

Full Length Research Paper

***Salvia miltiorrhiza* inhibits the expressions of transcription factor T-bet (T-bet) and tumor necrosis factor α (TNF α) in the experimental colitis in mice**

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Salvia miltiorrhiza (SM) is mainly used for the treatment of coronary heart disease in china and it also represses inflammation, but the mechanism is not well known. This study aimed to investigate the effects of SM powder for injection on the expressions of transcription factor T-bet (T-bet) and tumor necrosis factor α (TNF α) in the experimental colitis in mice. Mice were grouped and treated with SM powder for injection at the time of colonic instillation of trinitrobenzene sulfonic acid (TNBS). Expression studies were performed by real-time polymerase chain reaction (PCR), western blot (WB) and immunohistochemistry (IHC), and histology studies were performed by hematoxylin and eosin stain (H&E). The survival of mice was also monitored. The expressions of TNF α in the colon, T-bet messenger ribonucleic acid (mRNA) and T-bet protein in the spleen decreased in the groups treated with SM powder for injection. The inflamed colonic lesions were relieved and the survival of mice also increased in the treated groups. SM powder for injection repressed the expressions of T-bet and TNF α in the experimental colitis in mice, which could relieve the inflamed colonic lesions and elevate the survival of mice.

Key words: *Salvia miltiorrhiza*, T-bet, tumor necrosis factor α , colitis, mice, inflammatory bowel disease, Crohn's disease, ulcerative colitis.

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic inflammatory condition affecting nearly any part of the gastrointestinal tract. IBD comprises two different disease entities, Crohn's disease (CD) and ulcerative colitis (UC).

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Abbreviations: SM, *Salvia miltiorrhiza*; T-bet, transcription factor T-bet; TNF α , tumor necrosis factor α ; TNBS, trinitrobenzene sulfonic acid; PCR, polymerase chain reaction; WB, western blot; IHC, immunohistochemistry; H&E, hematoxylin and eosin stain; mRNA, messenger ribonucleic acid; IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; PBS, phosphate-buffered saline; OB, interleukin; OR, odds ratio.

It is regarded that IBD results from the interactions between genetic predisposition, bacterial microflora, environmental influences and immune system disorders (Braus and Elliott, 2009; Oliva-Hemker and Fiocchi, 2002; Siminovitch, 2006; Cho and Weaver, 2007). It has been proven that cytokines are crucially involved in the pathogenesis of IBD and drugs used for the treatment of IBD have significant effects on the expressions of cytokines. Corticosteroids, 5-aminosalicylates, azathioprine (6-mercaptopurine), methotrexate, thalidomide, monoclonal antibody to TNF α (Infliximab) are drugs for the pharmacological treatment of IBD (Abdel-Hady and Bunn, 2004). However, concern should be raised about the safety of the drugs since patients with IBD are often treated with long-term immunosuppressive therapies. It was reported that use of corticosteroids, azathioprine/6-mercaptopurine and infliximab were associated individually with significantly increased odds for opportunistic

infection, use of any one of these drugs yielded an odds ratio (OR) of 2.9, whereas use of 2 or 3 of these drugs yielded an OR of 14.5 for opportunistic infection. Immunosuppressive medications, especially when used in combination, and older age are associated with increased risk of opportunistic infections (Toruner et al., 2008), hence efforts to improve immunization status among patients with IBD are needed (Melmed et al., 2006). For this reason, finding efficient and safe drug for the treatment of IBD is necessary.

Salvia miltiorrhiza (SM), also known as Danshen, has been prepared as decoction, tablet, capsule, drop pill, injection and powder for injection for administration. It is effective in the management of coronary heart disease (Qin and Huang, 2009). Previous studies demonstrated that SM has the effect of anti-inflammation, which was due to inhibit the production of TNF α , interleukin-1 β (IL-1 β), IL-12, interferon- γ (IFN γ) and nuclear factor- κ B (Bai et al., 2008; Bok et al., 2000; Yang and Xue, 2007). This study was intended to investigate the effects of SM powder for injection on the expressions of T-bet and TNF α in the trinitrobenzene sulfonic acid (TNBS) induced colitis in mice.

MATERIALS AND METHODS

Experimental animals

Specific pathogen free, Balb/c female mice 6 to 8 weeks of age were purchased from the Laboratory Animals Center of China Medical University (Shenyang, Liaoning Province, China) and housed under standard conditions (25°C and 12-h light-dark cycle, 5 mice per 80 cm² cage) for at least 1 week before starting the experiments. Throughout, the mice were fed with standard pellet diet *ad libitum* except for special procedures. The experimental settings involving mice were approved by the local authority for Animal Care and Use. The study was performed in compliance with the animal welfare legislations of China Medical University (Shenyang, Liaoning Province, 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, Heilongjiang Province, China), it was extracted from about 1500 g dried root of SM with boiled distilled water and then with ethanol. Filtration and lyophilization were also used to obtain the 40 g brownish powder. The dry powder contained 8% sodium Danshensu (C₉H₉O₅Na) and 16% protocatechuic aldehyde (C₇H₆O₃) determined by colorimetric method (Ye, 2006). TNBS (5%) was purchased from Sigma-Aldrich Trading Co. Ltd. (Shanghai, China). Rabbit anti T-bet antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA). Rabbit anti TNF α antibody was purchased from Nanjing KeyGen Biotech. Co. Ltd. (Nanjing, Jiangsu Province, China). Occult blood (OB) test kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu Province, China). UltraSensitive™ S-P kit was purchased from Maixin-Bio. Co. Ltd. (Fuzhou, Fujian Province,

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 previously described (Neurath et al., 1995; Coquerelle et al., 2009). Briefly, after adaptively housed for 7 days, Balb/c mice were fasted for 24 h with free access to drinking. TNBS was administered intrarectally in a single dose of 100 mg/kg (1% TNBS contained in 30% ethanol solution) to the mice after been anaesthetized by 5% chloral hydrate (3 ml/kg, i.p.) via a polyethylene catheter of 5 cm length, 2 mm diameter. Mice were held in a vertical position for 30 s to ensure the distribution of TNBS within the entire colon and cecum.

