

Original Article

The effects of Qing Hua Chang Yin on the epithelial tight junctions of mice with inflammatory bowel disease

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Abstract: Intestinal barrier dysfunction is well described in inflammatory bowel disease (IBD) and has been studied as a target for IBD treatment. Qing Hua Chang Yin (QHCY) is a traditional Chinese medicine which has been used in the treatment of inflammatory bowel disease for many years. However, the underlying mechanisms of its therapeutic effects remain largely unknown. In the present study, the protective effects of QHCY on epithelial barrier dysfunction were investigated using a DSS-induced mice colitis model. We found that treatment with QHCY significantly ameliorated the clinical manifestations of colitis including the disease activity index (DAI), colon shortening, and the histological evidence of colitis in DSS-induced acute colitis in mice. The administration of QHCY profoundly reduced the DSS-induced increase of TNF- α levels in both colonic tissue and serum and MPO expression in colonic tissue, along with a decrease in serum SAA levels. Furthermore, QHCY significantly reversed the DSS-induced downregulation of ZO-1, occluding, and claudin-1 inversely and reduced the DSS induced increase in the phosphorylation of Elk-1. Overall, our findings indicate that QHCY regulates the expression of tight junctions in DSS-induced colitis, so this may be one of the mechanisms associated with its therapeutic effects on IBD.

Keywords: Qing Hua Chang Yin, traditional Chinese medicine, inflammatory bowel disease, intestinal barrier, tight junction

Introduction

Inflammatory bowel disease (IBD), which includes ulcerative colitis (UC) and Crohn's disease (CD), is a group of chronic gastrointestinal disorders characterized by intestinal inflammation and mucosal damage [1]. Both the prevalence and incidence has been increasing worldwide [2]. Although the pathogenesis of IBD remains elusive, environmental factors, genetic susceptibility, and immune dysfunction are considered the major contributing factors. Emerging evidence indicates that intestinal barrier dysfunction is also fundamental to its pathogenesis [3, 4]. The epithelial barrier consists of epithelial cells and intercellular junctional complexes [5] including the tight junction (TJ) proteins, namely occludin, claudin-1, interacting with the central plaque protein zona occludens (ZO)-1 [6, 7]. Th-

ese TJ complexes are well recognized as playing a pivotal role in maintaining the epithelial barrier. TJ disruption results in the functional disturbances of the paracellular barrier and an increase in intestinal epithelial paracellular permeability. Irregular and high paracellular permeability increases the intestinal penetration of potential harmful antigens and luminal bacteria, which can act as a trigger for initiating and accelerating the mucosal inflammation in IBD [8-11]. Elk-1 belongs to the ETS domain transcription factor family and the ternary complex factor subfamily and is a direct substrate of ERK1/2 [12, 13]. Serine phosphorylation of Elk-1 (Ser383 and Ser389) is necessary for the transcriptional regulation. ERK1/2 is known to induce the phosphorylation and activation of Elk-1. A previous study has shown that Elk-1 plays a regulatory role in TJ barrier modulation

The effect of Qing Hua Chang Yin on colitis in mice

[14]. Therefore, ameliorating intestinal barrier dysfunction may alleviate the development of inflammation or may accelerate the resolution of intestinal inflammation [10, 15].

Pharmacological intervention is regarded as one of the critical strategies for IBD treatment. For instance, corticosteroids, 5-aminosalicylic acid (5-ASA) derivatives, immune suppressants and immune modulators can relieve mucosal inflammation and IBD-related symptoms [16]. However, the long-term use of these therapies is often limited due to the loss of response in many patients and also the remarkable side effect profile, such as immune suppression [17]. Therefore, it is necessary to find new therapeutic strategies for IBD which are safe and capable of sustaining prolonged clinical remission while improving gut mucosal healing.

