Anti-inflammatory effects of *Fagopyrum cymosum* administered as a potential drug for ulcerative colitis

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Abstract. *Fagopyrum cymosum* (Trev.) Meisn (Fag), which belongs to the Polygonaceae family, has been widely used to treat inflammatory diseases. Previous studies have revealed that Fag components exhibit anti-inflammatory activities; however, their potential use in treating inflammatory bowel disease (IBD) has not been explored. In the present study, mice with 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis were used as a model of IBD. Fag extract was orally administered for 3 days following the induction of colitis and the conventional drug, salicylazosulfapyridine (SASP), was used as a control. The results revealed that Fag significantly ameliorated TNBS-induced body weight loss and colonic shortening in mice ($P<0.05$). Furthermore, Fag suppressed levels of proinflammatory cytokines and reduced macrophage infiltration into colonic tissues ($P<0.05$). To further verify the anti-inflammatory effects of Fag at the molecular level, a murine macrophage cell line, Raw264.7, was used. Nuclear translocation of nuclear factor (NF)-κB p65 and the phosphorylation of inhibitor of NF-κB (IκB) were assessed using western blotting. The results demonstrated that Fag inhibited the production of proinflammatory cytokines via inhibiting NF-κB p65 nuclear translocation and IκB phosphorylation ($P<0.05$). Furthermore, the clinical study results revealed that Fag had significantly fewer side effects ($P<0.05$) and served as a better anti-inflammatory drug for ulcerative colitis compared with SASP.

Introduction

Inflammatory bowel disease (IBD) is characterized by chronic intestinal inflammation and is predominantly comprised of Crohn's disease (CD) and ulcerative colitis (UC) (1,2). Approximately 3.6 million people are affected by IBD in the USA and Europe (2), and the number of patients with IBD in Asia has increased in recent years (3,4). Although the exact etiology of IBD remains unknown, it has been reported that dietary habits, environmental factors, genetic susceptibility and infectious microbes may contribute to its development (5-8). At the molecular level, the pathogenesis of IBD involves an imbalance between proinflammatory cytokines, including tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin (IL)-1β, IL-6 and IL-12, and anti-inflammatory cytokines, including IL-4, IL-10 and IL-11 (9-11). As one of the most important regulators of proinflammatory gene expression, nuclear factor (NF)-κB serves several functions (12). In the cytoplasm, it exists in a stable complex form with inhibitory κB (IκB). The NF-κB-IκB complex is disrupted by the phosphorylation of IκB, and NF-κB is subsequently translocated to the nucleus and bound to DNA binding sites, which inducesthe transcription of target genes associated with inflammatory responses (13). Attenuating the activity of NF-κB has been used to treat a variety of immune disorders, including autoimmune and inflammatory diseases (14).

Proinflammatory cytokines, including IL-1, IL-6, and TNF-α, are overexpressed in patients with IBD (12). Furthermore, a large population of infiltrated macrophages, which release inflammatory mediators including histamine, prostaglandin E2, nitric oxide and reactive oxygen species, may be detected in the patients' mucosa (3,4). Macrophages also secrete proteases that damage tissue by degrading the extracellular matrix (14). Immunoregulation of active macrophages may therefore be an efficient treatment for IBD (15).

It has been reported that limiting colonic inflammation via anti-inflammatory agents may reduce the risk of developing UC-associated cancer (2). Various anti-inflammatory agents, including 5-aminosalic acid, corticosteroids and immunosuppressive agents, are widely used to treat IBD (14,16,17). However, the serious side effects (including weight gain, osteoporosis, nausea and poor immunity) and high recrudescence rates of these agents limit their clinical applications (18). The conventional drug salicylazosulfapyridine (SASP) has been reported to induce chromosome aberrations and sister

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chromatid exchanges (19). Novel therapies and alternative medicines are therefore urgently needed. Herbal medicines have potential as treatments for IBD due to their low toxicity profiles and high patient compliance (7).

Fagopyrum cymosum (Trev.) Meisn (Fag), which is a herbal rhizome of the Polygonaceae family (20), and buckwheat species have been widely used to treat bacterial dysentery, lung disease (21), irritable bowel syndrome (IBS) and rheumatism (22-25). Previous chemical studies have revealed that Fag rhizomes contain compounds that are effective for the treatment of inflammatory disease, including phenolics, flavonoids, β-sitosterol, hecogenin, *p*-coumaric acid, ferulic acid, luteolin, dimericprocyanidin, glutinone, protocatechuic acid, epicatechin and shakuchirin (20,21,26).

