Chin J Integr Med 2022 Feb;28(2):124-129

Chinese Journal of Integrative Medicine

Available online at link.springer.com/journal/11655
Journal homepage: www.cjim.cn/zxyjhen/zxyjhen/ch/index.aspx
E-mail: cjim_en@cjim.cn

Original Article

Huangqin Decoction Attenuates DSS-Induced Mucosal Damage and Promotes Epithelial Repair via Inhibiting TNF-α-Induced NF-κB Activation*

GU Li-mei[1,2,3], LI Hui[1,2,3], XIA Jun-quan[1,2,3], PAN Cheng-yu[1,2,3], GU Chao[1,2,3], and TIAN Yao-zhou[1,2,3]

ABSTRACT **Objective:** To investigate the protective effect of Chinese herbal formula Huangqin Decoction (HQD) on ulcerative colitis mouse model induced by dextran sulphate sodium (DSS) and human intestinal epithelial cell injury induced by tumour necrosis factor-α (TNF-α). **Methods:** *In vivo*, 30 male C57BL/6 mice were divided into 5 groups using a random number table (*n*=6 per group), including control, DSS, 5-aminosalicylic acid (5-ASA), HQD low- (HQD-L) and high-dose (HQD-H) groups. The colitis mouse model was established by 3% (w/v) DSS water for 5 days. Meanwhile, mice in the HQD-L, HQD-H and 5-ASA groups were administrated with 100, 200 mg/kg HQD or 100 mg/kg 5-ASA, respectively, once daily by gavage. After 9 days of administration, the body weight, disease activity index (DAI) score and colon length of mice were measured, the pathological changes of colons were analyzed by hematoxylin-eosin staining (HE) staining, and the levels of serum interleukin (IL)-6, IL-1β and TNF-α were measured by enzyme linked immunosorbent assay. *In vitro*, the human colon epithelial normal cells (FHC cells) were exposed to HQD (0.6 mg/mL) for 12 h and then treated with TNF-α (10 ng/mL) for 24 h. The tight junction (TJ) protein expression levels of Claudin-4 and Occludin, and the protein phosphorylation levels of p65 and inhibitor of nuclear factor kappaB (NF-κB)-α (IκBα) were measured by Western blot. **Results:** *In vivo*, compared with the DSS group, HQD-H treatment attenuated the weight loss and reduced DAI score of mice on the 8th day ($P<0.05$). Moreover, HQD-H treatment ameliorated the colon shortening in the DSS-induced colitis mice ($P<0.05$). HE staining showed HQD attenuated the pathological changes of colitis mice, and the histological scores of HQD-H and 5-ASA groups were significantly decreased compared with the DSS group ($P<0.05$). Meanwhile, HQD-H and 5-ASA significantly decreased the serum IL-1β, IL-6 and TNF-α levels of mice ($P<0.05$). *In vitro* experiments showed that HQD up-regulated Occludin and Claudin-4 protein expressions and inhibited p-p65 and p-IκBα levels in FHC cells compared with the TNF-α group ($P<0.05$). **Conclusion:** HQD significantly relieved the symptoms in DSS-induced colitis mice by inhibiting pro-inflammatory cytokines expression and maintained the homeostasis of TJ protein in FHC cells by suppressing TNF-α-induced NF-κB activation.

KEYWORDS Huangqin Decoction, ulcerative colitis, tumour necrosis factor-α, nuclear factor kappaB pathway, tight conjunction protein, Chinese medicine

Ulcerative colitis (UC) is one of the common inflammatory bowel diseases (IBDs) producing chronic intestinal inflammation and tissue damage. One of the most important pathological features of UC is the leaky intestinal epithelial mucosal barrier that increases the intestinal permeability.[1] Tight junctions (TJs) components molecules, such as occludins, claudins, junctional adhesion molecule 1 (JAM-A), and zonula occludens-1 (ZO-1), are necessary for the barrier function of epithelia and preserve the intestinal epithelial mucosal barrier integrity.[2,3] Occludin is the first transmembrane protein identified, and may represent an important role in intestinal permeability regulation.[4] Meanwhile, claudin-4 can function as

*Supported by the Scientific Research Project of Jiangsu Provincial Administration of Traditional Chinese Medicine (No. JD2019SZXYB05), Natural Science Foundation of Nanjing University of Chinese Medicine (No. XZR2020030), and National Administration of Traditional Chinese Medicine: 2019 Project of Building Evidence-Based Practice Capacity for Traditional Chinese Medicine (No. 2019XZZX-XH007)

