Current Medical Science
DOI https://doi.org/10.1007/s11596-020-2262-5
40(5):910-916,2020

Anti-inflammatory Activity of Mollugin on DSS-induced Colitis in Mice*

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Summary: We aimed to explore the anti-inflammatory activity of mollugin extracted from *Rubia cordifolia* L, a traditional Chinese medicine, on dextran sulfate sodium (DSS)-induced ulcerative colitis (UC) in mice. Thirty C57BL/6 mice were divided into a control group (n=6), a model group (n=6), and three experimental groups (40, 20, 10 mg/kg of mollugin, n=6 each). DSS solution (3%) was given to mice in the model group and experimental groups from day 4 to day 10 to induce the mouse UC model. Mice in the experimental groups were intragastrically administrated mollugin from day 1 to day 10. Animals were orally given distilled water in the control group for the whole experiment time and in the model group from day 1 to day 3. The changes in colon pathology were detected by hematoxylin and eosin (HE) staining. Interleukin-1β (IL-1β) in the serum, and tumor necrosis factor-α (TNF-α) and interferon-γ (IFN) in the tissues were measured by enzyme linked immunosorbent assay. Expression levels of Toll-like receptor 4 (TLR4) and myeloid differentiation factor 88 in the colon tissues were detected by immunohistochemistry. Results showed that mollugin could significantly reduce weight loss and the disease activity index in the DSS-induced UC mouse model. HE examinations demonstrated that mollugin treatment effectively improved the histological damage (P<0.05). The overproduction of IL-1β and TNF-α was remarkably inhibited by mollugin treatment at doses of 20 and 40 mg/kg (P<0.05). Additionally, the levels of TLR4 in colon tissues were significantly reduced in mollugin-treated groups compared with the DSS group. Our findings demonstrated that mollugin ameliorates DSS-induced UC by inhibiting the production of pro-inflammatory chemocytokines.
Key words: mollugin; anti-inflammatory activity; DSS-induced colitis

Inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), is characterized by idiopathic, chronic and relapsing inflammation of the intestinal tract[1]. IBD has been more common in developed countries. In recent years, however, the incidence rate of IBD tends to rise in China. Epidemiological studies have shown that the incidence rate of IBD in China is as high as 3.44%, ranking first in Asia[2]. Currently, there is no cure for IBD. The high recurrence of IBD leads to a poor quality of life and a great loss of medical resources.

The pathogenesis of IBD was reported to be related to immunologic, genetic, environmental factors, and their interactions[3]. Drugs used for IBD treatment roughly fall into five categories: 5-aminosalicylic acid (5-ASA), glucocorticoids (GC), immunosuppressants (IS), biological therapeutic agents, and herbal medicines. Among them, anti-inflammatory drugs, immunomodulatory drugs and some biological therapeutic agents are commonly used for UC[4, 5]. 5-ASA (such as sulfasalazine, mesalamine, and olsalazine) was only used for treatment of mild-to-moderate UC, but its effects on moderate-to-severe UC are less clear[6]. The anti-inflammatory activity of GC (such as prednisone, hydrocortisone, and dexamethasone) has been reported to be attributed to the repression of pro-inflammatory genes through signal transduction by binding to their steroid receptor, the GC receptor (GR)[7]. The combination of biological therapeutic agents, such as infliximab (IFX) and IS therapy is more effective than IFX alone for achieving and maintaining clinical remission at 4–6 months in patients with moderate-to-severe UC, regardless of

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*This work was supported by the National Natural Science Foundation of China (No. 81703380), the Natural Science Foundation of Hubei Province (No. 2017CFB782), the Scientific Research Project of Health and Family Planning Commission of Hubei Province (No. WJ2017M077), and the Applied Basic Research Project of Wuhan Science and Technology Bureau (No. 2017060201010215).

prior IS use[8]. Approximately one-third of IBD patients in remission under anti-tumor necrosis factor (TNF) treatment relapsed 1 year after discontinuation[9]. Herbal medicines with biological activities have been widely used to treat UC. Studies have shown satisfactory therapeutic effects of several traditional herbal medicines on DSS-induced UC[10–12].

