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ORIGINAL ARTICLE

Influence of Shenqing Recipe on Morphology and Quantity of Colonic Interstitial Cells of Cajal in Trinitrobenzene Sulfonic Acid Induced Rat Colitis[△]

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Objective To observe the influence of Shenqing Recipe (SQR), a kind of Traditional Chinese Medicine, on the morphology and quantity of colonic interstitial cells of Cajal (ICC) in trinitrobenzene sulfonic acid (TNBS)-induced rat colitis, and to investigate the possible mechanism of SQR in regulating intestinal dynamics.

Methods Sixty rats were randomly divided into normal control, model I, model II, mesalazine, and high-dose, and low-dose SQR groups with 10 rats in each group. TNBS (10 mg) dissolved in 50% ethanol was instilled into the lumen of the rat colon of the latter five groups to induce colitis. On the 4th day after administration of TNBS, each treatment group was administered one of the following formulations by enteroclysis gavage once a day for 7 days: 600 mg·kg⁻¹·d⁻¹ mesalazine, 2.4 g·kg⁻¹·d⁻¹ SQR, and 1.2 g·kg⁻¹·d⁻¹ SQR. Model II rats received normal saline solution. After 7 days colonic samples were collected. While the colonic samples of model I group were collected on the 3rd day after TNBS administered. Ultrastructure of ICC in the damaged colonic tissues was observed with transmission electron microscope. Expression of c-kit protein in colonic tissue was determined by immunohistochemical staining and Western blot.

Results The ultrastructure of colonic ICC in the rat model of TNBS-induced colitis showed a severe injury, and administration of SQR or mesalazine reduced the severity of injury. Similarly, the expression of c-kit protein of TNBS-induced colitis rat model was significantly decreased compared with the normal control group ($P<0.05$). Treatment with SQR or mesalazine significantly increased the expression of c-kit protein compared with the administration of control formulations ($P<0.05$), especially the high-dose SQR group.

Conclusion SQR could alleviate and repair the injured ICC, and improve its quantity, which might be involved in regulating intestinal motility.

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ULCERATIVE colitis (UC) is an inflammatory disease of the rectal and colonic mucosa and seems to be the result of a series of complex interactions, such as susceptibility genes, environment, and the immune system. The definite pathogenesis of UC has not been elucidated. However, intestinal motility has been recognized to be associated with the pathogenesis of UC.¹ Interstitial cells of Cajal (ICC) serve as electrical pacemakers, active propagation pathways for slow waves, and mediators of enteric motor neurotransmission and are involved in abnormality of intestinal motility.² It is reported that the changes of number and structure in ICC are associated with the pathogenesis of UC.³

Our previous data indicated that the Shenqing Recipe (SQR), a kind of Traditional Chinese Medicine, could inhibit expressions of a multitude of inflammatory mediators in trinitrobenzene sulfonic acid (TNBS)-induced rat colitis.⁴ This paper observed the effects of SQR on the morphology and quantity of colonic ICC in TNBS-induced colitis rat model, and aimed to investigate the mechanism of SQR in regulating intestinal dynamics in UC.

MATERIALS AND METHODS

Animals

Sixty male Sprague-Dawley rats weighing 180-200 g, purchased from Shanghai SLAC Laboratory Animal Co. Ltd. (China), were used in the study. All rats were housed on wire mesh bottom cages in a room with a constant temperature of 22°C-24°C. Light cycles were provided with 12 hours of light and 12 hours of dark. Rats were fed a standard chow diet and free access to water. All experimental procedures described below were approved by our institutional animal research committees and were in accordance with nationally approved guidelines for the treatment of laboratory animals.

Experimental design

Sixty rats were randomly divided into normal control group, model I group, model II group, mesalazine group, high-dose SQR group, and low-dose SQR group with 10 rats in each group. Rats in the latter five groups were used to establish colitis model. The rats in normal control group received no specific interventions. Colonic samples in model I group were collected on the 3rd day after intracolonic administration of TNBS (Sigma, St. Louis, MO, USA) in order to observe whether the model was established successfully. While in the other five groups, samples were collected after treatment with corresponding drugs for 7 consecutive days. Model II group rats received 0.33 mL

normal saline solution by enteroclysis gavage once a day for 7 days. Mesalazine group rats received mesalazine solution (Jiamusi Luling Pharmaceutical Co., Ltd, China) with the dose of 600 mg·kg⁻¹·d⁻¹ by enteroclysis gavage once a day for 7 days. High-dose and low-dose SQR groups received SQR solution (Shanghai Jiahua Biological Pharmacy Co., China) with the dose of 2.4 g·kg⁻¹·d⁻¹ and 1.2 g·kg⁻¹·d⁻¹ respectively by enteroclysis gavage once a day for 7 days.

