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Therapeutic Efficacy of the Traditional Chinese Medicine Baishaoqiwu on TNBS-induced Colitis is Associated with Down-regulation of the TLR4/MyD88/NF-κB Signaling Pathway

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Abstract. *Background/Aim: The traditional Chinese medicine Baishaoqiwu (BSQW) has been previously used to clinically treat inflammatory bowel diseases. However, the mechanisms of it's therapeutic efficacy remain unclear. The aim of this study was to examine the efficacy of BSQW on ulcerative colitis (UC) and the TLR4/MyD88/NF-κB signaling pathway in a rat model of colitis. Materials and Methods: The colitis rat model was induced by anal instillation of 2,4,6-trinitrobenzene sulfonic acid (TNBS). The animals with induced colitis were treated with BSQW at a dose of 13.2 mg/kg daily, p.o. Mesalazin was used as a positive control and was given to the animals with induced colitis at a dose of 420 mg/kg daily, p.o. The untreated animals with induced colitis and normal animals served as model controls and normal controls, respectively. Macroscopic and histological assessments were performed after treatment. The expression of MyD88, NF-κB P65 and TLR4 were determined by immunohistochemical analysis. Results: Administration of BSQW or Mesalazin ameliorated TNBS-induced macroscopic and histological damage in the rats with induced colitis. The macroscopic score and total colitis index were significantly reduced in the BSQW- and Mesalazin-treated groups compared to the model control group ($p<0.05$). BSQW or Mesalazin significantly inhibited TNBS-induced expression of the TLR4, MyD88 and NF-κB P65 genes. No treatment-related toxicity was found in either the BSQW- or the Mesalazin-treated groups. Conclusion: Suppression of the TLR4/MyD88/NF-κB signaling pathway may be one of the mechanisms involved in the therapeutic efficacy of BSQW against UC.*

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Ulcerative colitis (UC) is an inflammatory bowel disease (IBD) and is associated with chronically-relapsing disorders of the gastrointestinal tract (1-3). Histologically, it is characterized by the presence in the gut of extensive areas of ulceration, pronounced infiltration of neutrophils and epithelial-cell necrosis. The incidence and prevalence of IBD are now increasing in China with westernization of lifestyle and industrialization (4). UC has been treated with 5-aminosalicylic acid derivatives, corticosteroids and immunosuppressants, such as azathioprine and cyclosporine. Immunomodulators are used in refractory or steroid-dependent UC patients. In addition, biological agents have shown efficacy in moderate to severe UC (1). Although many drugs have been used to treat UC, the adverse effects of first-line drugs limit their use. Therefore, the challenge remains to develop novel and specific therapies for IBD (5).

Natural products, such as those found in traditional Chinese medicine (TCM), have received much attention due to their anti-inflammatory potential (6, 7). Baishaoqiwu (BSQW) is a well-known TCM formula consisting of a combination of 7 herbs, including radix paeoniae alba, *Coptis*, scutellaria baicalensis,

cortex phellodendri, radix aucklandiae, angelica sinensis and pericarpium arecae (8). BSQW has shown anti-inflammatory and anti-oxidative effects, immunomodulation, improvement of the microcirculation and wound healing, thereby ameliorating clinical symptoms of UC (9-12). BSQW, therefore, has been used in China to clinically treat UC (6). It was reported that BSQW reduced platelet and serum nitric oxide in rats with UC (13). However, the mechanisms of the therapeutic effects of BSQW against UC remain largely unclear.

Recent studies have shown that the TLRs/MyD88/NF-κB signal-transducing pathway plays an important part in UC (14-16). In the present study, we evaluated the therapeutic effects of BSQW on an established rat model of colitis induced by 2,4,6-trinitrobenzene sulfonic acid (TNBS) and determined the expression of signal transduction proteins (TLR4, MyD88 and NF-κB p65) in the colon tissue before and after treatment.

Materials and Methods

Animals. Male Sprague-Dawley (SD) rats (weighing 200-220 g) were purchased from the Animal Department of the College of Medicine, Yangzhou University [certificate No. SCXK(Su) 20120004)]. All rats were maintained in a HEPA-filtered environment at 24-25°C and humidity was maintained at 50-60%. All animals were fed with autoclaved laboratory rodent diet. All animal experiments were approved by the Animal Committee of Nanjing Origin Biosciences, China.