1. Gastroenterology Department, Affiliated Hospital of Integrated Traditional Chinese and Western Medicine, Nanjing University of Chinese Medicine, Nanjing (210028), China; 2. Gastroenterology Department, Jiangsu Province Hospital on Integration of Chinese and Western Medicine, Nanjing (210028), China; 3. Jiangsu Province Academy of Traditional Chinese Medicine, Nanjing (210028), China
Correspondence to: Prof. TIAN Yao-zhou, E-mail: tianyaozhou1960@163.com

DOI: https://doi.org/10.1007/s11655-021-3343-4

paracellular sodium barrier, and down-regulation of claudin-4 may lead to altered TJ structure and impaired epithelial function in active UC.[5]

Pro-inflammatory cytokines are up-regulated and cause an increase intestinal permeability in UC.[6,7] Tumour necrosis factor-α (TNF-α) is up-regulated in UC patients and mice models such as chemical dextran sulphate sodium (DSS)-induced colitis. Meanwhile, TNF-α-mediated nuclear factor kappaB (NF-κB) signal pathway activities play a key role in the intestinal barrier impairment.[8] Targeting TNF shows a benefit for some patients in reducing symptoms of IBD and relieving inflammation in the intestinal tract. Therefore, DSS-induced colitis mouse model and human colon epithelial normal cells (FHC cells) treated by TNF-α as *in vitro* model are very useful to screen potential drugs for treating IBDs such as UC.

Huangqin Decoction (黄芩汤, HQD) is a classic Chinese herbal formula. It has been applied to treat gastrointestinal diseases such as diarrhea, abdominal spasms, vomiting, and nausea.[9] The pharmacodynamics studies showed that the main bioactive ingredients of HQD include baicalin, wogonoside, baicalein, wogonin, oroxylin-A, paeoniflorin, liquiritin, and other herb components.[10,11] Although HQD has been widely applied for treating gastrointestinal diseases in China and achieved significantly clinical efficacy, the underlying mechanisms are still unclear. In this study, we investigated whether HQD had an effect on UC and its underlying mechanisms using both DSS-induced colitis mice *in vivo* and FHC cells treated by TNF-α *in vitro*.

METHODS

Drugs and Reagents

DSS were purchased from MP Biomedicals (Cat No. 160110, USA). RPMI-1640, fetal bovine serum (FBS, lot No. 1640958), penicillin and streptomycin were purchased from GIBCO BRL (15240-062, lot No. 2211107, USA). Recombinant human TNF-α (70-EK282, lot No. 28210345), interleukin (IL)-1β (70-EK201B, lot No. A201B10452), and IL-6 (EK206/3-96, lot No. A20600721) mouse enzyme linked immunosorbent assay (ELISA) kits were purchased from MultiSciences (China). Primary antibodies for occludin (ab167161, lot No. GR119583-71) were purchased from Abcam (USA). p65 (8242, lot No. 13), phosphorylation (p)-p65 (3033, lot No. 14), inhibitor of NF-κBα (IκBα, 4814, lot No. 17), p-IκBα (2859, lot No. 17), GAPDH (5174, lot No. 7), and secondary antibodies were obtained from Cell Signaling Technology (USA). Primary antibodies for claudin-4 (sc-376643, lot No. K2017) were obtained from Santa Cruz Biotechnology (USA). The 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, M2128, lot No. MKCK3153) was purchased from Sigma-Aldrich (USA), and 5-aminosalicylic acid (5-ASA, HY-15027, lot No. 14055) was purchased from MedChem Express (USA).

HQD Preparation

HQD, which consists of the roots of *Scutellaria baicalensis* Georgi, *Paeonia lactiflora* Pall, *Glycyrrhiza uralensis* Fisch, and the fruit of *Ziziphus jujuba* Mill, was supplied and authenticated from Pharmacy of Traditional Chinese Medicine at Jiangsu Province Hospital on Integration of Chinese and Western Medicine (China). The mixtures of formular medicinal materials were extracted with distilled water at a weight ratio of 1:8. Ten times of one dose of formula mixture was weighted and incubated in distilled water for 30 min at room temperature. Then the mixture was further extracted at 100 ℃ for 120 min twice. The extracts were combined and filtered. The filtrate was concentrated to 1.2 g/mL of crude drug by rotary evaporator, and precipitated by ethanol addition up to 70% alcohol content and maintained at 4 ℃ overnight. The supernatant was collected by filtration and concentrated by rotary evaporator at 60 ℃ to obtain the concentrated solution. The concentrated extract mixture was aliquoted and stored in –80 ℃ freezer before use. A part of concentrated extract was lyophilized for 24 h. The lyophilized powder was stored in the refrigerator at 4 ℃.