Induction of colitis

Colonic inflammation was induced using a technique described by Morris *et al.*⁵ Fasting rats was lightly anesthetized by intraperitoneal injection of ketamine (2 mL/kg), and a polyethylene catheter was inserted into the anus with its proximal edge 8 cm from the anal verge. TNBS dissolved in 50% ethanol (10 mg in a volume of 0.25 mL) was instilled in the colon through the cannula to induce colitis. Following the instillation of TNBS solution, rats were maintained in a head-down position for a few minutes to prevent leakage of the intracolonic instillation. On the 4th day after administration of TNBS, the latter four groups began to receive corresponding drug for 7 consecutive days. The rats were monitored daily for general conditions and survival. After treatment of 7 days, rats were sacrificed and the distal 8 cm of the colon was excised free of adherent adipose tissue, rinsed with ice-cold saline, and opened longitudinally for further analysis.

Assessment of macroscopic and histological damage in colitic rats

The macroscopic damage was scored on a scale from 0 to 5 as previously described.⁶ Scoring of macroscopic colon damage in TNBS-induced colitis was as follows: (0) no damage; (1) hyperemia and no ulcer; (2) linear ulcers and no colonic wall thickening; (3) linear ulcers and colonic wall thickening in one area; (4) colonic ulcers in multiple areas; and (5) severe ulceration and perforation.

These tissue samples were processed routinely and embedded in paraffin. Sections were stained with haematoxylin and eosin. The histological damage were observed and scored by two independent researchers blinded to the identity of samples, and graded according to a modified histopathological criteria: (a) infiltration of acute inflammatory cells: (0) none, (1) mild, (2) severe; (b) infiltration of chronic inflammatory cells: (0) none, (1) mild, (2) severe; (c) fibrin deposition: (0) negative, (1) positive; (d) submucosal edema: (0) none, (1) focal, (2) diffuse; (e) necrosis of epithelial cells: (0) none, (1) focal, (2) diffuse; (f) mucosal ulceration: (0) negative, (1) positive.⁷

Myeloperoxidase (MPO) activity assay

Tissue samples were thawed, weighed, and homogenized in 5 mL of 50 mmol/L phosphate buffer (pH 7.4) at 4°C and centrifuged at 15 000 *g* for 10 minutes at 4°C. The deposit was suspended in 3 mL 0.5% hexadecyltrimethylammonium bromide in 50 mmol/L phosphate buffer (pH 6.0) at 25°C. The homogenate was then subjected to three cycles of freezing. An aliquot of the homogenate (50 μ L) was added to a 0.5-mL reaction solution containing 80 mmol/L phosphate buffer (pH 5.4), 0.5% hexadecyltrimethylammonium bromide, 1.6 mmol/L tetramethylbenzidine, and 0.3 mmol/L H₂O₂ to spectrophotometrically assay MPO activity at 655 nm (UV-6100PCS, Shanghai Lengguang Technology Co. Ltd., China). One unit of MPO activity was defined as that degrading 1 μ mol peroxide in one minute at 25°C. Results were expressed as U per g protein. Total protein was determined using Bradford method. MPO activity kit was obtained from Nanjing Jiancheng Biological Engineering Institute (Nanjing, China).

Transmission electron microscopy

A short segment of the proximal colon was removed and fixed with 3% glutaraldehyde and 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.3) for 2 hours at room temperature. After being rinsed in the same buffer, the specimens were fixed in 1% osmium tetroxide for 2 hours at 4°C. The specimens were then rinsed in distilled water, block-stained with uranyl acetate solution for 3 hours, dehydrated in a graded series of ethanol, and embedded in epoxy resin. Ultrathin sections were cut with a Reichert microtome, double-stained with uranyl acetate and lead citrate, and observed with a Philips Tecnai-12 electron microscope.