Natural products such as baicalin and astragalosides have been used in traditional Chinese medicine (TCM), and these compounds have acquired global attention because of their potent anti-inflammatory role [18-20]. Qing Hua Chang Yin (QHCY), a well-known traditional Chinese formula which is composed of *Coptis chinensis* Franch, *Herba et Gemma Agrimoniae*, *Radix Sanguisorbae*, *Magnolia officinalis*, *Radix Paeoniae Rubra*, *Elettaria cardamomum*, *Semen Coicis*, *Artemisia capillaris Thunb*, *Semen Dolichoris Album*, *Herba Eupaatorii Fortunei*, and *Poria cocos*. In TCM, it is believed that these products synchronously work together which confer QHCY the following functional properties: elimination of heat and dampness, augmentation of the functions of the spleen, and thereby an overall increase in the vitality of life. In the TCM system, accumulation of toxic dampness and heat is one of the critical causative factors in ulcerative colitis (UC) pathogenesis, so clearing heat and dampness is an effective strategy for the treatment of UC. Thus, QHCY has been successfully used in China for the management for UC for many years [21-26].

Our previous studies of the DSS-induced colitis mice model have indicated that QHCY successfully alleviates the clinical manifestations of colitis, such as weight loss, blood in the stool, histological evidence of colitis, and the release of inflammatory cytokines modulating via the TLR4/NF- κ B and IL-6/STAT3 signaling pathways [27-29]. Previous research demonstrated that a defective intestinal barrier plays a fundamental

role in the pathogenesis of IBD, but there is little evidence showing whether QHCY can protect or boost the intestinal epithelial barrier function. In order to further understand the therapeutic mechanism of QHCY, in this present study, we investigated the effects of QHCY on intestinal epithelial barrier function and the expression of the TJ protein in mice with colitis induced by dextran sulfate sodium (DSS).

Materials and methods

Materials and reagents

DSS (molecular weight: 36-50 kDa) was obtained from MP Biochemicals (Solon, OH, USA). The mouse serum amyloid A (SAA) ELISA kit and the protein extraction and BCA assay kit were from Thermo Fisher Scientific (Grand Island, NY, USA). ZO-1 and Elk-1 antibodies were purchased from GeneTex (Irvine, CA, USA), and the phospho-Elk-1 and MPO antibodies were supplied by Abcam (Cambridge, MA, USA). Claudin-1 and occludin antibodies were obtained from Proteintech (Chicago, IL, USA). β -actin antibody and horseradish peroxidase-(HRP) conjugated secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). The mouse TNF- α ELISA kit was provided by BioLegend (San Diego, CA, USA). All the other chemicals were provided by Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted.

Preparation of QHCY

In total, 33 g dehydrated *Coptis chinensis* Franch, 220 g dehydrated *Herba et Gemma Agrimoniae*, 110 g dehydrated *Radix Paeoniae Rubra*, 100 g dehydrated *Radix Sanguisorbae*, 110 g dehydrated *Magnolia officinalis*, 56 g dehydrated *Elettaria cardamomum*, 110 g dehydrated *Herba Eupaatorii Fortunei*, 110 g dehydrated *Artemisia capillaris Thunb*, 110 g dehydrated *Semen Dolichoris Album*, 220 g dehydrated *Semen Coicis* and 220 g dehydrated *Poria cocos* were extracted with boiling water 3 times in 2 L of distilled water. The extracts were then filtered and concentrated by boiling them down to a final volume of 1,000 ml. The stock concentration of QHCY was 1.4 mg/ml.

Establishment of the mouse model of colitis and QHCY treatment

All animal protocols used in this study were approved by the Institutional Animal Care and

The effect of Qing Hua Chang Yin on colitis in mice

Table 1. Disease activity index score

Weight loss (%)	Stool consistency	Rectal bleeding	Score
0	Normal	Normal	0
1-5			1
6-10	Loose	Hemoccult-positive	2
11-15			3
>15	Diarrhea	Gross bleeding	4

The disease activity index was calculated as the sum of the scores for weight loss, stool consistency, and rectal bleeding.

Use Committee of Fujian University of Traditional Chinese Medicine. Fifteen Male BALB/cJ mice (6-8 weeks; 25-28 g) were purchased from the Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and acclimatized for 5 days with ad libitum access to water and food prior to the experiment. The mice were maintained under standardized conditions with 12-h light/dark cycles and 5 animals per cage. After they were acclimatized for 5 days, the mice were randomly divided into three groups (n=5): the control group, the DSS-induced UC group (DSS group), and the DSS-induced UC group treated with QHCY (DSS+QHCY group). Colitis was induced in the mice with 3% DSS (weight to volume ratio dissolved in drinking water) for 7 days. The control mice were given drinking water alone. The test agents were administered via oral gavage once a day for 12 days after the initial DSS administration.