Groups and treatments

Mice with induced colitis were divided into 5 groups (5 per group) randomly. After the induction of colitis drugs were administered daily for 7 days and the treatments of groups were as follows: Group A: none; Group B: sterile normal saline 10 ml/kg by intraperitoneal injection daily; Group C: 2% SM normal saline 10 ml/kg by intraperitoneal injection daily; Group D: 4% SM normal saline 10 ml/kg by intraperitoneal injection daily; Group E: 6% SM normal saline 10 ml/kg by intraperitoneal injection daily. The normal mice were also studied as Group N when necessary.

Disease activity index

In all protocol studies, mice were monitored for the appearance of activity, grooming behavior, mean food consumption, diarrhea, loss of body weight, and overall mortality for 7 days after TNBS instillation. For scoring colitis activity, weight changes were recorded daily. Faeces samples of each animal were visually inspected for signs of diarrhea and rectal bleedings. The disease activity index (DAI) was calculated by summarizing the scores for weight loss, stool consistency, OB test positivity or gross bleedings as described in Table 1 (Bank et al., 2006).

Colon sampling for histology and immunohistochemistry

Mice were sacrificed by 10% chloral hydrate (3 ml/kg, i.p.) 7 days after colonic instillation of TNBS. The entire colon was dissected and the colon content was removed by gently rinsing with cold phosphate-buffered saline (PBS); 3 sections of about 0.5 cm were obtained from the distal, transversal and proximal segment of the colon respectively. Specimens were fixed in 4% paraformaldehyde about 24 h then paraffin embedded 5 μ m sections were stained with hematoxylin and eosin (H&E) for routine histology. Immunohistochemistry was performed on 5 μ m paraffin sections according to the manual of UltraSensitive™ S-P kit: deparaffinization, rehydration, heat assisted antigen retrieval, endogenous peroxidase inhibition and incubation with tumor necrosis factor α (TNF α) antibody. The antibody was used at 1:100 final dilutions. After repeated washings with PBS, sections were incubated with biotin-IgG and then streptavidin-peroxidase. After washing with PBS, the reaction product was revealed by 0.6% 3,3-diaminobenzidine. Slides were counterstained with hematoxylin. Negative controls were incubated with PBS and yielded no staining. The optical density was measured by Image-Pro Plus 6.0 as the expression level of TNF α .

Table 1. Scores for disease activity index.

Scores	Weight loss (%)	Stool consistency	Rectal bleeding
0	< 5	Well formed pellets	No
1	5 - 10	-	-
2	10 - 15	Pasty, semiformed stool	Hemoccult positive
3	15 - 20	-	-
4	> 20	Liquid stool	Visible gross bleeding

The disease activity index (DAI) was the summarizing scores for weight loss, stool consistency, occult blood (OB) test positivity or gross bleedings.

Spleen sampling for quantitative RT-PCR

Spleen specimens were stored in RNase free tube, then put in liquid nitrogen immediately and stored at -80°C for quantitative real-time polymerase chain reaction (RT-PCR) of T-bet mRNA. The frozen spleen tissue was split into 3 parts and the RNAsimple Total RNA Kit was used for the extraction of total RNA following the protocol described by the manufacturer. RNA concentration was determined by ultra violet (UV)-spectroscopy and the integrity was assessed by denaturing agarose gel electrophoresis. TIANScript RT Kit was used for the reverse transcription of total RNA, and 1.5 µg of total RNA was transcribed into cDNA in a 14.5 µL reaction containing 1 µL oligo (dT)₁₅, 1 µL random, 2 µL dNTP (2.5 mM each), ddH₂O added to 14.5 µL, incubated at 70°C for 5 min, 0°C for 2 min, the product was mixed with 4 µL 5 × First-Strand Buffer, 0.5 µL RNasin and 1 µL (200 U) TIANScript M-MLV and then incubated at 42°C for 50 min, 95°C for 5 min to get 20 µL cDNA. The product of cDNA 1 µL mixed with 0.5 µL forward primers, 0.5 µL reverse primers, 10 µL SYBR Green mastermix and 8 µL ddH₂O were used for the real-time fluorescent quantitative PCR in the Exicycler™ 96(Bioneer Corporation, Korea), an initial denaturation/activation step (10 min 95 °C) was followed by 40 cycles (10 s at 95°C, 20 s at 60°C and 30 s at 72°C), and a final held at 4°C for 5 min. The gene specific primers were: T-bet: forward 5'-AAGTTCAACCAGCACCAGACAG -3' and reverse 5'-CACCAAGACCACATCCACAAAC -3'; β-actin: forward 5'-ACGTTGACATCCGTAAGAC -3' and reverse 5'-GAAGGTGGACAGTGAGGC -3'.