Immunohistochemistry

Colon specimens were fixed in 10% neutral buffered formalin solution and embedded in paraffin. Sections (5 μ m thickness) were incubated with a rabbit derived antibody against c-kit (CD117, 1:100 dilution, Santa Cruz Biotechnology Inc., CA, USA) for 16 hours at 4°C and biotinylated anti-rabbit polyclonal IgG (1:1000 dilution, Wuhan Boster Bio-engineering Co., Ltd., Wuhan, China) for 15 minutes at room temperature. The reaction products were visualized by diaminobenzidine (DAB) solution (Wuhan Boster Bio-engineering Co.Ltd.,Wuhan, China) sequentially. Nuclear staining was done with hematoxylin. The percentage of labeled cells was determined, according to the following equation: number of labeled cells/total counted cells $\times$ 100%. The intensity of immunostaining was rated as 0-4: over 75%, 4+; 50%–75%, 3+; 25%–50%, 2+; and below 25%, 1+.

Western blot

A 1-2 cm segment was isolated from a region of the inflamed colon and homogenized. And the tissue protein was extracted according to the manufacturer's instructions (Wuhan Boster Bio-engineering Co. Ltd.). Equal amount of proteins (40 μ g) was loaded onto 12% SDS-PAGE and separated by electrophoresed for each sample. Then proteins were transferred to polyvinylidene difluoride (PVDF) membrane. Nonspecific binding sites in the membranes were blocked with 5% (w/v) nonfat dry milk in phosphate-buffered saline (PBS, pH7.4). PVDF membrane was incubated with rabbit anti-c-kit polyclonal antibody (1:200 dilution, Santa Cruz Biotechnology Inc.) for 16 hours at 4°C. Following incubation, membranes were washed with PBS containing 0.01% Tween 20, then exposed to a horseradish-peroxidase-conjugated goat antiserum against rabbit IgG diluted in 1:600 (Wuhan Boster Bio-engineering Co., Ltd.,). Immunoreactive bands were detected by chemiluminescence. Relative densities of the bands were quantitated using the Quantity One 4.3.1 imaging system (Bio-Rad, Shanghai, China).

Statistical analysis

Parametric data were presented as $\bar{x} \pm s$ and statistical analyses were performed using two-way analysis of variance (ANOVA) or *t*-test with the software package SPSS 11.5. Results were considered significant at a *P* value less than 0.05.

RESULTS

General conditions

All of the TNBS-induced colitis rats developed diarrhea. One rat died in model I group and low-dose SQR group respectively. Colon adhesion was found when we anatomized them, so we excluded them from analysis.

Colonic damage and MPO activity

Macroscopic and histological examination of the colon revealed that the inflammation score of model I group was significantly increased compared with the normal control group (*P*<0.05). Treatment with high-dose SQR, low-dose SQR or mesalazine resulted in a significant decrease in severity of macroscopic and histological injury compared with model II group (all *P*<0.05, Table 1).

MPO activity of colonic tissues was significantly higher in normal control group than that in model I group (*P*<0.05). Compared with model II group, the MPO activity was significantly decreased in high-dose SQR group, low-dose SQR group, and mesalazine group (all *P*<0.05, Table 1).

Ultrastructure of ICC

In normal control group, ICC was spindle-shaped with a huge ovate nucleolus, caveolae, and numerous free ribosomes. A mass of mitochondria, smooth and rough endoplasmic reticulum were present in the cytoplasm. ICC located around the nerve fibers, and contacted with neighboring smooth muscle cells by such as intermediate junctions. In model I and model II groups, the junctions between ICC and other cells were absent; and the structure of ICC was unclear with endoplasmic reticulum dilated and threshed, mitochondrial swollen and vacuolated, vacuole formed in cytoplasm, and concave karyotheca widen. In mesalazine group, the configuration of ICC was almost normal. The mitochondria slightly swelled and the ridge of mitochondria existed. The rough endoplasmic reticulum lesion was not obvious. In high-dose SQR group and low-dose SQR group, the configuration of ICC was normal with intact connections between cells, and the ridge of mitochondria existed (Fig. 1).