Animals

Thirty male C57BL/6 mice (weighing 22–24 g, 6–8 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd., China [license No. SCXK (Beijing) 2016-0006]. Animals were housed under specific pathogen free (SPF) condition at controlled temperature (22±2 ℃), humidity (50%±10%) and light (12 h light/dark cycle) with food and water *ad libitum*. The mice were acclimatized for 7 days before the experiment, and the experiments were performed in accordance with the Guidelines of Animal Ethics Committee of Nanjing University of Chinese Medicine.

Chin J Integr Med 2022 Feb;28(2):124-129

Grouping, Modeling and Treatment

Thirty mice were divided into 5 groups using a random number table (n=6 per group), including control, DSS, 5-ASA, HQD low-dose (HQD-L) and HQD high-dose (HQD-H) groups. The control group received distilled water, and the other 4 groups were given 3% (w/v) DSS water for 5 days to induce UC.[12] During the DSS challenge, mice in the HQD-L, HQD-H and 5-ASA groups were administrated with 100, 200 mg/kg HQD or 100 mg/kg 5-ASA by gavage, respectively, once a day, for consecutive 9 days; while the control and DSS groups were given distilled water only by gavage. All the mice were freely to access diet and drink.

Evaluation of UC and Sample Collection

The general condition of mice was observed every morning, the body weight was recorded daily. Weight loss, severe diarrhea, and colonic bleeding were monitored and evaluated daily according to description for symptoms and score categories for the disease activity index (DAI, Appendix 1).[13] On the 9th day, the terminal time, the mice were anesthetized with isoflurane and then sacrificed by cervical dislocation. The whole blood samples were collected via heart puncture, and the serum was separated and stored at –80 ℃ for ELISA analysis. The colorectum was harvested and length of colon was measured. The colons were cut into segments, and one was fixed in 4% paraformaldehyde for hematoxylin-eosin (HE) staining with a slice of 5-μm thickness. Evaluation of the scores of colon histological changes (Appendix 2) were performed by 2 trained persons independently according to the standards. The other segments were snap frozen in liquid nitrogen for Western blot or cytokines detection, respectively.

Quantification of Serum IL-1β, IL-6 and TNF-α by ELISA

At the end of treatment, whole blood samples of mice were collected and serum was separated by centrifugation at 2,500 r/min for 15 min at 4 ℃. Then the supernatants were collected for measurement of TNF-α, IL-1β, and IL-6 levels by the ELISA kits according to the product directions.

Assessment of TJs-Related Protein Expression by Western Blot *in vitro*

In vitro, the FHC cells, obtained from the BeNa Culture Collection (China), were divided into 4 groups, including control group, TNF-α group, HQD group, and TNF-α+HQD group. The TNF-α+HQD group was exposed to HQD (0.6 mg/mL) for 12 h before treatment with TNF-α (10 ng/mL) for 24 h. FHC cells were homogenized in 500 μL radio immunoprecipitation assay (RIPA) lysis buffer. The concentration of protein in the supernatant after centrifugation (15,000 r/min for 10 min at 4 ℃) was determined by bicinchoninic acid assay (BCA) protein kit (lot No. 080719191115, Beyotime, China). Then 20 μg protein was separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE-GEL) according to standard procedure. The proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane, which was blocked with 2% bovine serum albumin (BSA) in phosphate buffered saline+Tween-20 (PBS-T) for 1 h at room temperature. Subsequently, the primary antibodies against Claudin-4, Occludin, p65, p-p65, IκBα, p-IκBα and GAPDH were incubated, respectively, for overnight at 4 ℃, and then horseradish peroxidase (HRP) conjugated secondary antibodies were applied and visualized with electrochemiluminescence (ECL) detection reagents (Thermo Fisher Scientific, USA). The band intensity was quantified by the Image J software (IJ153-win-java8, National Institutes of Health, USA).