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

Spleen sampling for western blot

Spleen specimens were stored in sterile tube, put into liquid nitrogen immediately and stored at -80°C for western blot (WB) of T-bet. In brief, spleen tissue was lysed in ice-cold radio immunoprecipitation assay (RIPA) 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, 1.0% phenylmethylsulfonyl fluoride), and the amount of protein was determined by the bicinchoninic acid assay. A 30 µg aliquot of protein was loaded in each lane and subjected to 10% sodium dodecyl sulfate-

polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, protein was transferred to polyvinylidene difluoride (PVDF) membrane. The primary antibody was rabbit anti T-bet (1:500 dilution), while sheep anti rabbit IgG conjugated to horseradish peroxidase was used as secondary antibody, and the reaction was developed with the electro-chemiluminescence (ECL) solution. Blots were stripped and analyzed for β-actin, as an internal loading control, using a rabbit anti β-actin (1:5000 dilution). The optical density of bands was measured by Image-Pro Plus 6.0, the value obtained was reported as relative level with respect to the β-actin in the same sample, the value of normal mouse was considered the unit value.

Statistical analysis

All data were presented as mean ± standard deviation ($\bar{x} \pm SD$) and differences between groups were analyzed with parametric test (t test) or nonparametric test (χ^2 test). Software SPSS version 13.0 was used for the analysis when appropriate and $p < 0.05$ was considered significant.

RESULTS

Manifestation of mice

The mice appeared depressed, lazy, anorexia, had diarrhea and their body hair was in disorder and lack of gloss at day 2 after a single intracolonic administration of TNBS ethanol solution at 100 mg/kg. Bloody stool and weight loss occurred at day 3 as well as some death. The mice got better after day 4 and the total survival ratio at day 7 was 68%, but the survival ratio of group D was 91%, which was higher significantly compared with group A (50%, $p < 0.05$).

However, the differences of group C (64%) and E (73%) compared with group A were not significant, and it was also not significant to compare between group A and group B (64%).

The DAI scores of group D and E were (3.20 ± 1.09) and (2.8 ± 1.10) at day 7, which decreased significantly compared with group A (4.80 ± 1.09 , $p < 0.05$) and B (4.40 ± 0.89 , $p < 0.05$), but the difference was significant for group N (0.6 ± 0.55) compared with each other group (Figure 1).