Evaluation of clinical manifestations

The progress of DSS-induced colitis was assessed daily in a blinded fashion. The clinical manifestations noted included an estimation of body weight, the consistency of the stools, and the status of hematochezia in the stool as described by others [30]. The disease activity index (DAI) score was used to evaluate the sum of the weight loss, rectal bleeding and stool consistency scores and is shown in **Table 1**.

Sample collection

After finishing the experiments, the mice were anesthetized with 45 mg/kg of pentobarbital sodium and a blood sample was acquired through the eye socket vein of the mice and left to clot on ice. Serum was acquired by centrifugation at 5,000 rpm for 5 min in 4°C and stored at -80°C until analysis. The colons, from the cecum to the anus, were separated, and we measured the lengths with a ruler. Next, an

approximately 5 mm section of each distal colon was fixed in 10% (v/v%) formalin solution and given a histological examination. The remaining ones were stored at -80°C for other analyses.

Histopathological evaluation

The colon tissues were fixed and processed in paraffin. Next, the colon tissues 4 µm thick were stained with hematoxylin and eosin (H&E). The histologic score was examined with the sum of the inflammation severity, inflammation extent, and crypt damage divided by the percentage involvement following the scoring system shown in **Table 2**. The histopathological score was examined in a blinded fashion according to the criteria described by Sann et al. [31].

Measuring the level of TNF-α and SAA by ELISA assay

Prior to the ELISA evaluation, the colon tissues were homogenized in an ice-cold RIPA lysis buffer with 1% protease inhibitor cocktail and 1% phosphatase inhibitor and centrifuged at 14,000 rpm for 15 min in 4°C. The supernatant was transferred to 1.5 ml tubes for the ELISA analysis. The levels of TNF-α and SAA in the serum and colon tissue were evaluated with the commercially available ELISA kits of TNF-α and SAA in accordance with the manufacturer's protocol, respectively. The absorbance was measured at 570 nm using an ELISA reader (Bio-Tek, Model ELX800, USA). All samples were assessed in triplicate.

RNA extraction and qPCR analysis

The total RNA from the colon samples was isolated with a TRIzol reagent in accordance with the manufacturer's instructions. The reverse transcription of mRNA was processed with a PrimeScript RT reagent kit according to the manufacturer's protocol. The obtained cDNA was performed to examine the expression of ZO-1, occludin and claudin-1 by qPCR with SYBR green dye using the ABI7500 fast sequence detection system. GAPDH was used as an internal control. The primer sequences used in the qPCR are listed in **Table 3**.

Western blot analysis

Colon tissues were selected randomly from each group, homogenized in the T-PER lysis

The effect of Qing Hua Chang Yin on colitis in mice

Table 2. The histologic scores

Inflammation Severity	Inflammation extent	Crypt damage	Percent involvement	Score
None	None	None	0	0
Mild	Mucosa	Basal 1/3 damage	1-25%	1
Moderate	Submucosa	Basal 2/3 damage	26-50%	2
Severe	Muscle layer	Crypt lost; surface epithelium present	51-75%	3
	Transmural	Crypt and surface epithelium lost	76-100%	4

Table 3. List of primers used in this study

Gene	Primer sequence	Primer length (bp)
ZO-1	Forward 5'-CCTGTGAAGCGTCACTGTGT-3'	20
	Reverse 5'-CGCGGAGAGACAAGATGT-3'	20
Occludin	Forward 5'-CATAGTCAGATGGGGGTGGA-3'	20
	Reverse 5'-ATTATGATGAACAGCCCC-3'	20
Claudin-1	Forward 5'-ATGCCAATTACCATCAAGGC-3'	20
	Reverse 5'-AGCACCGGGCAGATACAGT-3'	19
GAPDH	Forward 5'-TTGATGGCAACAATCTCCAC-3'	20
	Reverse 5'-CGTCCCGTAGACAAAATGGT-3'	20