Statistical Analysis

SPSS Statistics 25.0 software (IBM, USA) was used for statistical analysis. All data were presented as mean ± standard deviation ($\bar{x} \pm s$), and one-way analysis of variance was used for comparison between multiple groups. P<0.05 was considered statistically significant.

RESULTS

HQD Ameliorated DSS-Induced Colitis in Mice

Continual DSS treatment for up to 5 days induced mouse colitis formation with significant body weight loss and other symptoms such as bleeding and diarrhea, whereas the control mice were healthy without any treatment. HQD treatment significantly improved the disease symptoms and partially restored body weight loss. As shown in Figure 1A, compared with the control group, the body weight of DSS group was significantly decreased (P<0.05). On the 8th day, HQD-H and 5-ASA treatments attenuated the weight loss compared with the DSS group (P<0.05). Moreover, the DAI scores of the HQD-H and 5-ASA groups were reduced (P<0.05, Figure 1B).

Furthermore, HQD-H treatment ameliorated colon shortening in the DSS-induced colitis model mice (P<0.05, Figure 2).

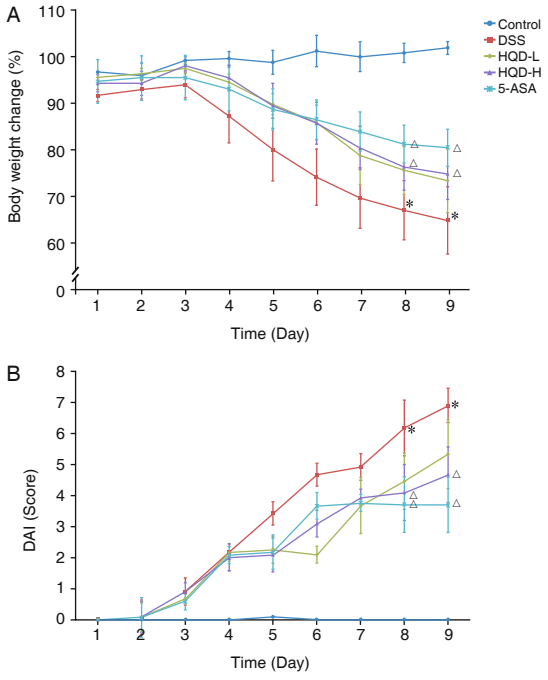

Figure 1. Effect of HQD on Body Weight and DAI in DSS-Induced Colitis Mice ($\bar{x} \pm s$, n=6)

Notes: DAI: disease activity index. *P<0.05 *vs*. control group; △P<0.05 *vs*. DSS group

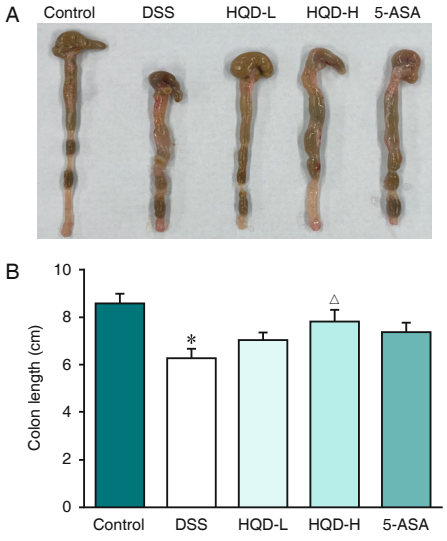

Figure 2. Effect of HQD on Colon Length Change in DSS-Induced Colitis Mice ($\bar{x} \pm s$, n=6)

Notes: *P<0.05 *vs*. control group; △P<0.05 *vs*. DSS group

HQD Relieved Colitis-Related Histological Injury in Mice

In Figures 3A–3E, the control group showed integrated and clear structures, while the DSS group exhibited loss of mucosa and severe inflammatory and injury symptoms, including neutrophil infiltration, bowel wall thinning, mucosal erosions. However, treatment of HQD significantly attenuated the pathological changes. As shown in Figure 3F, the histological score of DSS group was significantly higher than the control group (P<0.05). Compared with the DSS group, the histological scores of HQD-H and 5-ASA groups were significantly decreased (P<0.05).