Mollugin is a major bioactive component isolated from *Rubia cordifolia* L, which has been used as a traditional Chinese medicine for centuries and is officially listed in the Chinese Pharmacopoeia[13]. In Chinese Medicine, the root of *Rubia cordifolia* L can stop bleeding, promote blood circulation to remove blood stasis[14], and it is commonly used to treat arthritis, hematorrhea, hemostasis, and dysmenorrhea[15]. Mollugin was reported for its several pharmacological effects, such as anti-inflammatory[16], antitumor[17], antibacterial and antioxidant activities[18]. Recently, Kim and Zhang reported that mollugin might be a new drug candidate for the treatment of inflammatory diseases, such as colon inflammation, and tumor diseases[19, 20]. However, the anti-inflammatory mechanisms of mollugin are still unclear. Our previous study[21] reported that aqueous extract of *Rubia Cordifolia's* aerial part has a preventive effect on dextran sulfate sodium (DSS)-induced UC. In the present study, we further evaluated the anti-inflammatory activity of mollugin on DSS-induced UC.

1 MATERIALS AND METHODS

1.1 Reagents

Mollugin was purchased from the National Institutes for Food and Drug Control (NIFDC, Beijing, China) with a purity of, at least, 98% in HPLC analysis, and its structure is shown in fig. 1A. DSS (MW 36000-50000) was from MP Biomedicals, USA. Sodium carboxyl methyl cellulose (CMC-Na) was procured from Wuhan Kerui Biotechnology Co., Ltd., China.

1.2 Animals

Thirty male C57BL/6 mice, aged 6–8 weeks and weighing 22±2 g, were purchased from the Laboratory Animal Center of Hubei (license number: SCXK (Liao) 2015-0018). Animals were housed in plastic cages under standard laboratory conditions on a 12:12 h light-dark cycle at room temperature (22±2°C) and at a relative humidity of 50%–60%. Animals had free access to pellet food and distilled water under specific pathogen-free conditions. All study protocols were approved by the Animal Care and Use Committee of Tongji Medical College.

1.3 Induction of UC and Drug Administration

Mice were randomly divided into five different groups: a control group, a model group, and three mollugin-treated groups (40, 20, 10 mg/kg of mollugin). An acute experimental UC model was induced with DSS as previously described, with minor modifications[22]. Briefly, mice were given *ad-libitum* drinking access to a 3% DSS solution (3 g DSS powder in 100 mL of distilled water) from day 4 to day 10 in the model group and three mollugin-treated groups. The drinking water was changed every two days. All mice were fed adaptively for 1 week before the experiment, and drugs or distilled water was intragastrically administered using a syringe for all the mice. Prophylactic administration of mollugin (40, 20, and 10 mg/kg, dissolved in 1% CMC-Na) was done from day 1 to day 3 in mollugin-treated groups. Mice in the control group and DSS group were given the same volume of distilled water during the same period of time. On day 4, mice in the DSS group and mollugin-treated groups were administered 3% DSS drinking water and the control group was given distilled water. The body weights, stool consistency, and blood in the stool were monitored every day to calculate the disease activity index (DAI) score.

1.4 Sample Collection

At the end of the experimental period (day 10), all mice were fasted overnight. Blood was taken from the eyeball. Then, mice were sacrificed using cervical dislocation. After standing for 30 min, blood was centrifuged at 3500 r/min for 10 min. The serum was separated and kept at –80°C for further analysis. After gross examination, the distal colon tissue was fixed in 10% neutral-buffered formalin for histological and immunohistochemical analyses. Another colon tissue sample (from the ileocecal junction to the anal verge) was obtained for further experiments.

1.5 Histological Analysis

Paraffin-embedded samples were cut into 5 μm sections and then stained with hematoxylin and eosin (HE) for light microscopic examination. The sections of the colon were photographed using a Nikon EclipseE100 (Japan) photomicroscope. Histological scoring[23] was based on three parameters as described below: (a) severity of inflammation: 0 = no inflammation; 1 = mild; 2 = moderate; 3 = severe; (b) depth of inflammatory involvement: 0 = no inflammation; 1 = mucosa; 2 = mucosa and submucosa; 3 = transmural; (c) crypt damage: 0 = intact crypts; 1 = loss of the basal one-third of the crypts; 2 = loss of the basal two-thirds of the crypts; 3 = entire crypt loss but intact epithelial surface; 4 = entire crypt loss and change of epithelial surface with erosion. The histological score was calculated by adding the three evaluations and a maximal score of 10 was given. Histological score of colons was independently completed by two pathologists in a blinded way, and the average score in each group was calculated.