buffer solution with 1% phosphatase inhibitor and 1% protease inhibitor cocktail, and then centrifuged at 14,000 rpm for 15 min in 4°C. The supernatant was collected and the concentrations were performed with the BCA protein assay kit. Equal amounts of protein from each sample were resolved on 12% Tris-glycine gels and transferred onto a nitrocellulose membrane with semi-dry transfer at 25V for 7 minutes. The membranes were blocked for 1 h with SuperBlock buffer (ThermoFisher Scientific, NY, USA) and incubated with the indicated primary antibodies against, MPO (rabbit, monoclonal, 1:1,000, Abcam, ab208670), ZO-1 (rabbit, polyclonal, 1:1,000, Genetex, GTX108613), occludin (rabbit, polyclonal, 1:1,000, proteintech, 13409-1-AP), claudin-1 (rabbit, polyclonal, 1:1,000, proteintech, 13050-1-AP), p-Elk-1 (rabbit, polyclonal, 1:1,000, Abcam, ab218133), Elk-1 (rabbit, polyclonal, 1:1,000, Genetex, GTX-101263) and β -actin (rabbit, polyclonal, 1:2,000, CST, 4967) at 4°C overnight and then with appropriate anti-rabbit IgG, HRP-linked antibody (1:5,000, CST, 7074) followed by enhanced chemiluminescence detection.

Statistical analysis

Data were performed using the SPSS 22.0 software (Chicago, IL, USA). The data was analyzed using the Student's *t*-test and one-way ANOVA,

followed by LSD's test or Dunnett's test. All measurements in this study are expressed as the means \pm SD. *P*-values <0.05 indicates statistically significant differences.

Results

QHCY ameliorates the clinical symptoms of DSS-induced acute colitis in mice

To investigate whether QHCY has a beneficial effect on the progress of colitis, mice were treated with 3% DSS to induce acute colitis and then treated with or without QHCY. The clinical symptoms, including body weight changes, the consistency of the stool, and the status of rectal bleeding, were evaluated and the DAI were calculated. Compared with the control group, the mice treated with DSS showed body weight loss on day 11, and the administration of QHCY significantly improved the body weight as compared with DSS group (100.602 \pm 2.733, 78.686 \pm 7.701 and 92.337 \pm 2.646%, respectively, **Figure 1A**). Consistent with these findings, QHCY could significantly reduce the DAIs as compared with the DSS group (0.400 \pm 0.548, 9.600 \pm 1.673 and 4.400 \pm 1.517, respectively **Figure 1B**). Meanwhile, QHCY could profoundly neutralize the colon length shortening of DSS-induced mice (8.033 \pm 0.551, 6.275 \pm 0.629 and 7.240 \pm 0.623 cm, respectively **Figure 2**). These results are similar with those of our previous studies [29] and further confirm that QHCY has therapeutic efficacy against UC.

QHCY ameliorates colon histological injury of DSS-induced acute colitis in mice

The histological and morphological characteristics of the colonic mucosa were evaluated by H&E staining. The colons of the control group showed intact epithelium, well defined gland