Induction of colitis. All animals were allowed to acclimate for 1 week and fasted for 24 h prior to induction of colitis, with free access to water. Rats were anaesthetized with 10% chloral hydrate (3 ml/kg) *via* intraperitoneal injection. Rats were fixed in a supine position, and a rubber tube (diameter 2.0 mm, length 15 cm), lubricated with paraffin oil, was inserted 8 cm proximal to the anus. Then 2,4,6-trinitrobenzene sulfonic acid (TNBS, 100 mg/kg) and absolute ethyl alcohol 1:1) mixture were slowly instilled into the rat *via* the rubber tube. The normal control animals were injected with saline. After keeping the rats in an inverted head-down-hip-high position for about 2 min in order to prevent anal leakage of TNBS, rats were allowed to lie and recover from anethesia with free access to food and water.

Treatment. All animals were randomly assigned to 4 groups of 8 mice each, 12 days after TNBS instillation. Normal control animals were assigned to Group 1 and received distilled water. The animals with induced colitis were assigned to Groups 2-4. Group 2 served as the model control and received distilled water. Group 3 served as the positive control and received Mesalazin at a dose of 420 mg/kg daily. Group 4 received BSQW granules at a dose of 13.2 g/kg daily. All treatments were carried out *via* oral gavage and continued daily for 14 days. Animal body weights and clinical signs were recorded daily during the experiments.

Macroscopic assessment. All animals were sacrificed 14 days after treatment initiation. The distal colon was removed and opened longitudinally. Afterwards, the colonic segment was cleaned of fecal content, fat and mesentery and then processed for assessment by macroscopic and histological techniques. Macroscopic colonic damage was assessed using a magnifying glass by an independent observer and was scored. The scale for macroscopic damage ranged from 0-10 and was based on the appearance of ulceration, thickening of the bowel wall, sites of ulceration and sites of inflammation (17). One segment in the colon was then collected and fixed in 10% formalin for histological and immunohistochemical analysis.

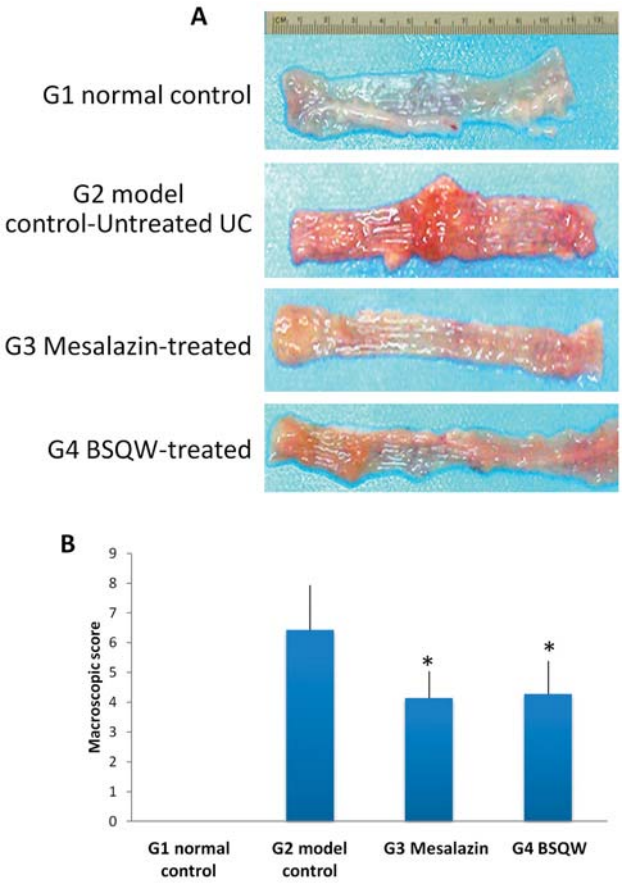

Figure 1. *BSQW reduces colonic macroscopic damage in rats with TNBS-induced colitis. A: Representative images of macroscopic intestinal changes. B: Macroscopic pathological scores. Data are represented as mean ± SD of 8 animals of each group. *p<0.05, when compared to the model-control group. BSQW: Baishaoqiwu. TNBS: 2,4,6-trinitrobenzene sulfonic acid.*

Histological assessment. For histological examination, the colonic tissue was fixed in 10% formalin, dehydrated, paraffin embedded, processed, sliced into 4 μm-thick sections and stained with haematoxylin and eosin (H&E). Histological damage was evaluated and scored by a pathologist who was blinded to the experimental groups, according to previously-described criteria (18). The total colitis index was then obtained from an evaluation score based on inflammation severity, inflammation extent, and crypt damage visualized in H&E-stained sections.