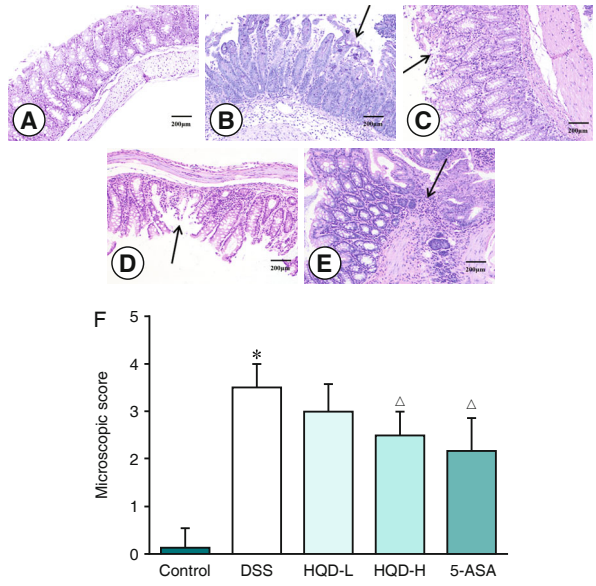

Figure 3. Effect of HQD on Histological Changes in DSS-Induced Colitis Mice ($\bar{x} \pm s$, n=6)

Notes: A–E: paraffin sections of colonic tissue stained by HE (×40); A: control group: normal structures of intestinal mucosa; B: DSS group: multiple acute and chronic inflammatory cell infiltration and surface erosion were observed in the whole intestinal mucosa; C: HQD-L group: acute and chronic inflammatory cell infiltration was observed in the whole intestinal wall, mainly neutrophils, and mucosal erosion on the surface of the lesion; D: HQD-H group: chronic inflammatory cell infiltration in intestinal wall, and mucosal gland repair were observed; E: 5-ASA group: less intestinal wall inflammation, mucosal muscle was thickened, accompanied by chronic inflammatory cell infiltration. F: microscopic scores of histological changes. *P<0.05 *vs*. control group; △P<0.05 *vs*. DSS group

HQD Decreased Secretion of Pro-inflammatory Cytokines in Colitis Mice

Compared with the control group, DSS group exhibited significant increases in the serum levels of IL-1β, IL-6 and TNF-α (P<0.05), whereas HQD-H and 5-ASA significantly reversed these responses compared with the DSS group (P<0.05, Figure 4).

HQD Up-regulated TJs-Related Protein Expressions Induced by TNF-α in FHC Cells

As shown in Figures 5A–5C, compared with the control group, the protein expressions of Claudin-4 and Occludin were decreased in the TNF-α group (P<0.05),

Chin J Integr Med 2022 Feb;28(2):124-129

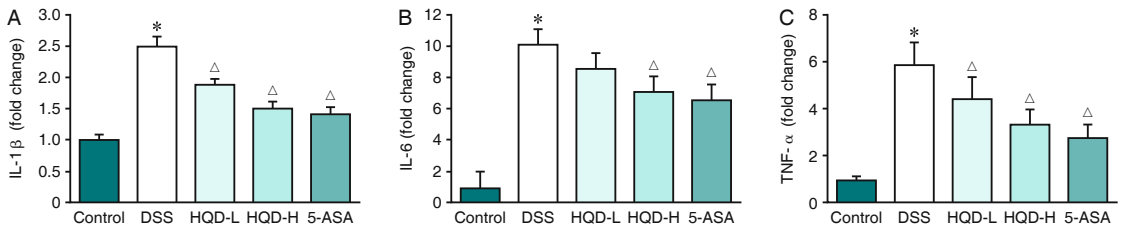

Figure 4. Effect of HQD on Serum Levels of IL-1 β , IL-6 and TNF-α in DSS-Induced Colitis Mice by ELISA ($\bar{x} \pm s$, n=6)

Notes: IL-6: interleukin-6; TNF-α : tumour necrosis factor-α . *P<0.05 *vs*. control group; △P<0.05 *vs*. DSS group

whereas they were increased in the presence of HQD regardless of TNF-α treatment (P<0.05).