The effect of Qing Hua Chang Yin on colitis in mice

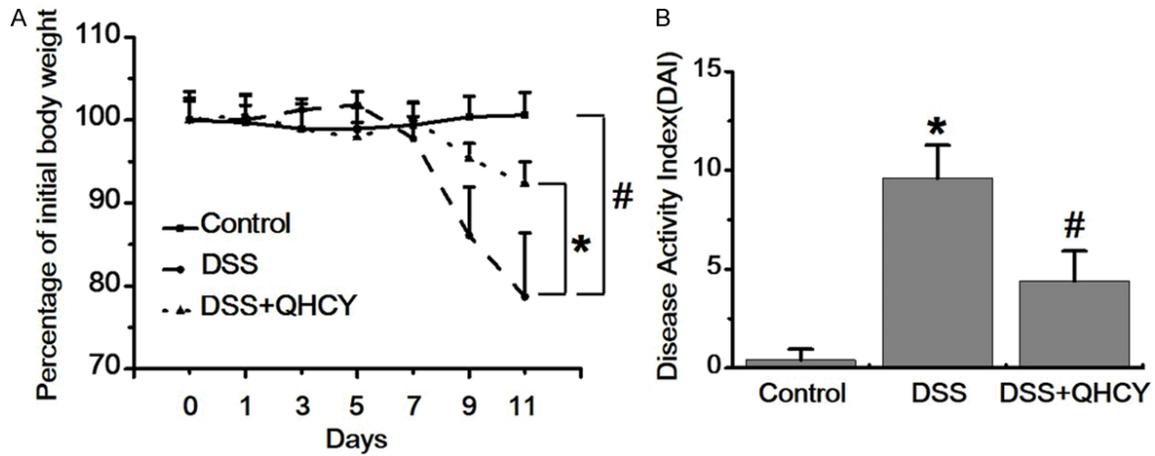


Figure 1. The effect of Qing Hua Chang Yin (QHCY) on the clinical manifestations in the dextran sulfate sodium (DSS)-induced ulcerative colitis mouse model. Colitis was induced by the administration of DSS. On the first day of model construction, the animals received an intragastric administration of saline or QHCY daily for 12 days. A. Body weight was measured daily and expressed as the body weight change (%) for 2 days. B. The progression of DSS-induced colitis was monitored daily in a blinded manner, including the observation of changes in body weight, stool consistency and rectal bleeding. The disease activity index (DAI) was calculated as described. The data shown are the means \pm SD (error bars) from 5 individual mice/group. * $P < 0.05$, vs. control group; # $P < 0.05$, vs. DSS group.

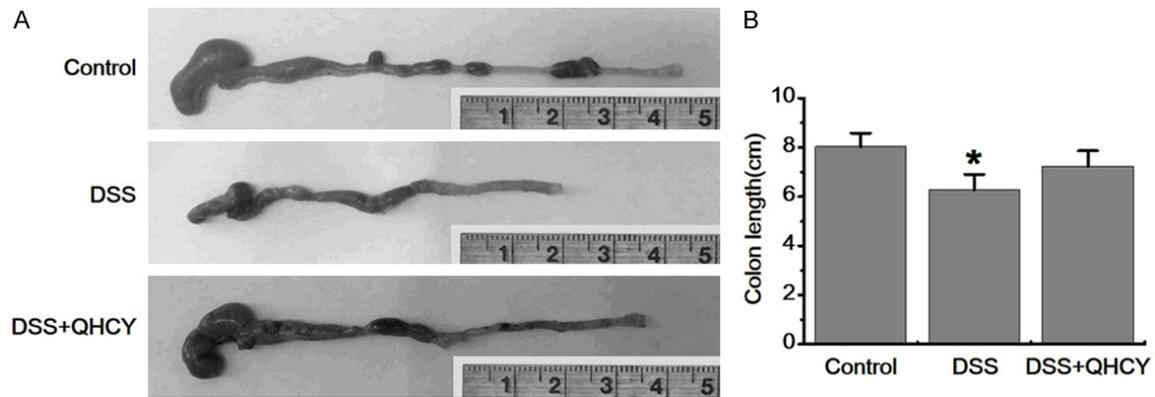


Figure 2. The effect of Qing Hua Chang Yin (QHCY) on colon length in the dextran sulfate sodium (DSS)-induced ulcerative colitis mouse model. At the end of the experiments, the animals were anesthetized. The colons were excised from from the cecum to the anus. A. Colon length was measured as an indirect marker of inflammation. B. The macroscopic appearance of colons. Data shown are the means \pm SD (error bars) from 5 individual mice/group. * $P < 0.05$, vs. control group; # $P < 0.05$, vs. DSS group.

lengths, and without leukocyte infiltrated substantial goblet cells. However, the colons of DSS group demonstrated serious ulcers in the colon membranes, crypt distortion, the infiltration of inflammatory cells, and a hyperplastic epithelium, resulting in a high histological score. Compared with the DSS group, QHCY ameliorated the mucosal ulceration and reduced the neutrophil cell infiltration, causing a low histological score (9.200 ± 2.049 vs. 5.200 ± 0.837 , respectively; **Figure 3**). These results were consistent with our previous reports [29]

further indicating that QHCY could ameliorate tissue injury induced by DSS.

QHCY reduces the levels of TNF- α , SAA and MPO in DSS-induced UC mice

Inflammatory cytokines including TNF- α and SAA are important mediators of the mucosal inflammation during UC [32]. We therefore investigated the levels of TNF- α in the serum and colon tissue, and the levels of SAA in serum using an ELISA assay. As shown in **Figure 4**, the

