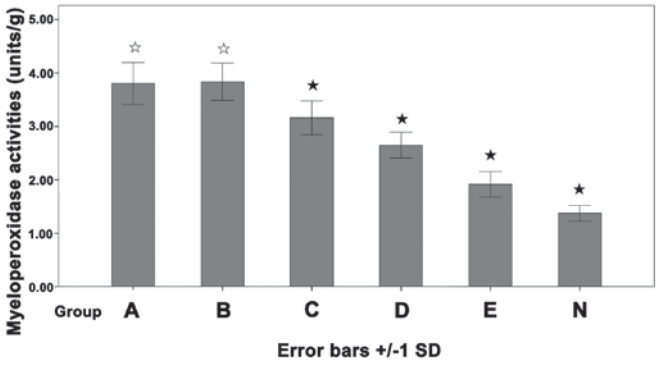

Figure 2. Myeloperoxidase activity of the colon tissue. Balb/c mice were instilled with trinitrobenzene sulfonic acid (TNBS; 100 mg/kg) intracolonically and treated with daily injections of 2, 4 and 6% *Salvia miltiorrhiza* (SM; 10 ml/kg), normal saline (NS; 10 ml/kg) or none. Group A, TNBS; B, TNBS + NS; C, TNBS + 2% SM; D, TNBS+ 4% SM; E, TNBS + 6% SM; N, normal mice. White star, P>0.05, group A vs. group B; black star, P<0.05 vs. group A and B.

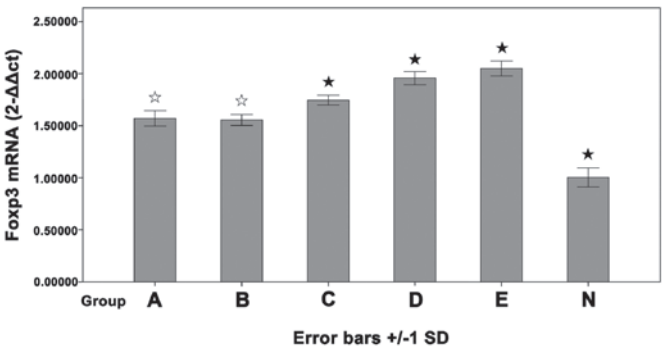

Figure 3. Expression of Foxp3 mRNA in the spleen. Balb/c mice were instilled with trinitrobenzene sulfonic acid (TNBS, 100 mg/kg) intracolonically and treated with daily injections of 2, 4 and 6% *Salvia miltiorrhiza* (SM; 10 ml/kg), normal saline (NS; 10 ml/kg) or none. Group A, TNBS; B, TNBS + NS; C, TNBS + 2% SM; D, TNBS + 4% SM; E, TNBS + 6% SM; N, normal mice. White star, P≥0.05, group A vs. group B; black star, P<0.05 vs. group A and B.

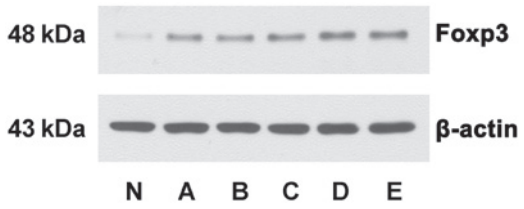

Figure 4. Western blot analysis of Foxp3 protein in the spleen. Balb/c mice were instilled with trinitrobenzene sulfonic acid (TNBS; 100 mg/kg) intracolonically and treated with daily injections of 2, 4 and 6% *Salvia miltiorrhiza* (SM; 10 ml/kg), normal saline (NS; 10 ml/kg) or none. Group N, normal mice; A, TNBS; B, TNBS + NS; C, TNBS + 2% SM; D, TNBS + 4% SM; E, TNBS + 6% SM.

group N (1.37±0.15 units/g) was lower than in any other group. The difference was not significant between groups A and B (P>0.05, Fig. 2).