C-kit expression in colonic tissue

Immunohistochemistry showed the strongest positive staining of c-kit expression was brown or tan, normally located in submucosal border, myenteric plexus and circular muscle layers in normal control group. C-kit expression was decreased in model I group than that in normal group (1.23 ± 0.17 vs. 3.65 ± 0.21 , $P<0.05$). C-kit expression was increased in high-dose SQR (2.97 ± 0.30), low-dose SQR (2.21 ± 0.19) and mesalazine group (2.35 ± 0.25) compared with model II group (1.86 ± 0.12 , all $P<0.05$) (Fig. 2).

Western blot analysis indicated c-kit expression in the colonic tissues was significantly higher in normal control group than that in model I group (1.19 ± 0.15 vs. 0.40 ± 0.03 , $P<0.05$). Compared with model II group (0.49 ± 0.05), c-kit expression was significantly increased in high-dose SQR (0.99 ± 0.11), low-dose SQR (0.70 ± 0.10), and mesalazine groups (0.73 ± 0.07 , all $P<0.05$)(Fig. 3).

Table 1. Scoring of macroscopic and histological damage and MPO levels measured in TNBS-treated rats after administration of mesalazine or SQR[§]

Group	n	Macroscopic damage score	Histological damage score	MPO level (U/g protein)
Normal control group	10	0	0	2.78±0.26
Model I group	9	8.28±0.15 [#]	6.89±0.13 [#]	0.56±0.20 [#]
Model II group	10	7.36±0.27	4.45±0.20	2.03±0.33
Low-dose SQR group	9	2.56±0.15 [*]	3.25±0.11 [*]	1.57±0.27 [*]
High-dose SQR group	10	3.21±0.19 [*]	3.14±0.22 [*]	1.03±0.23 [*]
Mesalazine group	10	3.18±0.10 [*]	3.18±0.18 [*]	1.59±0.12 [*]

§: Plus-minus values are means±SD.
MPO: myeloperoxidase; TNBS: trinitrobenzene sulfonic acid; SQR: Shenqing Recipe.
^{*} $P<0.05$ compared with model II group; [#] $P<0.05$ compared with normal control group.

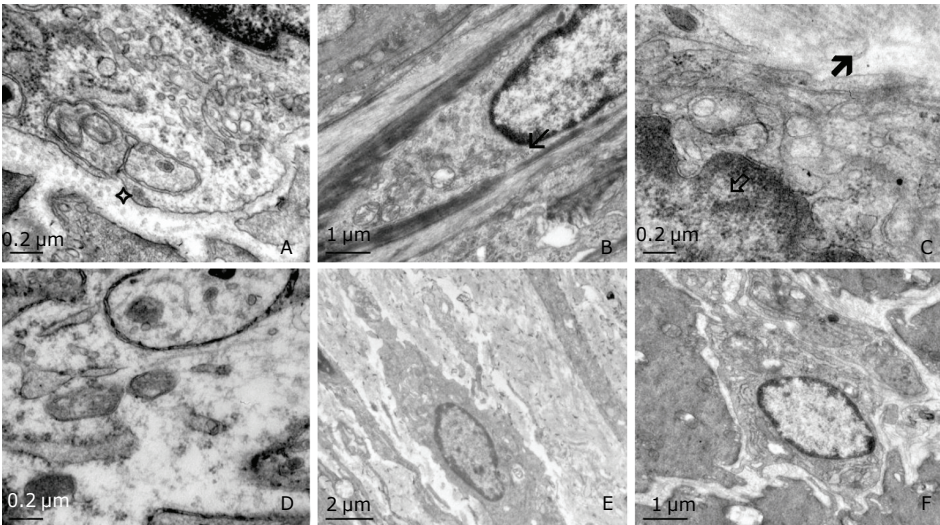


Figure 1. Transmission electron micrographs of interstitial cells of cajal (ICC) in the region of rat proximal colon. A. normal control group (×43 000); B. model I group (×43 000); C. model II group (×43 000); D. mesalazine group (×43 000); E. low-dose SQR group (×6000); F. high-dose SQR group (×11 500).
◆ shows a huge ovate nucleolus and concave karyotheca; ⚡ shows nuclear atrophy and widened concave in karyotheca; ⚡ shows vacuole formed in cytoplasm; ⚡ shows swollen and vacuolated mitochondria.