The effect of Qing Hua Chang Yin on colitis in mice

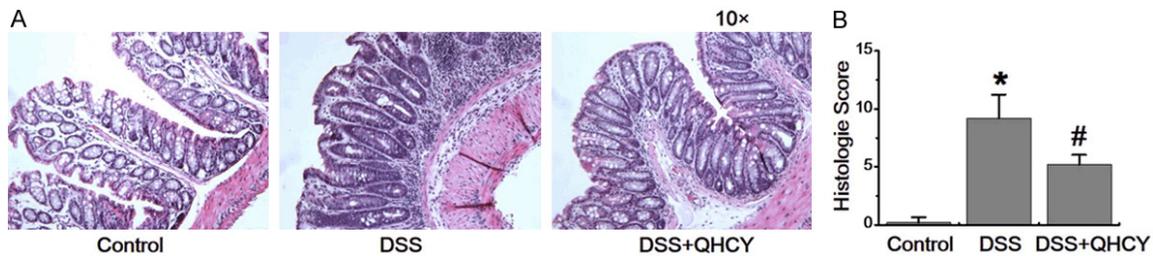


Figure 3. The effect of Qing Hua Chang Yin (QHCY) on histological damages in colon tissues in the dextran sulfate sodium (DSS)-induced ulcerative colitis mouse model. The colon tissues were stained with hematoxylin and eosin. A. Histopathological changes were observed under a microscope. B. Histopathological scores of colon tissues. Representative images are shown (magnification, $\times 100$). Data shown are the means \pm SD (error bars) from 5 individual mice/group. * $P < 0.05$, vs. control group; # $P < 0.05$, vs. DSS group.

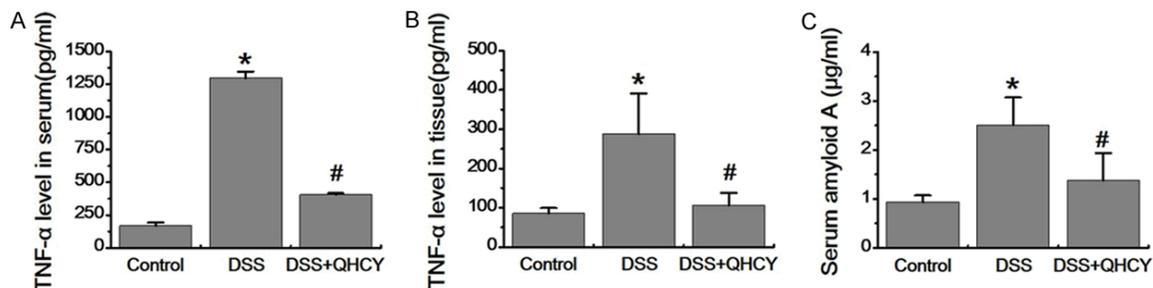


Figure 4. The effect of Qing Hua Chang Yin (QHCY) on the level of TNF- α in serum and colon tissue and the level of serum amyloid A (SAA) in the dextran sulfate sodium (DSS) induced ulcerative colitis mouse model. The levels of TNF- α in serum (A) and colon tissue (B) and SAA in sera (C) were determined by ELISA. Data shown are the means \pm SD (error bars) from 5 individual mice/group. * $P < 0.05$, vs. control group; # $P < 0.05$, vs. DSS group.

DSS-induced mice showed a dramatic increase in the TNF- α levels in both the serum and colon tissue, which were significantly reduced after treatment with QHCY. Similarly, QHCY also profoundly reduced the high serum level of SAA in the DSS-induced mice. We next measured the effect of QHCY on the expression of MPO in the UC model using WB. Compared with the control group, the DSS-induced colitis mice group showed a significant increase in the protein levels of MPO, which was also reversed by the administration of QHCY (**Figure 6**).

QHCY increases the mRNA expression of ZO-1, occludin and claudin-1 in the DSS-induced UC mouse mode

Intestinal barrier function plays a key role in maintaining normal bowel function in multiple ways, such as preventing the entry of harmful microbes, antigens, and toxins. The disruption of the intestinal barrier function is critically involved in the progression of colitis [33]. Thus, we investigated the effect of QHCY on the expression of ZO-1, occludin and claudin-1 in

the DSS-induced colitis mice model by qPCR. As shown in **Figure 5**, the expression of ZO-1, occludin and claudin-1 in the DSS-induced colitis mice were significantly decreased compared to the mice in the control group ($P < 0.05$), while treatment with QHCY significantly reversed the effect of DSS on the expressions of ZO-1, occluding, and claudin-1 ($P < 0.05$).