1.6 Enzyme-linked Immunosorbent Assay (ELISA) for Cytokines in Serum and Tissue

Tissues from mice in each group were taken out

and stored at –80°C. They were weighted and lysed using lysis buffer to extract the total protein. The total protein amount in each lysate was determined using an Enhanced BCA Protein Assay Kit (Aspen Biotechnology, China). The concentration of IL-1β in the serum, and TNF-α and IFN-γ in the tissue were measured by an ELISA kit (ELK Biotechnology, China) in strict accordance with the manufacturer's instructions.

1.7 Immunohistochemistry of Toll-like receptor 4 (TLR4) and Myeloid Differentiation Factor 88 (MYD88)

The immunohistochemical method was adopted as previously described[24] with some modifications. Primary antibodies (TLR4 dilution: 1:1000, and MYD88 dilution: 1:400 with PBS) were stored at 4°C overnight, and the second antibody was diluted (1:200) in goat anti-rabbit labeled with horseradish peroxidase (HRP).

1.8 Statistical Analysis

The data were analyzed using GraphPad Prism version 8.0.1 software (GraphPad Software, Inc., USA) and were processed using the SPSS version 17.0 statistical analysis software (SPSS Inc., USA). Data were expressed as mean±standard deviation (SD). All *P* values were two tailed, and a *P* value lower than 0.05 was considered to be significant.

2 RESULTS

2.1 General Observations

To evaluate the therapeutic effect of mollugin on DSS-induced UC, the weight loss and DAI scores of mice in all groups were determined (fig. 1B and 1C). As expected, the body weight of mice in the DSS group was significantly reduced as compared with that in the control group. The body weight changes in mice treated with mollugin at 10 or 20 mg/kg were similar to those observed in the DSS group. The body weight decrease of mice treated with mollugin at the dose of 40 mg/kg was significantly increased on day 8 (P<0.05) and day 10 (P<0.01) as compared with that in the DSS group.

DAI scores (body weight loss, stool consistency, and blood in the stool) in the DSS group were significantly increased when compared with those in the control group. Administration of mollugin at different doses (10, 20, and 40 mg/kg) markedly decreased the DAI scores, especially at the dose of 40 mg/kg (P<0.05, fig. 1C).

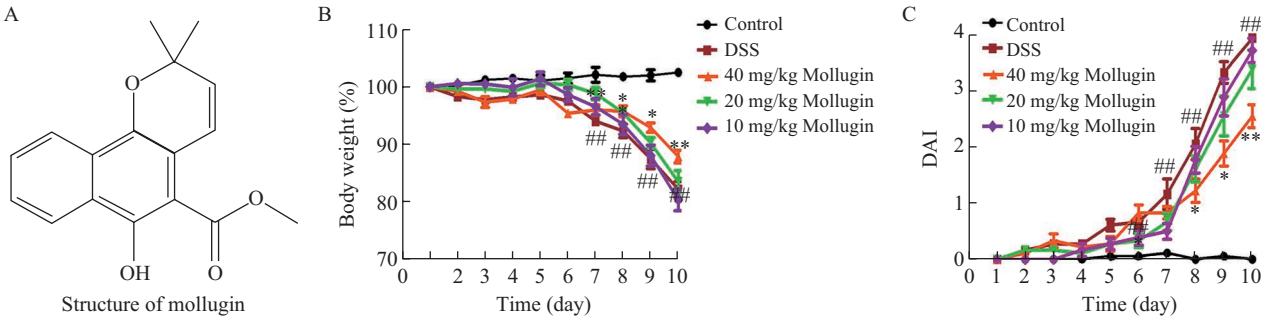

Fig. 1 Mollugin ameliorates dextran sulfate sodium (DSS)-induced experimental UC in mice.
A: structure of mollugin; B: body weight changes in each group; C: comparison of DAI scores. All values are presented as mean±SD (n=6). $^{\#}P$<0.05, $^{\#\#}P$<0.01 *vs.* control group; $^{*}P$<0.05, $^{**}P$<0.01 *vs.* DSS group