Immunohistochemical analysis. Paraffin-embedded colonic tissue sections were fixed in 4% paraformaldehyde, de-paraffinized and dehydrated through graded ethanol. The sections were washed three times with PBS for 5 min each, blot dried, and then treated with 3% hydrogen peroxide for 30 min at room temperature to block

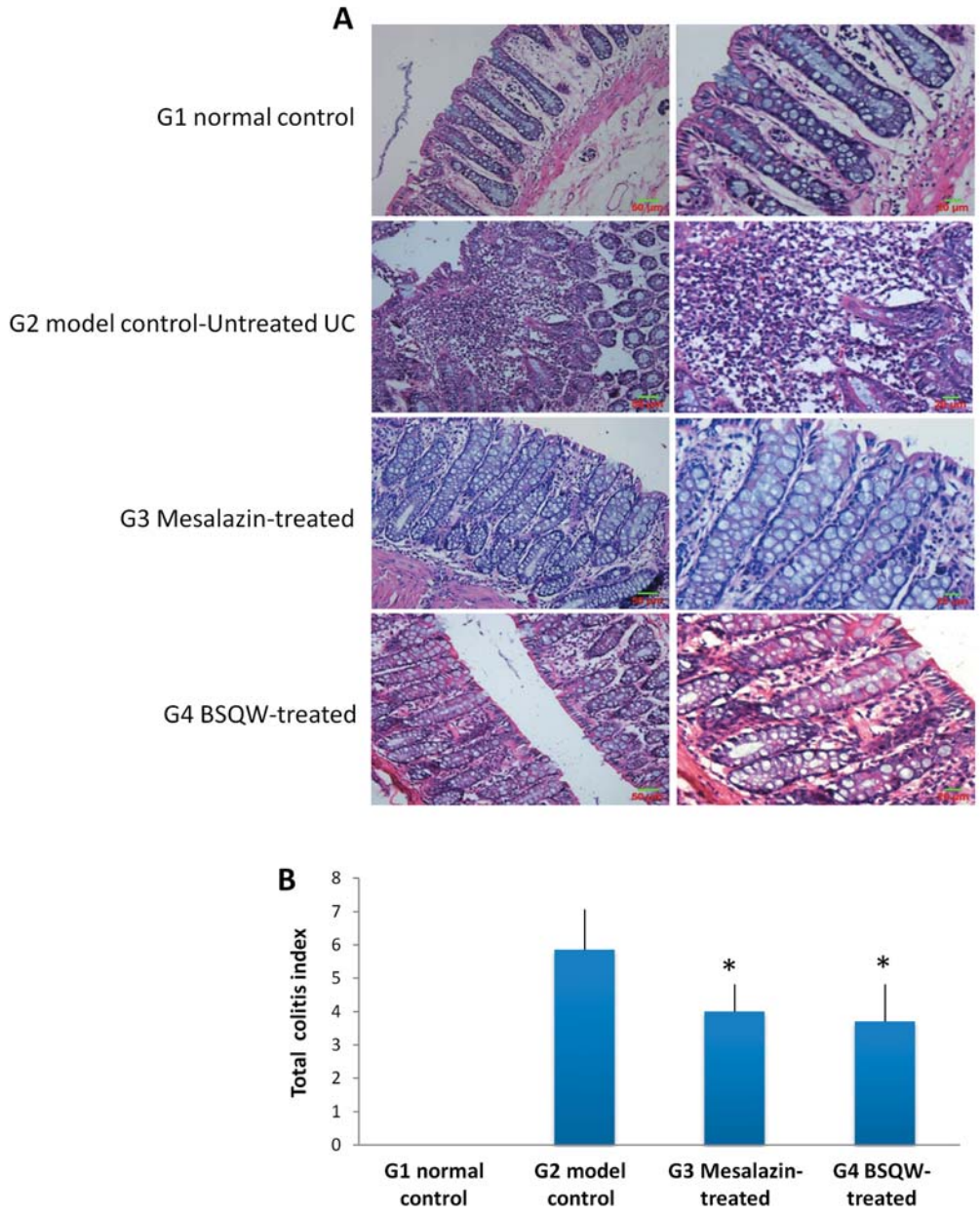

Figure 2. *Effect of BSQW on histological damage in colon tissues in the TNBS-induced colitis rat model. A: Representative histological images of H&E staining of colon tissues (magnification: left panel ×100, right panel ×200). B: Total colitis index of colonic samples of each group. Data are represented as mean ± SD of 8 animals in each group. *p<0.05, when compared to the model group. BSQW: Baishaoqiwu. TNBS: 2,4,6-trinitrobenzene sulfonic acid.*