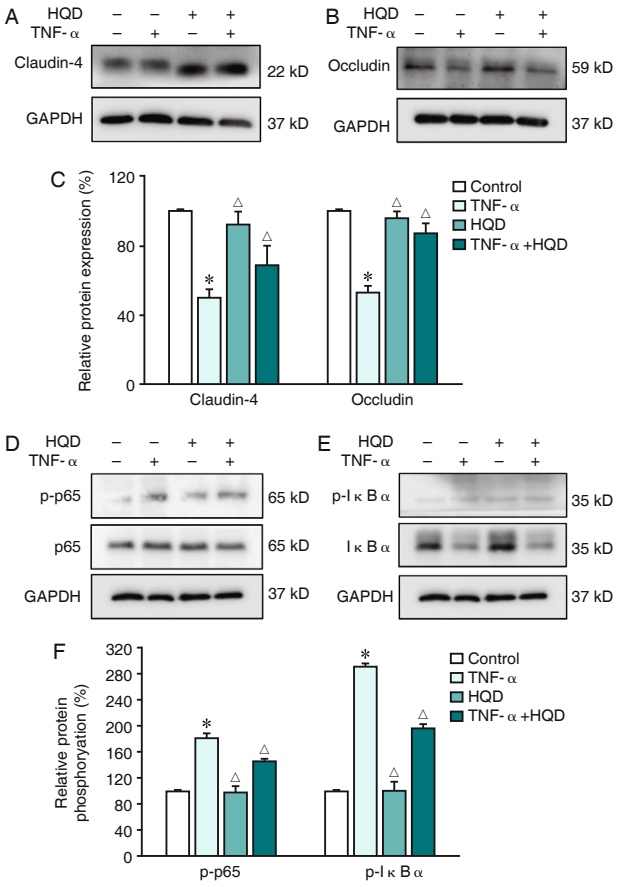

Figure 5. Effect of HQD on Regulating TJs-Related Protein Expressions and NF-κ B Cell Signal in FHC Cells by Western Blot ($\bar{x} \pm s$, n=3)

Notes: TNF-α : tumour necrosis factor-α ; p-I κ B α : phosphonated inhibitor of nuclear factor kappaB-α . *P<0.05 *vs*. control group; △P<0.05 *vs*. TNF-α group

HQD Inhibited NF-κ B Cell Signal Induced by TNF-α in FHC Cells

As shown in Figures 5D–5F, compared with the control group, the levels of p-p65 and p-I κ B α in the TNF-α group were significantly increased (P<0.05). However, HQD potently inhibited p-p65 and p-I κ B α levels induced by TNF-α compared with the TNF-α group (P<0.05).

DISCUSSION

UC has been increasing in population with poor morbidity, mortality and symptomatic burden for individual life and the whole society as well. To date, the interventions for UC focus on anti-inflammation, immunosuppression or alleviating the corresponding symptoms without cure.[14] The DSS-induced colitis model has been used extensively to study colonic inflammation in animal experiments.[15] HQD has been applied in gastrointestinal diseases in China for several centuries, which are effective in clinical application. Our results showed that treatment with HQD prominently decreased disease symptoms and DAI, and increased the length of colons. More importantly, HQD could attenuate the gastrointestinal structure injure in colon tissue. All these strongly suggests that HQD can alleviate the colitis features induced by DSS.

The TJ proteins include occludins, claudins, JAMs, and cytoplasmic accessory proteins such as ZO-1, 2, 3,[16,17] which play vital roles in maintaining permeability of intestinal barrier in colon. Some pro-inflammatory cytokines are up-regulated and have a potential correlation with the expression of TJ proteins in UC.[18] In consistent with a previous study,[18] our study indicated that TNF-α decreased the expressions of Claudin-4 and Occludin. By contrast, HQD treatment increased the expressions of Claudin-4 and Occludin. All these results indicated that HQD could maintain or protect the intestinal mucosal barrier integrity by increasing the protein expression of TJs.

Activation of NF-κ B proinflammatory signaling pathway has been demonstrated in mucosa of UC patients.[19] Proinflammatory cytokines including TNF-α could activate NF-κ B signal.[20] In this study, HQD significantly attenuated inflammation pathway by modulating NF-κ B signaling pathways in FHC cells. Furthermore, HQD inhibited the activity of NF-κ B

signal by decreasing phosphorylation and degradant of IκBα.

In summary, this study revealed protective effect and potential molecular mechanism of HQD against UC in DSS-induced colitis model. It may be attributed to renovation on the intestinal mucosal barrier, which would be possibly benefited from regulation of NF-κB proinflammatory signaling pathway in intestinal epithelial cells. Taken together, the anti-inflammatory activity of HQD in the DSS-induced colitis model highlights a promising application in clinical treatment of UC.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Gu LM, Li H, and Pan CY conceived and carried out the experiments. Xia JQ and Gu C conceived the experiments and analyzed data. Tian YZ designed the experiment. Gu LM and Tian YZ wrote the manuscript. All authors read and approved the final version.