2.2 Mollugin Inhibits DSS-induced Colon Injury

As shown in fig. 2A, the mucosal epithelium of mice in the control group was complete and continuous, mucosal villi and glands were orderly arranged, and a small number of lymphocytes were expressed in lamina propria without inflammatory cell infiltration. The colonic mucosal epithelial cells disappeared continuously, the glands were missing, and a large number of inflammatory cells were infiltrated into the submucosa in the DSS group. Histological score analysis confirmed the protective effects of mollugin (fig. 2B). The mice treated with mollugin at different doses (10, 20, and 40 mg/kg) presented distinct layers of mucosal tissue, less disorganized arrangement of mucosal villi and glands, and significantly less infiltration of inflammatory cells, when compared to those of the DSS group (P<0.01 for all).

2.3 Mollugin Decreases Pro-inflammatory Cytokines Production in DSS-induced UC

ELISA (fig. 3) showed that mollugin treatment at 20 or 40 mg/kg could significantly decrease the production of IL-1β and TNF-α, when compared with that in the DSS group (P<0.05), but the decrease was not in a dose-dependent manner. Meanwhile, mollugin at the dose of 10 mg/kg slightly decreased IL-1β and TNF-α levels, and no significant differences were found. The levels of IFN-γ were reduced in the control group and mollugin-treated groups at 20 or 40 mg/kg compared with those in the DSS group, and there were no significant differences.

2.4 Mollugin Inhibits the Expression of TLR4 and increases the Expression of MYD88

The expression of TLR4 was significantly increased in DSS group compared to the control

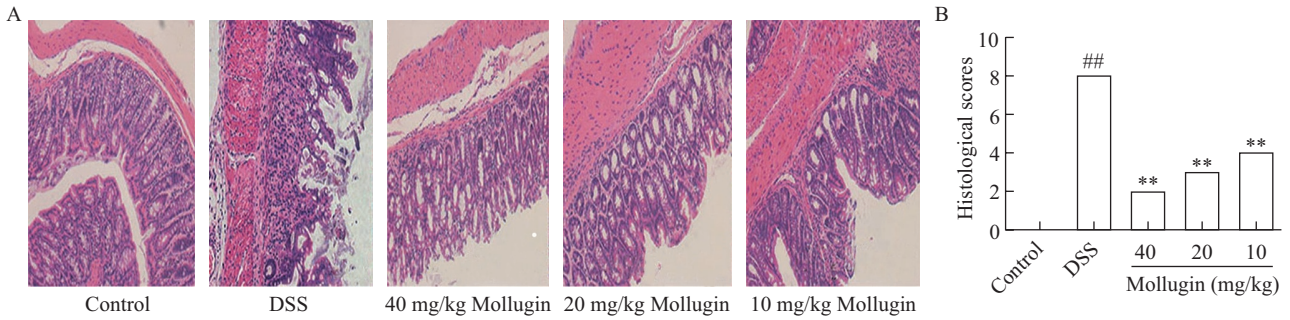

Fig. 2 Mollugin inhibits the histological changes in DSS-induced colon injury of mice in each group.
A: representative HE staining of the colonic mucosal epithelium of mice in each group (×200); B: histological scoring. Pathological changes were scored on a 0–10 scale. Data are represented as mean±SD. $^{\#\#}P<0.01$ *vs.* control group, $^{**}P<0.01$ *vs.* DSS group