endogenous peroxidase activity. The sections were immersed in antigen-retrieval solution (citrate buffer, pH 6.0) for 10 min. This was followed by rinsing with PBS. After blocking with normal goat serum for 30 min at 37˚C, sections were co-incubated with primary anti-MyD88, NF-κB p65 and TLR4 antibodies (1:150 dilution in PBS) overnight and then with a peroxidase-conjugated anti-rabbit IgG secondary antibody for 1 h at room temperature. Thereafter, the sections were incubated with 3,3-diaminobenzidine (DAB) reagents for 10 min, counterstained with hematoxylin, dehydrated and mounted for microscopy. The slides were viewed at 400x magnification and positive cells were recognized by the appearance of a brown stain. Expression levels were quantified by the average optical density (AOD) of the positive cells in 5 fields/sample with Image-Pro Plus 6.0 software.

Statistical analysis. Statistical analysis was performed using SPSS16.0 software (SPSS Inc., Chicago, IL, USA). All results are expressed as mean ± SD. Comparisons between two or multiple groups were made with the Student's *t*-test or ANOVA. $p<0.05$ was considered significant.

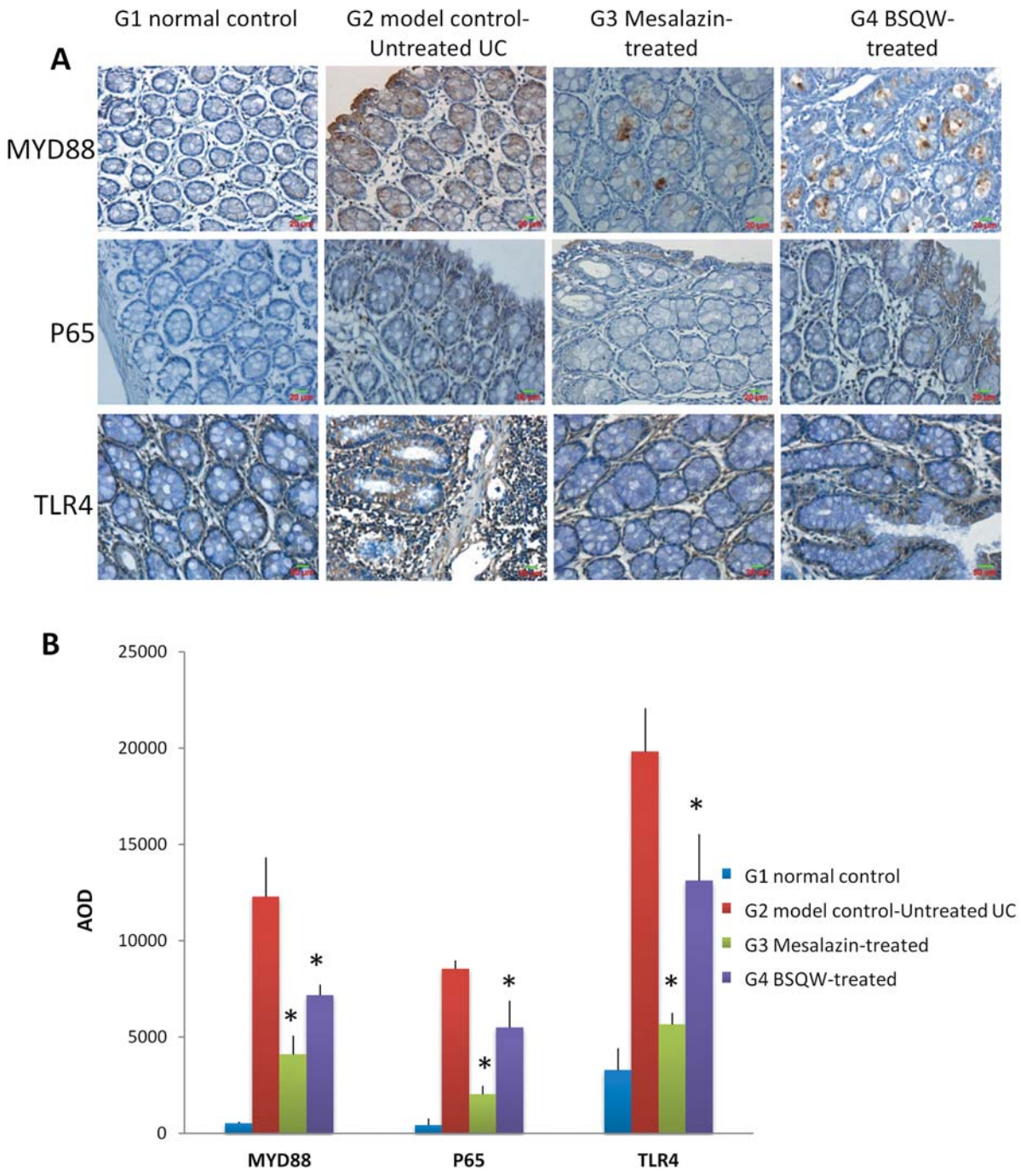

Figure 3. *Effect of BSQW on expression of MyD88, NF-κB P65 and TLR4 in colon tissues in the TNBS -induced colitis rat model. A: Representative images of immunostaining of colon tissues (magnification, 400x). B: AOD of immunostaining samples of colon tissues. Data are mean ± SD of 8 animals in each group. *p<0.05, when compared to the model group. BSQW: Baishaoqiwu. TNBS: 2,4,6-trinitrobenzene sulfonic acid. AOD: average optical density.*

Results and Discussion

BSQW reduces colonic macroscopic damage in rats with TNBS-induced colitis. Normal control animals showed no colonic damage and the colonic damage score was zero. Rats in the model control group displayed severe colonic damage with hyperemia, thickening of the bowel, necrosis, inflammation, and a large area of ulceration. The macroscopic

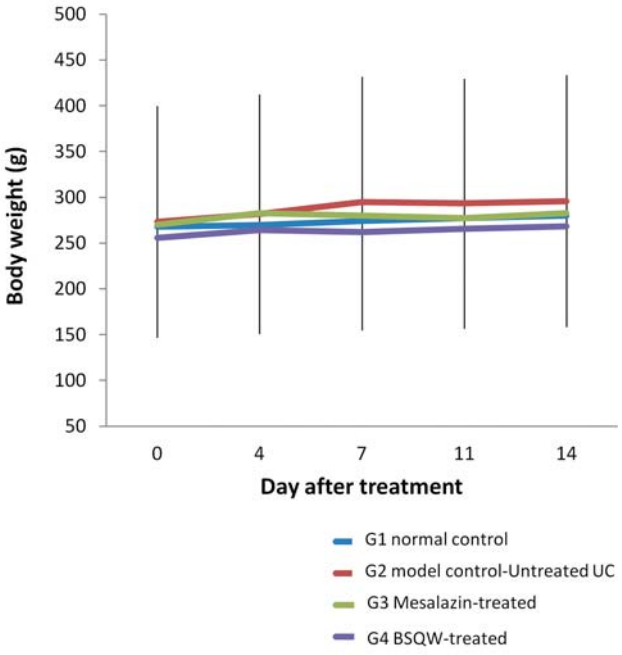

Figure 4. *Effect of BSQW on body weight in the rat TNBS-induced colitis model. No significant body weight loss was found in BSQW- and mesalazine-treated rats.*