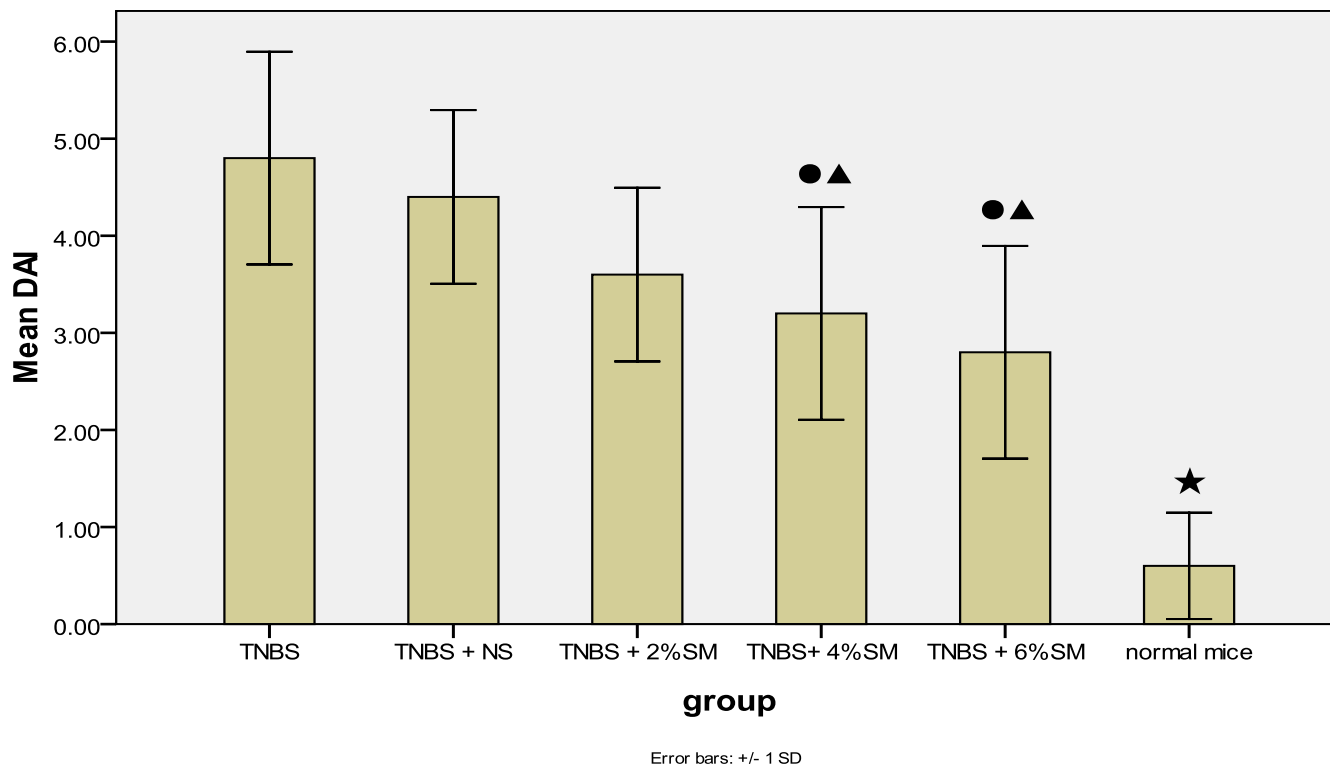


Figure 1. The disease activity index (DAI) scores of colitis. Balb/c mice were instilled with trinitrobenzene sulfonic acid (TNBS, 100 mg/kg) intracolonicly and treated with daily injections of 2, 4 and 6% *Salvia miltiorrhiza* (SM, 10 ml/kg), normal saline (NS, 10 ml/kg) or not. Group A, TNBS; B, TNBS + NS; C, TNBS + 2% SM; D, TNBS+ 4% SM; E, TNBS + 6% SM; N, normal mice. ● $p < 0.05$ vs group A, ▲ $p < 0.05$ vs group B, ★ $p < 0.05$ vs group A, B, C, D and E.

Histological evaluation of colitis

Necrobiosis and loss of epithelial cells, mucosal erosion, hyperemia, hemorrhage, granulomas with infiltration of inflammatory cells, crypt abscess, ulceration, disorder of gland, goblet cells loss were the enteropathy in all groups administered with TNBS. The enteropathy of group D and E were significantly relieved compared with group A and B (Figure 2).

Expression of TNF α in colon

TNF α was expressed in the mucous membrane of colon, mainly in the cytoplasm of intestinal villus and gland epithelium (Figure 3). The mean OD values of TNF α were $(21.39 \pm 4.65) \times 10^{-3}$ and $(19.39 \pm 6.54) \times 10^{-3}$ in SM treated group D and E, respectively which decreased significantly ($p < 0.05$) compared with group A $(29.28 \pm 4.7) \times 10^{-3}$ and B $(31.81 \pm 9.68) \times 10^{-3}$. The difference, however, was not significant for group N $(15.67 \pm 3.78) \times 10^{-3}$ compared with group D and E (Figure 4).

Expression of T-bet mRNA in spleen

The relative levels with respect to normal mice of T-bet

mRNA were 1.64 ± 0.04 , 1.46 ± 0.09 and 1.25 ± 0.11 in group C, D and E, respectively which decreased significantly compared with group A (2.13 ± 0.06 , $p < 0.05$) and B (2.13 ± 0.20 , $p < 0.05$) (Figure 5).

Expression of T-bet protein in spleen

The relative levels of T-bet OD values with respect to normal mice were 2.25 ± 0.10 , 1.90 ± 0.16 and 1.31 ± 0.08 in treated group C, D and E respectively which decreased significantly compared with group A (2.66 ± 0.16 , $p < 0.05$) and B (2.72 ± 0.07 , $p < 0.05$) (Figures 6 and 7).

DISCUSSION

IBD is a chronic gastrointestinal immune disorder characterized by intestinal inflammation and mucous membrane damage. Different animal models have been used for the studies of IBD. Colitis induced by intrarectal injection of TNBS ethanol solution was widely used for the testing of pharmacological molecules or agents that could lead to a possible cure for IBD. It was demonstrated that ethanol could destruct the mucosal barrier which enhanced TNBS

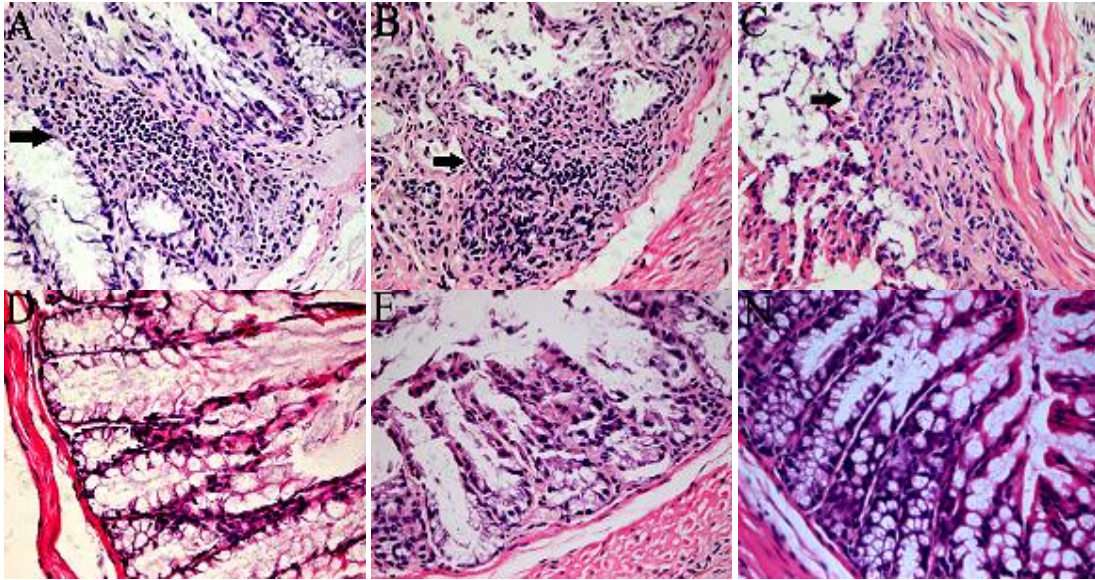


Figure 2. Hematoxylin-eosin (HE) stain of colon. Balb/c mice were instilled with trinitrobenzene sulfonic acid (TNBS, 100 mg/kg) intracolonicly and treated with daily injections of 2, 4 and 6% *Salvia miltiorrhiza* (SM, 10 ml/kg), normal saline (NS, 10 ml/kg) or not. Arrows indicated that the colon walls were thickened and granulomas with infiltration of inflammatory cells were seen in the colons of group A (TNBS), B (TNBS + NS) and C (TNBS + 2%SM). Colon lesions were significantly improved in treated group D (TNBS+ 4%SM) and E (TNBS + 6%SM). N is the colon of normal mouse. Original magnification was X 400.