Expression of Foxp3 mRNA in the spleen. The relative levels of Foxp3 mRNA in treated groups C (1.75±0.05), D (1.96±0.06) and E (2.05±0.07) were increased significantly compared

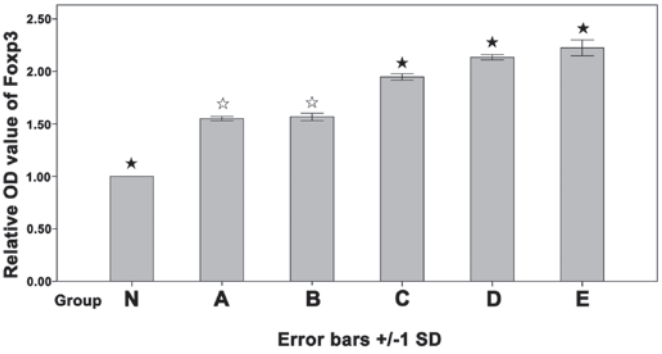

Figure 5. Expression of Foxp3 protein in the spleen. Balb/c mice were instilled with trinitrobenzene sulfonic acid (TNBS; 100 mg/kg) intracolonically and treated with daily injections of 2, 4 and 6% *Salvia miltiorrhiza* (SM; 10 ml/kg), normal saline (NS; 10 ml/kg) or none. Group N, normal mice; A, TNBS; B, TNBS + NS; C, TNBS + 2% SM; D, TNBS + 4% SM and E, TNBS + 6% SM. White star, P>0.05, group A vs. group B; black star, P<0.05 vs. group A and B.

with groups A (1.57±0.07) and B (1.56±0.05). In addition, the difference was not significant between group A and B, but the levels in group N were lower than in any other group (P<0.05, Fig. 3).

Expression of Foxp3 protein in spleen. The relative levels of Foxp3 optical density values in treated groups C, D and E were (1.95 ± 0.03), (2.13 ± 0.02) and (2.22 ± 0.08), respectively, which were increased significantly compared with group A (1.55 ± 0.02) and B (1.57 ± 0.04), but the difference was not significant between group A and B (Fig. 4 and 5).

Discussion

IBD is a chronic inflammatory condition that can affect almost any part of the gastrointestinal tract. IBD comprises two different disease entities, Crohn's disease (CD) and ulcerative colitis. Colitis can be induced in mice by treatment with a TNBS-ethanol enema (18). Granulomas with infiltration of inflammatory cells in all the layers of the intestine were observed in this model (Fig. 1). The isolated macrophages produce large quantities of IL-12, and the lymphocytes produce large quantities of IFN-γ and IL-2. This evidence indicates that the colitis in this model is induced by a Th type-1 response, constituting a CD model (14). This model is widely used for testing pharmacological molecules or agents that may lead to a possible cure for IBD. To maintain the intestinal homeostasis, natural $CD4^+CD25^+$ Tr are are hypothesized to exhibit a significant role in self-tolerance and the downregulation of inflammation in the intestine. Tr cells inhibit the antigen-specific T-cell responses mainly by cell-cell contact (19-21). Foxp3 is a member of the forkhead-winged helix family. Foxp3 is a master regulatory gene for the development and function of $CD4^+CD25^+$ Tr cells. It is specifically expressed in natural $CD4^+CD25^+$ Tr cells and can be used as a reliable marker for these cells in mice and in humans (22-25). In the present study, SM increased the expression of Foxp3 mRNA and protein in the spleen (Fig. 3-5), inhibited the infiltration of inflammatory cells (Fig. 1) and decreased the myeloperoxidase activity in the colon (Fig. 2). Notably, the expression of the T-box family transcription factor T-bet and TNF-α were also

suppressed by SM in the previous study (26). T-bet is a marker of Th1 cells and it is essential for Th1 differentiation from naive T cells (27). These results indicated that SM and Tr cells induced by SM inhibited the Th type 1 response. This effect of SM in increasing the expression of Foxp3 *in vitro* (12) and *in vivo*, which to the best of our knowledge has not been mentioned previously. SM may be effective for the treatment of inflammatory disease; however, further studies are required to reveal the mechanism and signaling pathway of the anti-inflammatory effects of SM, which may enable us to obtain an improved understanding of the bioactivity of SM and to implement future therapeutical approaches.

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