QHCY increases the protein expression levels of ZO-1, occludin, claudin-1 and reduces the phosphorylation of Elk-1 in the DSS-induced UC mouse model

Transcription factor Elk-1, is phosphorylated by MAP kinase and promotes gene expression in order to respond to inflammatory progression [34]. We also determined the effect of QHCY on the expression of ZO-1, occludin, claudin-1, Elk-1 and the phosphorylation of Elk-1 in a DSS-induced UC mouse model using western blotting. Compared with the control group, the DSS-induced colitis mice group showed a significant reduction in protein levels of the TJ proteins including ZO-1, occludin and claudin-1,

The effect of Qing Hua Chang Yin on colitis in mice

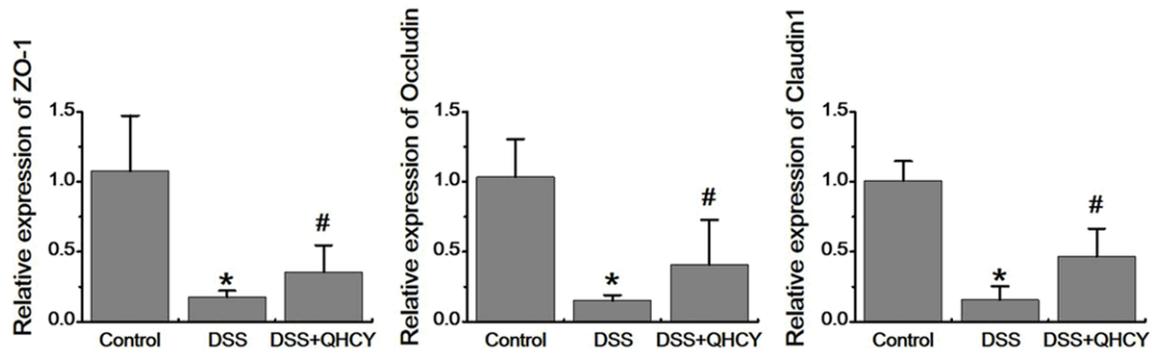


Figure 5. Effect of Qing Hua Chang Yin (QHCY) on the mRNA expression of ZO-1, occludin and claudin-1 in the dextran sulfate sodium (DSS)-induced ulcerative colitis mouse model. ZO-1, occludin and claudin-1 in colon tissues were determined by qPCR. GAPDH was used as the internal control. Data shown are the means \pm SD (error bars) from 5 individual mice/group. * $P < 0.05$, vs. control group; # $P < 0.05$, vs. DSS group.