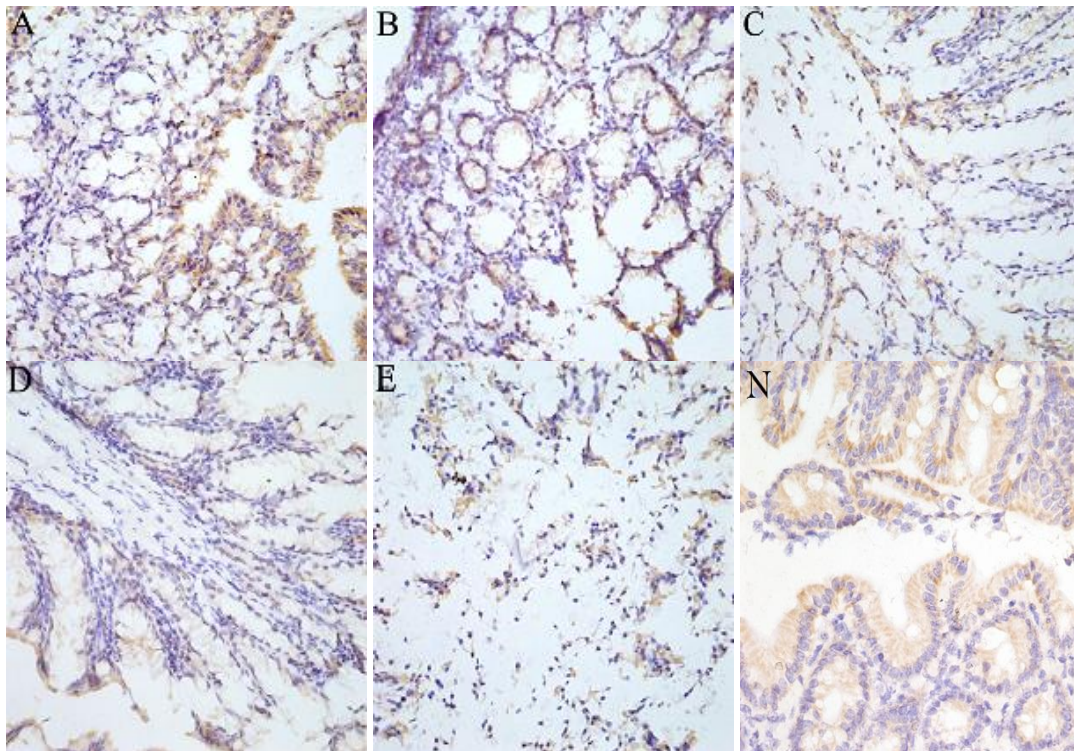


Figure 3. Immunohistochemistry (IHC) stains of tumor necrosis factor α (TNF α) in the colon. Balb/c mice were instilled with trinitrobenzene sulfonic acid (TNBS, 100 mg/kg) intracolonicly and treated with daily injections of 2%, 4%, 6% *Salvia miltiorrhiza* (SM, 10 ml/kg), normal saline (NS, 10 ml/kg) or not. TNF α was expressed in the mucous membrane of colon, mainly in the cytoplasm of intestinal villus and gland epithelium (yellow to brown in the pictures). Group A: TNBS; B: TNBS + NS; C: TNBS + 2%SM; D: TNBS+ 4%SM; E: TNBS + 6%SM; N: normal mouse. Original magnification was X 400.