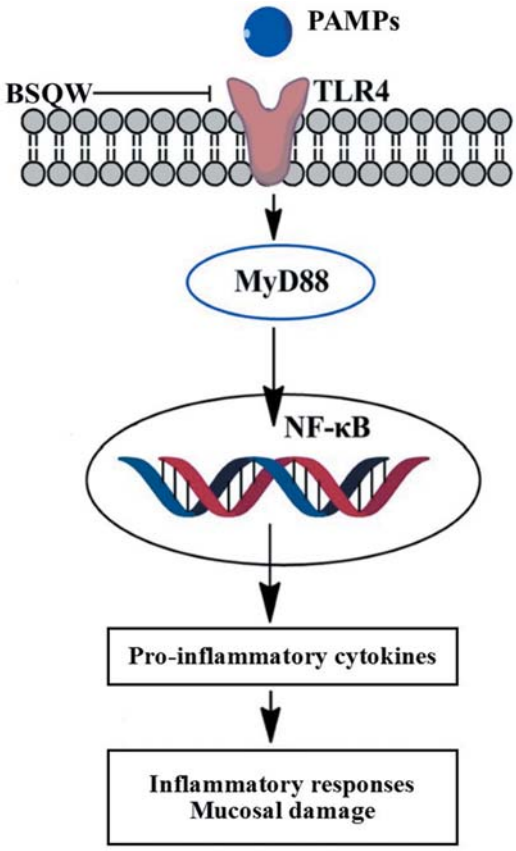

Figure 5. *Schematic diagram of the inhibitory effect of BSQW on the TLR4/MyD88/NF-κB signaling pathway. BSQW may inhibit the expression of TLR4, which in turn, down regulates MyD88 and blocks the activation of NF-κB, leading to the attenuation of inflammatory responses and mucosal damage in TNBS-induced colitis. PAMPs: Pathogen-associated molecular patterns.*

colon damage score was significantly increased. The severity of colonic destruction was markedly ameliorated after Mesalazin or BSQW treatment, with reduced areas of inflammation and ulceration. The macroscopic colon damage score was significantly reduced (Figure 1A and B).

BSQW improves TNBS-induced histopathological changes. There was no histopathological change in the colons of control rats. Histologic evaluation of the rats with TNBS-induced colitis showed transmural inflammation involving all layers of the bowel. The inflammatory process was associated with patchy ulceration, epithelial cell loss, pronounced depletion of goblet cells, distortion of the tubular glands, numerous inflammatory cell infiltration, and dilated crypts. These histologic signs were much improved in the rats treated with Mesalazin or BSQW. Mesalazin- and BSQW-treated groups showed significant reduction of inflammatory infiltration and transmural lesions and less goblet-cell depletion compared with the model control group (Figure 2A). The total colitis index score was significantly reduced in Mesalazin- and BSQW-treated groups compared with the model control group (Figure 2B).

BSQW suppresses the activation of the TLR4/MyD88/NF-κB signaling pathway in TNBS-induced colitis. To elucidate the mechanisms of therapeutic efficacy of BSQW against UC, we determined its effects on the activation of the TLR4/MyD88/NF-κB pathway in colon tissues of mice with UC. As shown in Figure 3A and B, the expression of MyD88, NF-κB p65 and TLR4 was significantly increased in the TNBS-induced UC model group compared with the normal control group. However, these expression levels were significantly reduced following treatment with Mesalazin and BSQW compared to the model control group.

The TLR4/MyD88/NF-κB signaling pathway is one of the major pathways mediating inflammatory responses. NF-κB is a critical signaling molecule in the inflammatory process, which facilitates the expression and secretion of pro-inflammatory cytokines, and then leads to a series of inflammatory responses and mucosal damage (19-21). MyD88 signal is an upstream signal molecular of the NF-κB signaling pathway. It has been shown that inhibiting the activation of NF-κB, by blocking the MyD88 signal, will

reduce the release of proinflammatory cytokines, alleviate the inflammatory response and achieve a therapeutic effect (22). TLR4 is activated by recognizing pathogen-associated molecular patterns (PAMPs) in bacteria, which in turn triggers signaling cascades leading to the activation of NF-κB (23). Thus, the TLR4/MyD88/NF-κB pathway has become a major target for the treatment of inflammatory diseases, including UC (Figure 5).

Effect of BSQW on body weight and toxicity. Clinical observation and body weight measurement of animals during the study were performed to assess toxicity of Mesalazin and BSQW treatment. No physical or behavioral signs that indicated adverse effects due to either treatment were observed. As shown in Figure 4, a stable body weight in all treated groups indicated no obvious toxicity.

In conclusion, we demonstrated, for the first time, that BSQW prevents the development of UC in vivo through suppression of the TLR4/MyD88/NF-κB signaling pathway. Therefore, the TLR4/MyD88/NF-κB signaling pathway is expected to become a new target for the treatment of UC.

Conflicts of Interest

None of the Authors have a conflict of interest in regard to this study.

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