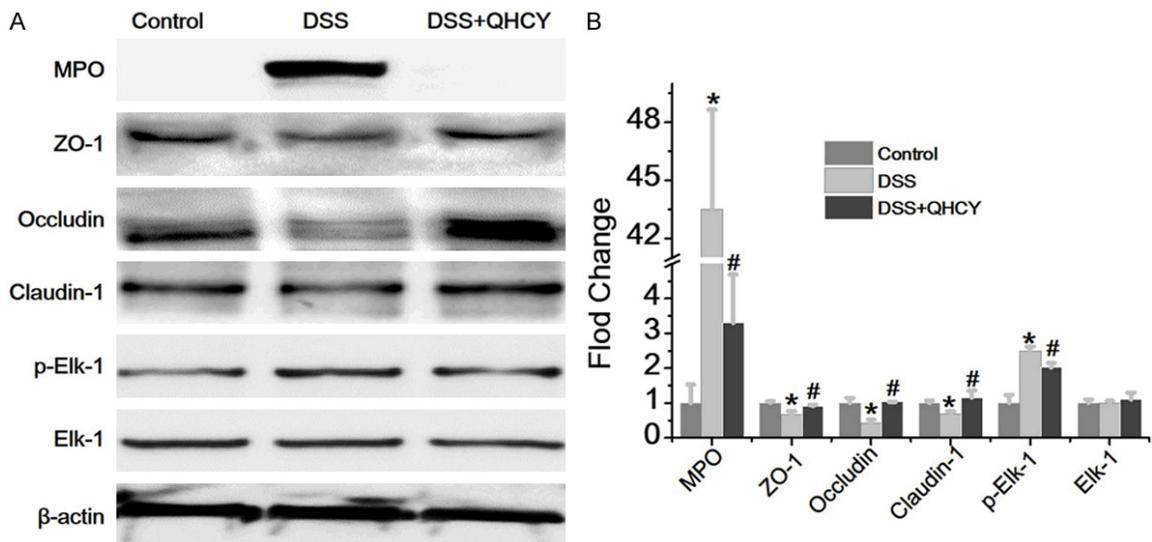


Figure 6. Effect of Qing Hua Chang Yin (QHCY) on the protein expression levels of MPO, ZO-1, occludin, claudin-1, Elk-1 and phosphorylation of Elk-1 in the dextran sulfate sodium (DSS)-induced ulcerative colitis mouse model. MPO, ZO-1, occludin and claudin-1, as well as the phosphorylation of Elk-1 expression in colon tissues were determined by western blot analysis (A). β -actin in was used as the internal control. Quantification of the levels of the indicated proteins relative to β actin as described in (B). Blots are representative of colon tissues randomly selected from 3 individual mice in each group. * $P < 0.05$, vs. control group; # $P < 0.05$, vs. DSS group.

which was also reversed by the administration of QHCY. However, the administration of QHCY significantly reversed the DSS-induced increase in the phosphorylation of Elk-1 (Figure 6).

Discussion

Epidemiological data have indicated that IBD is a chronic disease with intermittent exacerbations characterized by the acute worsening of severe inflammation during those flareups [35, 36]. In the current study, DSS-induced colitis in mice was utilized as a recognized preclinical

IBD model. The DSS successfully established profound colitis and the DSS treated mice exhibited all the manifestations characteristic of IBD including blood in the stool, diarrhea, weight loss, and the pathological features of gastrointestinal inflammation, loss of crypts, and ulceration [37, 38]. Our present study showed QHCY significantly relieved the DSS-induced clinical symptoms, including weight loss, blood in the stool, diarrhea, the DAI score, colon shortening, and colon histological injury (Figures 1-3), consistent with our previous study [29]. These results further confirmed that QHCY

has a therapeutic efficacy against the development of UC.

Although the exact etiology of IBD has yet to be fully elucidated, much evidence has demonstrated that inflammatory cytokines, both systemic and localized to the gut, play a major role in the pathogenesis of IBD [39, 40]. A prior study showed that TNF- α -induced TJ disruption improved with the expression of DRA with a subsequent reduction in the exacerbation of DSS-induced colonic inflammation [41]. SAA, an acute-phase protein, is induced in various inflammatory conditions including the IBD. An increased level of myeloperoxidase (MPO) was also documented in the intestinal tissues of patients with UC [42, 43]. In our experiment, DSS induced a significant colonic inflammation as indicated by the remarkable upregulation of the level of SAA in serum and TNF- α in both the serum and in colonic tissues. The upregulation of SAA and TNF- α were significantly attenuated by QHCY treatment (**Figure 4**), which corresponded well with the decreased MPO expression after QHCY treatment when compared with the DSS group (**Figure 6**).

The barrier function of colonic mucosa is maintained by the tight junctions and their molecular components, such as occludin and claudin-1, and an increase in the intestinal permeability is often documented in intestinal barrier dysfunction [44, 45]. The changes in the expression of these molecular components may lead to the dysfunction of tight junctions which in turn may result in defective mucosal function in UC patients. Our present murine study indicates that the administration of QHCY effectively attenuates the DSS-induced decrease in the mRNA levels of ZO-1, occludin and claudin-1, and the corresponding protein levels thereby maintain their expression. Elk-1, a critical transcription factor, could be regulated by JNK in the inflammatory response, which on activation also modifies the expression of inflammatory factors such as IL-6 and TNF- α [46]. We further found that treatment with QHCY remarkably reduced the phosphorylation level of Elk-1 (**Figures 5 and 6**). Overall, this is the first study to investigate QHCY's role in maintaining the intestinal barrier by regulating the expression of tight junctions in DSS-induced colitis.

In conclusion, our study further confirmed that QHCY ameliorates clinical manifestations such

as body weight reduction, colonic inflammation, and pathological damage in a DSS-induced colitis model. QHCY improves the intestinal barrier function by regulating tight junction proteins, including ZO-1, occludin and claudin-1. This further suggests that by improving the intestinal barrier function through the regulation of the mucosal tight junctions, QHCY may serve as a potential therapeutic agent for patients with IBD.

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Disclosure of conflict of interest

None.

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The effect of Qing Hua Chang Yin on colitis in mice

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