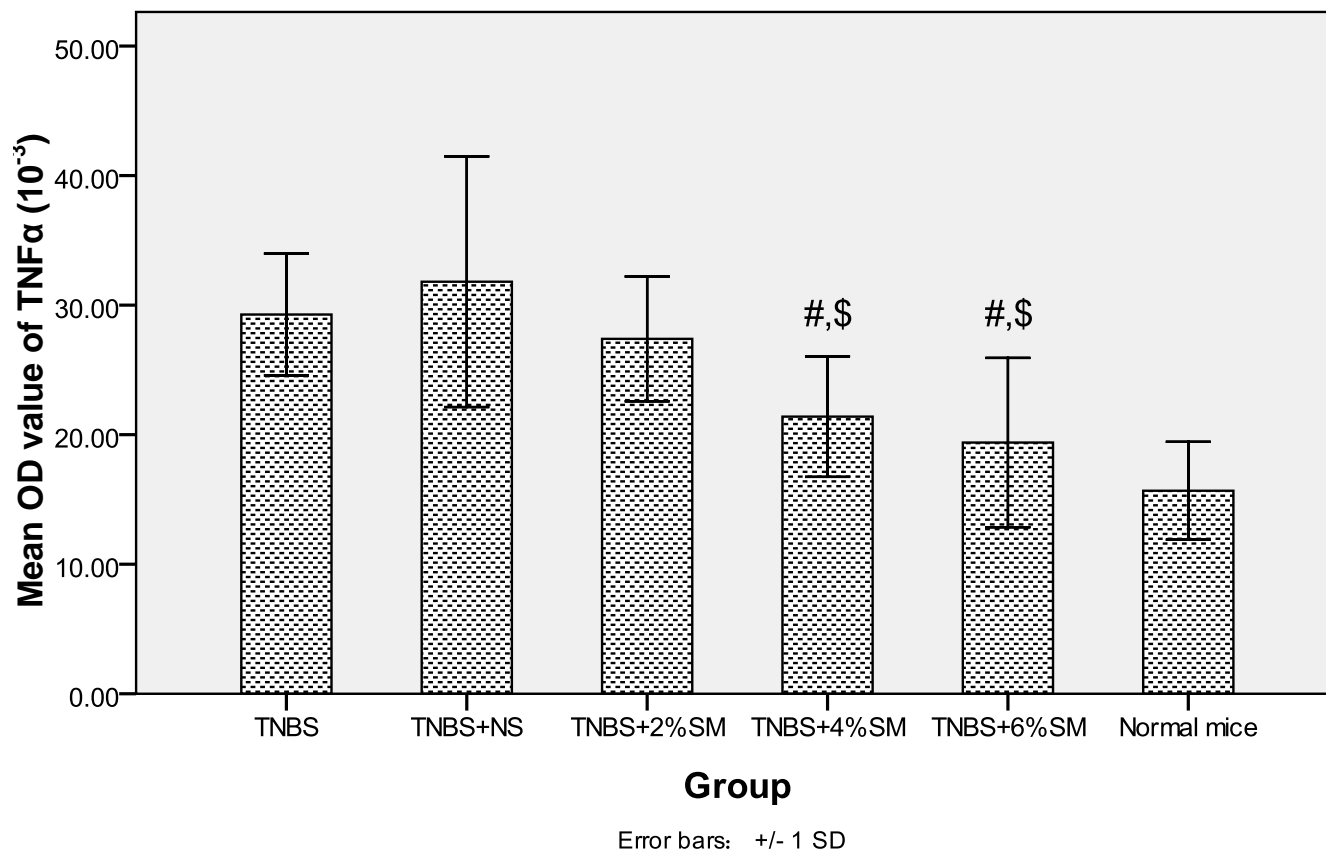


Figure 4. Mean OD value of tumor necrosis factor α (TNF α) in the colon. Balb/c mice were instilled with trinitrobenzene sulfonic acid (TNBS, 100 mg/kg) intracolonicly and treated with daily injections of 2, 4 and 6% *Salvia miltiorrhiza* (SM, 10 ml/kg), normal saline (NS, 10 ml/kg) or not. Group A: TNBS; B: TNBS + NS; C: TNBS + 2%SM; D: TNBS+ 4%SM; E: TNBS + 6%SM; N: normal mice. # $p < 0.05$ vs. group A, \$ $p < 0.05$ vs. group B.

as hapten combined with intestinal protein to form a complete antigen and evoke a delayed type hypersensitivity, which proceeds to develop chronic colitis (Hibi et al., 2002). In our study, colitis was induced successfully, the therapeutic dose of SM was 400 mg/kg (group D), which was 50 times as much as for human. Murine colitis treated with SM showed that the survival ratio was higher, the DAI scores were decreased, the enteropathy was relieved significantly, and especially the formation of granulomas was inhibited (Figure 2). However, the survival ratio did not increase with dose changed in group E (75 times as much as for human) and the colon mucosa in group E was thin, rarefaction (Figures 2 and 4). The dysplastic of mucosa was due to the anti-fibrosis effect of SM, the expressions of collagen gene and collagen were both reduced by SM (Wasser et al., 1998; Hu et al., 2009), and so further study should be carried out to investigate whether the effects of anti-inflammation and anti-fibrosis of SM were dose dependent.

It was also demonstrated that large amount of interleukin-12 (IL-12) was produced by macrophages and large amounts of interferon- γ (IFN γ) and IL-2 were

produced by lymphocytes which suggested that the colitis induced by TNBS was a Th type-1 response, constituting a CD model (Neurath et al., 1995). The similarities between the human CD and colitis induced by TNBS were allowed to address the immune system involvement in the pathogenesis of IBD (Ilan et al., 2002). The Th type-1 response was mediated by Th1/ TNF pathways, TNF, IFN- γ , IL-12, IL-1 and IL-6 were produced to initiate the inflammation. The Crohn's-like IBD, induced by the overexpression of TNF in an experimental mice model indicated that TNF was a key proinflammatory factor for inflammation (Kontoyiannis et al., 1999). Infliximab, a chimeric mouse-human monoclonal IgG1 antibody, is a powerful and selective anticytokine drug directed against TNF α and has been used worldwide. It is effective in the treatment of refractory luminal and fistulising CD (Rutgeerts et al., 2004; Sands et al., 2004).