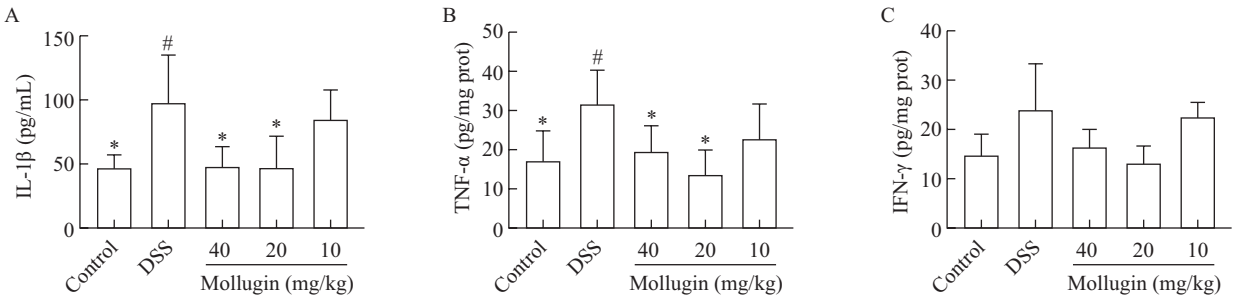

Fig. 3 ELISA analysis of the levels of IL-1β in the serum, and TNF-α and IFN-γ in the tissue
A: expression level of IL-1β; B: expression level of TNF-α; C: expression level of IFN-γ. Data are represented as mean±SD ($n\geq4$). $^{\#}P<0.05$ *vs.* control group, $^{*}P<0.05$ *vs.* DSS group

group. Mollugin (20, 40 mg/kg) could profoundly decrease the expression of TLR4 (fig. 4). In contrast, the expression of MYD88 was decreased in DSS group when compared with the control group. The expression of MYD88 was increased in 10, 40 mg/kg mollugin group when compared with the DSS group.

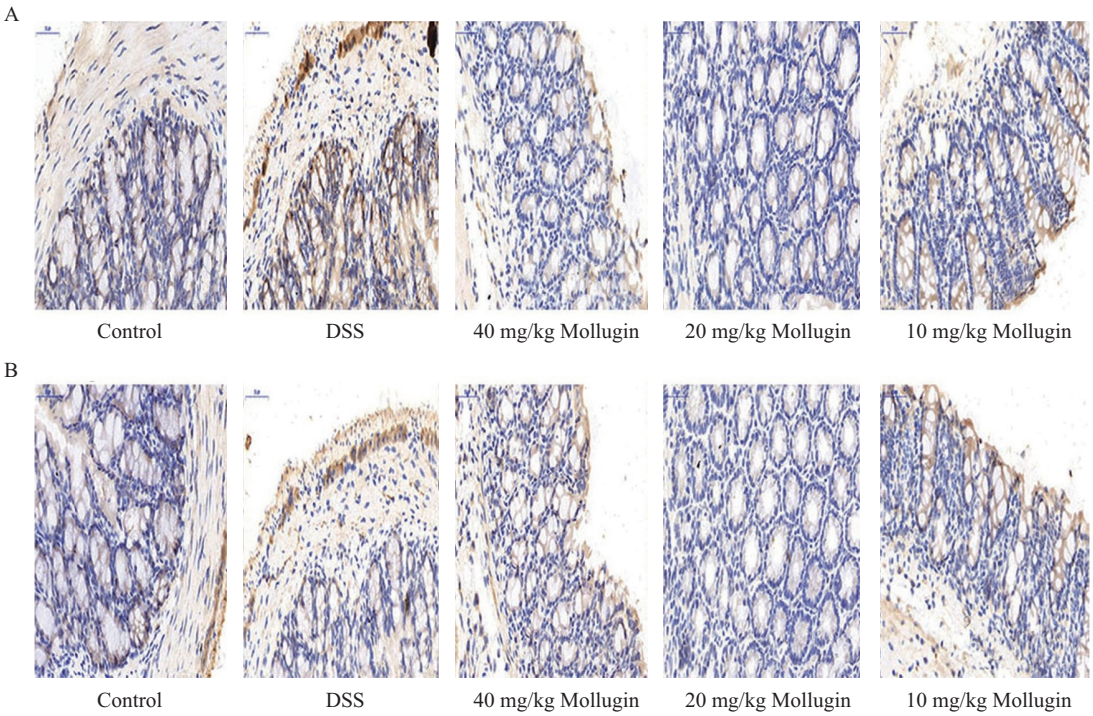

Fig. 4 Protein expression of TLR4 and MYD88 in colon tissues using immunohistochemistry (×400)
A: decreased positive expression of TLR4 in mollugin-treated groups; B: effects of mollugin treatment on MYD88 expression

3 DISCUSSION

UC is a chronic and non-specific inflammatory disease with high incidence and prevalence rates in developed countries. It can affect the rectum, or can progress proximally to involve part of, or the entire colon. Clinical symptoms include diarrhea, abdominal pain, gastrointestinal bleeding, and weight loss[25, 26]. The current goals of IBD therapy are the improvement of mucosal healing, normalization of biomarkers, histological healing, and healing on abdominal imaging[27, 28]. Although 5-ASA drugs have a better effect in clinical practice[29], their use does not lead to good clinical responses in many patients, and the best therapeutic IBD agents remain unknown. At present, oral administration of DSS is the most common method to induce experimental UC. DSS-induced experimental UC has similar pathological and clinical manifestations as UC[30]. Therefore, our study selected a DSS-induced UC model to evaluate the effects of mollugin.