Electronic Supplementary Material: Supplementary material (Appendixes 1 and 2) is available in the online version of this article at https://doi.org/10.1007/s11655-021-3343-4.

REFERENCES

1. Alipour M, Zaidi D, Valcheva R, Jovel J, Martinez I, Sergi C, et al. Mucosal barrier depletion and loss of bacterial diversity are primary abnormalities in paediatric ulcerative colitis. J Crohns Colitis 2016;10:462-471.
2. Awad WA, Hess C, Hess M. Enteric pathogens and their toxin-induced disruption of the intestinal barrier through alteration of tight junctions in chickens. Toxins (Basel) 2017;9:60.
3. Sawada N. Tight junction-related human diseases. Pathol Int 2013;63:1-12.
4. van Itallie CM, Anderson JM. Architecture of tight junctions and principles of molecular composition. Semin Cell Dev Biol 2014;36:157-165.
5. Oshima T, Miwa H, Joh T. Changes in the expression of claudins in active ulcerative colitis. J Gastroenterol Hepatol 2008;23(Suppl 2):S146-S150.
6. Al-Sadi RM, Ma TY. IL-1beta causes an increase in intestinal epithelial tight junction permeability. J Immunol 2007;178:4641-4649.
7. Capaldo CT, Nusrat A. Cytokine regulation of tight junctions. Biochim Biophys Acta 2009;1788:864-871.
8. Lee B, Moon KM, Kim CY. Tight junction in the intestinal epithelium: its association with diseases and regulation by phytochemicals. J Immunol Res 2018;2018:2645465.
9. Lam W, Jiang Z, Guan F, Huang X, Hu R, Wang J, et al. PHY906 (KD018), an adjuvant based on a 1800-year-old Chinese medicine, enhanced the anti-tumor activity of Sorafenib by changing the tumor microenvironment. Sci Rep 2015;5:9384.
10. Wang C, Tang X, Zhang L. Huangqin-Tang and ingredients in modulating the pathogenesis of ulcerative colitis. Evid Based Complement Alternat Med 2017;2017:7016468.
11. Zuo F, Zhou ZM, Zhang Q, Mao D, Xiong YL, Wang YL, et al. Pharmacokinetic study on the multi-constituents of Huangqin-Tang decoction in rats. Biol Pharm Bull 2003;26:911-919.
12. Wirtz S, Popp V, Kindermann M, Gerlach K, Weigmann B, Fichtner-Feigl S, et al. Chemically induced mouse models of acute and chronic intestinal inflammation. Nat Protoc 2017;12:1295-1309.
13. Yang R, Liao Y, Wang L, He P, Hu Y, Yuan D, et al. Exosomes derived from M2b macrophages attenuate DSS-induced colitis. Front Immunol 2019;10:2346.
14. Dai YC, Zhang YL, Wang LJ, Guo Q, Yang K, Ye RH, et al. Clinical presentation and treatment strategies for ulcerative colitis: a retrospective study of 247 inpatients. Chin J Integr Med 2016;22:811-816.
15. Eichele DD, Kharbanda KK. Dextran sodium sulfate colitis murine model: an indispensable tool for advancing our understanding of inflammatory bowel diseases pathogenesis. World J Gastroenterol 2017;23:6016-6029.
16. Heinemann U, Schuetz A. Structural features of tight-junction proteins. Int J Mol Sci 2019;20:6020.
17. Forster C. Tight junctions and the modulation of barrier function in disease. Histochem Cell Biol 2008;130:55-70.
18. Capaldo CT, Farkas AE, Hilgarth RS, Krug SM, Wolf MF, Benedik JK, et al. Proinflammatory cytokine-induced tight junction remodeling through dynamic self-assembly of claudins. Mol Biol Cell 2014;25:2710-2719.
19. Merga YJ, O'Hara A, Burkitt MD, Duckworth CA, Probert CS, Campbell BJ, et al. Importance of the alternative NF-kappaB activation pathway in inflammation-associated gastrointestinal carcinogenesis. Am J Physiol Gastrointest Liver Physiol 2016;310:G1081-G1090.
20. Kato A. Group 2 innate lymphoid cells in airway diseases. Chest 2019;156:141-149.

(Accepted April 23, 2021; First Online December 7, 2021)
Edited by YU Ming-zhu