Results from a single centre cohort indicated that patients with CD receiving long-term treatment with infliximab were very efficacious to maintain improvement during a median follow-up of almost 5 years and changed disease outcome by decreasing the rate of hospitalizations and surgery (Schnitzler et al., 2009). Long-term

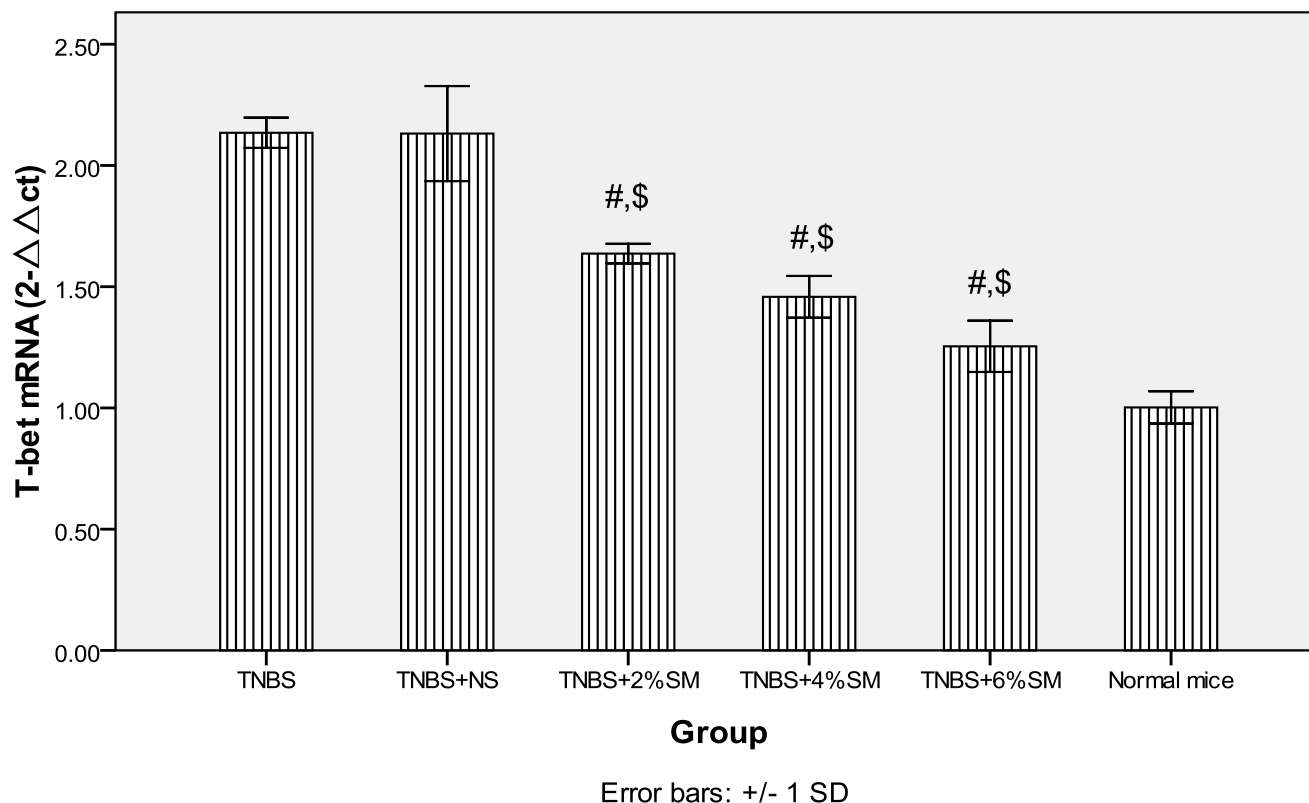


Figure 5. Expression of T-bet mRNA in spleen. Balb/c mice were instilled with trinitrobenzene sulfonic acid (TNBS, 100 mg/kg) intracolonicly and treated with daily injections of 2, 4 and 6% *Salvia miltiorrhiza* (SM, 10 ml/kg), normal saline (NS, 10 ml/kg) or not. Group A: TNBS; B: TNBS + NS; C: TNBS + 2%SM; D: TNBS+ 4%SM; E: TNBS + 6%SM; N: Normal mice. # $p < 0.05$ vs. group A, \$ $p < 0.05$ vs. group B.

infliximab treatment had a good overall safety profile in the patient with IBD (Fidder et al., 2009). Intracolonic administration of antisense oligonucleotide against TNF α resulted in a significant reduction of colonic TNF α and amelioration of histopathology in mice with colitis induced by TNBS (Zuo et al., 2010). In our study, SM inhibited the expression of TNF α in the experiment colitis significantly and showed a good therapeutic effect for the colitis, which was the same result as previous study (Bai et al., 2008).

In the differentiation of Th1 lymph cells, T-box family transcription factor T-bet (T-bet) is the specific hallmark for Th1 cells and it is essential for Th1 differentiation from naive T cells. Without the expression of T-bet, Th1 cells cannot be generated either *in vitro* or *in vivo*; however, overexpression of T-bet enable T cells to follow Th1 programming (Szabo et al., 2002). The ectopic expression of T-bet also transactivated the *IFN- γ* gene and induced endogenous *IFN- γ* production. Gene transduction of *T-bet* into polarized Th2 primary T cells redirected them into Th1 cells and expressed *IFN- γ* instead of *IL-4* or *IL-5*; T-bet is the master regulator controlling the destiny of Th1 cells (Szabo et al., 2000). It was also revealed that both T-bet and the transcription factor *Runx3* bond to the promoter of *IFN- γ* gene and the

silencer of *IL-4* gene resulted in the maximal production of *IFN- γ* and silencing of the gene encoding *IL-4* in Th1 cells. T-bet did not repress *IL-4* in *Runx3*-deficient Th2 cells and the *Runx3* was induced in Th1 cells in a T-bet-dependent manner, too (Djuretic et al., 2007).