Currently, a growing number of studies show a potential therapeutic effect of mollugin in UC treatment. Recent studies have shown that mollugin was highly permeable in rat intestinal segment, with absorption in each segment, while the specific absorption existed in the colon segment[31]. Mollugin and its synthetic derivatives inhibit TNF-α induced expression of inflammatory molecules via nuclear factor kappa beta (NF-κB)[32]. In the present study, we found that mollugin administration can improve DAI score and weight loss, and reduce colon injury.

Inflammatory mediators, such as pro-inflammatory cytokines, play a decisive role in the disease process. IL-1β and TNF-α are pro-inflammatory cytokines which can cause the onset of UC; they are secreted by inflammatory cells, initiate, and exaggerate inflammation responses. The production of IL-1β in the intestinal mucosa of UC patients is different from that of normal people; it was reported that IL-1β level in UC patients is significantly increased, while the IL-1β level in healthy people is very low[33, 34]. The contents of IL-1β, TNF-α and IFN-γ increased in IBD patients and rodents like mice[35, 36]. TNF-α, also known as cachectin, is a strong pro-inflammatory cytokine which plays an important role in the immune system during inflammation initiation, cell proliferation, differentiation and apoptosis[37, 38]. The overproduction of TNF-α associated with IBD has caused the most attention and investigation. Previous studies found that UC murine models have high levels of TNF-α[39]. In our experiment, 20 and 40 mg/kg mollugin remarkably suppressed the enhanced levels of IL-1β and TNF-α when compared with those in the DSS group. IFN-γ is critical in the regulation of multiple immune functions, such as antigen presentation, cellular proliferation, leukocyte trafficking, microbicidal effector activation, and pathogen recognition, which make it a primary pro-inflammatory cytokine involved in CD pathogenesis[40, 41]. Moreover, IFN-γ was found to be a major mediator involved in intratracheal chronic ovalbumin-induced colitis in IFN-γ deficient mice[42]. In the present study, we noted that the levels of IFN-γ in the control group and mollugin-treated (20, 40 mg/kg) groups were reduced as compared with those in the DSS group, but no significant differences were found.

Toll-like receptors (TLRs) are transmembrane protein family receptors that play a key role in nonspecific or innate immune defense[43]. TLRs are a growing family of molecules involved in innate immunity and they consist of, at least, ten highly homologous subtypes (10 in humans and 12 in mice)[44]. A study showed that in an immune reaction, TLR family stimulates the activation of the downstream MyD88 to form complexes[45]. The TLR4 pathway is primarily divided into MyD88-dependent and MyD88-independent signaling pathways based on the need for adaptor protein MyD88. In MyD88-dependent pathway, TLR recruits and activates downstream NF-κB[46]. Subsequently, the downstream NF-κB is activated, further leading to the transformation and secretion of pro-inflammatory factors. In order to investigate the mechanisms by which mollugin suppresses the inflammation in UC, we detected the expression of TLR4 and MyD88 by using immunohistochemistry. We found that mollugin treatment strongly decreased the expression of TLR4, but not that of MyD88. Perhaps, the anti-inflammatory activity of mollugin does not occur via the TLR4/MYD88-dependent pathway.

In summary, our current study suggests that mollugin can ameliorate DSS-induced UC by inhibiting the levels of pro-inflammatory cytokines *in vivo*. This study provides a novel promising therapeutic agent for UC and its active mechanisms are well worth further studies.

Conflict of Interest Statement

The authors declare that there is no conflict of interest with any financial organization or corporation or individual that can inappropriately influence this work.

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(Received Oct. 22, 2019; accepted Mar. 28, 2020)