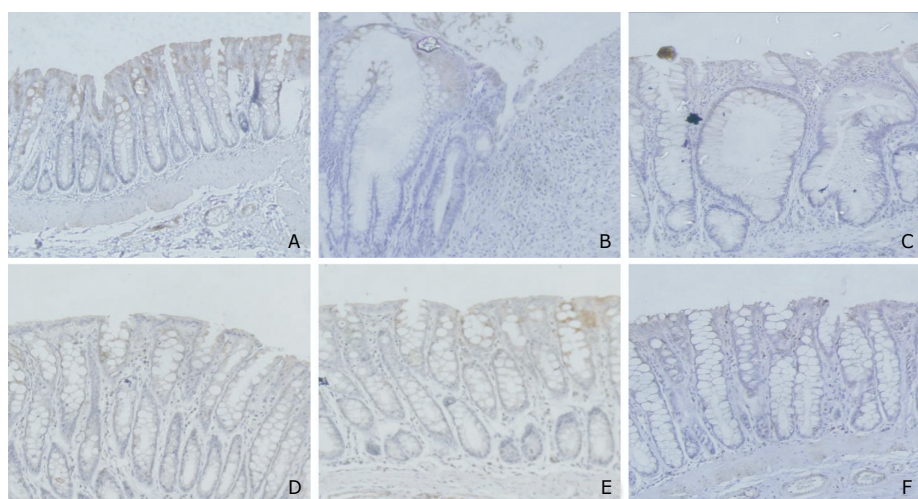


Figure 2. Immunohistochemical pictures of c-kit expression in the rat colon. DAB $\times 200$

A. normal control group; B. model I group; C. model II group; D. mesalazine group; E. low-dose SQR group; F. high-dose SQR group.



Figure 3. Result of Western blot analysis of c-kit expression in the rat colon.

A. normal control group; B. model I group; C. model II group; D. mesalazine group; E. low-dose SQR group; F. high-dose SQR group.

DISCUSSION

MPO abundantly presents in neutrophil granulocytes. It is a lysosomal protein stored in azurophilic granules of the neutrophil.⁸ It could serve as a sensitive predictor for inflammation in TNBS-induced murine colitis.⁹ In our study, for all TNBS-treated rats, colonic MPO activity was significantly increased compared with that in normal control group. After treatment with SQR, colonic MPO activity was significantly decreased compared with that in model II group. It suggested that SQR had anti-inflammatory effects.

ICC are a population of cells in the gastrointestinal tract that play an important role in controlling intestinal motility. They act as intestinal pacemakers, enteric neural intermediaries and spatial coordinators of smooth muscle activity.^{10,11} It was recognized that ICC express the gene product of c-kit, a proto-oncogene that encodes the receptor of tyrosine kinase. C-kit plays important roles in development and maintenance of the ICC network.² Kinoshita *et al*¹² found ICC network disappeared from the

inflamed region of model rats with TNBS-induced colitis, thereby ICC might play an important role in inducing intestinal dysmotility. So c-kit expression level could indirectly reflect the quantity of ICC. In this study, we found ultrastructure of colonic ICC was markedly injured, and the expression of c-kit protein was significantly decreased in TNBS-induced colitis rat model compared with the normal control group. SQR could improve the ultrastructure of ICC and increase the expression of c-kit protein, especially high-dose SQR. Patients with UC often experience symptoms such as bloody diarrhea, cramps, and tenesmus, which relate to intestinal dysmotility.¹³ SQR can alleviate the severity of ICC damage, repair the injured ICC, and resume its quantity, which might be involved in regulation of intestinal motility.

Mesalazine is effective for maintenance and induction of remission of UC, and maintenance therapy is of particular value in patients with UC.¹⁴ So in this study, mesalazine was as experiment parameter for SQR. But mesalazine has dose-dependent adverse effects,¹⁵ such as hypersensitivity rash, photosensitivity, and idiosyncratic reactions including fever, rash, agranulocytosis, hemolytic anemia, hepatitis, pancreatitis and pneumonitis, and male infertility. While Traditional Chinese Medicine may serve as a valid and safe alternative therapy with low side effects and low price in treatment of UC.^{16, 17}

The present study suggests that the ultrastructure of colonic ICC is injured with the quantity of ICC reduced secondarily in TNBS-induced rat colitis. SQR can alleviate and repair the injured ICC, and resume its quantity, which might be involved in regulating intestinal motility.

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