In this report, we showed that colitis induced by TNBS was a Th type-1 response. The expressions of T-bet mRNA and T-bet protein in the spleen were inhibited by SM greatly and as a result the expression of TNF α was also reduced significantly, which may be one of the mechanisms of anti-inflammation effect of SM that has not been previously mentioned. Further studies should be carried out to reveal the mechanism and signal pathway of the anti-inflammatory effect as well as the inhibition of Th1 cells differentiation of SM, which will enable to make a better understanding of Th1 inflammatory diseases and to implement future therapeutic approaches.

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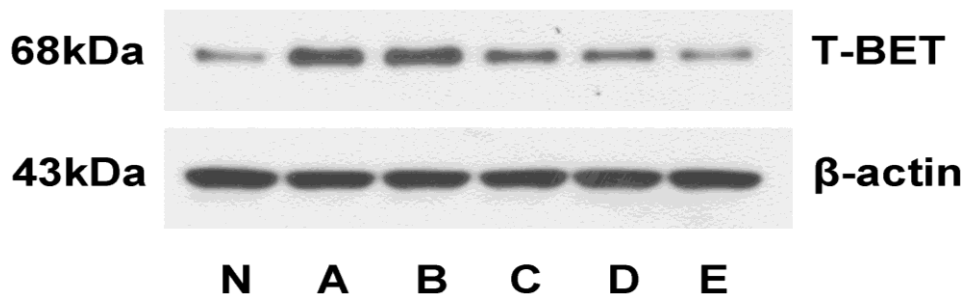


Figure 6. Western blot of T-bet protein in spleen. Balb/c mice were instilled with trinitrobenzene sulfonic acid (TNBS, 100 mg/kg) intracolonicly and treated with daily injections of 2, 4 and 6% *Salvia miltiorrhiza* (SM, 10 ml/kg), normal saline (NS, 10 ml/kg) or not. Group N: normal mice; A: TNBS; B: TNBS + NS; C: TNBS + 2%SM; D: TNBS+ 4%SM; E: TNBS + 6%SM.

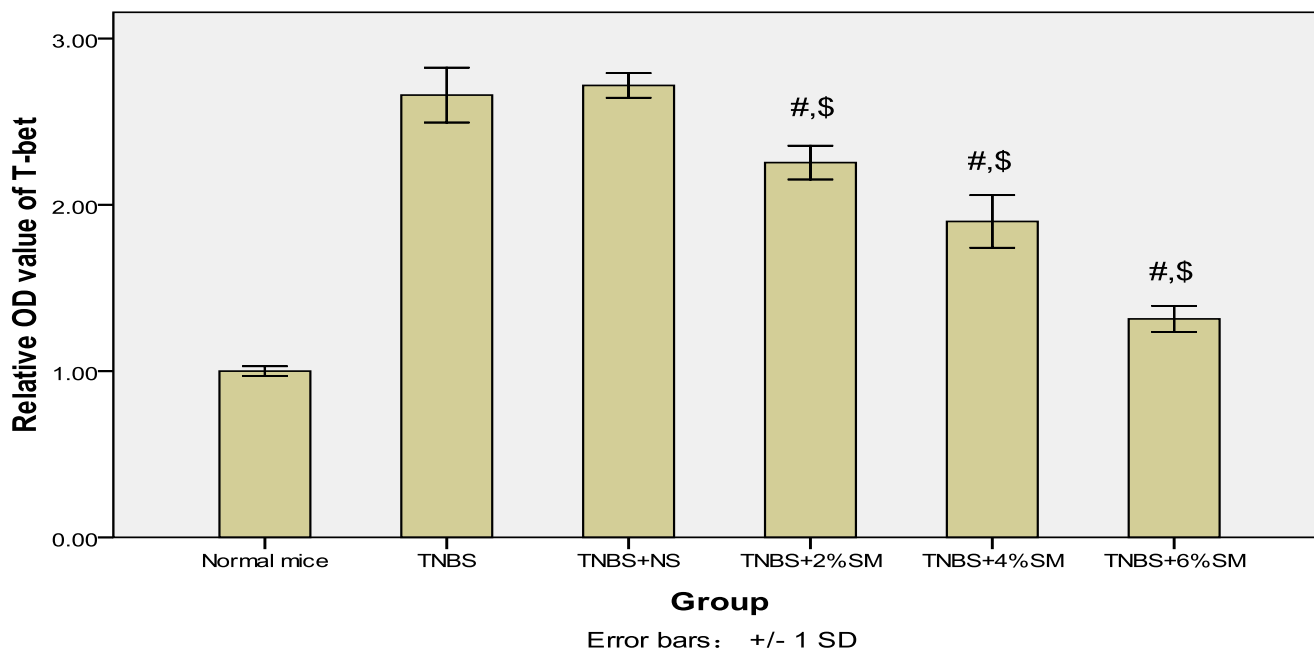


Figure 7. Expression of T-bet protein in spleen. Balb/c mice were instilled with trinitrobenzene sulfonic acid (TNBS, 100 mg/kg) intracolonicly and treated with daily injections of 2, 4 and 6% *Salvia miltiorrhiza* (SM, 10 ml/kg), normal saline (NS, 10 ml/kg) or not. Group N: normal mice; A: TNBS; B: TNBS + NS; C: TNBS + 2%SM; D: TNBS+ 4%SM; E: TNBS + 6%SM. # $p < 0.05$ vs. group A, \$ $p < 0.05$ vs. group B.

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