A previous study demonstrated that Fag is able to ameliorate hyperalgesia in rats with IBS (25). The hypothesis is that Fag reduces intestinal inflammation and enhances the function of mucosal epithelium via regulating the structure and function of tight junctions. To the best of our knowledge, the therapeutic effects of Fag on IBD have not previously been explored. In the present study, a 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced model of acute murine colitis was used to assess the therapeutic effects of Fag extract on IBD. To further investigate the anti-inflammatory effects of Fag at the molecular level, the phosphorylation of IκB and the nuclear concentration of NF-κB were measured in lipopolysaccharide (LPS)-induced RAW264.7 macrophages *in vitro*.

Materials and methods

Plant materials and reagents. Fagopyrum cymosum was purchased from Jiangsu Traffic Hospital (Nanjing, China). All samples were identified by Dr Shengjin Liu (Department of Medicinal Plants, Nanjing University of Chinese Medicine, Nanjing, China). TNBS was obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). SASP was supplied by Aladdin Shanghai Biochemical Technology Co., Ltd. (Shanghai, China). LPS was purchased from Sigma-Aldrich (Merck KGaA).

Extraction of Fag. Fag was extracted twice (100 g in 800 ml 50% ethanol) by conventional refluxing for 2 h. The extracts were combined, filtered and concentrated under reduced pressure in a vacuum at 60˚C to form a residue (9.48 g). The residue was resolved in 0.5% sodium carboxyl methylcellulose (Sigma-Aldrich; Merck KGaA) for animal experiments and *in vitro* testing.

High-performance liquid chromatography (HPLC) analysis. The Agilent 1260 HPLC system (Agilent Technologies, Inc., Santa Clara, CA, USA), consisting of a quaternary pump, autosampler, column oven and diode array detector, was utilized to analyze the samples. Chromatographic separation was performed using a SepaxGP-C18 column (5.0 μm, 4.6x250 mm; Sepax Technologies, Inc., Newark, DE, USA) at 40˚C. The flow rate was 1.0 ml/min and the injection volume was 20 μl. The mobile phase was a mixture of phosphoric acid solution (pH 3.0; A) and acetonitrile (B; A:B=8:92). The chromatμograms were recorded at 220 nm. For quantitative analysis, the Fag extract and the standard solution mixtures of protocatechuic acid, catechin, epicatechin, procyanidins B_1 and procyanidins B_2 were analyzed under the conditions described above (40˚C). Compounds were run at six different concentrations (protocatechuic acid, 13.47-215.52 μg/ml; catechin, 10.21-163.36 μg/ml; epicatechin, 12.35-197.60 μg/ml; procyanidins B_1, 6.16-98.56 μg/ml; and procyanidins B_2, 5.99 to 95.84 μg/ml) with a 20 μl injection volume. Compound content in the extract was assessed using linear regression analysis and was demonstrated to be linear in the range with a correlation coefficient of 0.997-0.999.

Animal experiments. A total of 58 male BALB/c mice (8 weeks old, 18-22 g) were obtained from Nantong University (Nantong, China; certificate no. SCXK-2008-0010). The mice were housed in an air-conditioned room at 20-22˚C and 80% humidity with a 12-h light/dark cycle and were provided with *ad libitum* access to standard laboratory chow and water. Animal experiments were conducted in accordance with protocols approved by the Animal Ethic Committee of the Nanjing University of Chinese Medicine. Mice were randomly divided into the following six groups: Control group (n=8), TNBS-induced colitis groups treated with different concentrations of Fag (n=10 per group; 0, 0.57, 1.14 and 2.28 g/kg) and the 200 mg/kg SASP group (n=10). Acute colitis was induced in BALB/c mice as previously described with modifications (27). Briefly, mice were fasted for 24 h and anesthetized with pentobarbital (50 mg/kg; Sigma-Aldrich; Merck KGaA). TNBS solution (2.5% w/v; 100 μl) in 50% ethanol was administered into the colon via a thin catheter 4 cm proximal to the anus. The control group received vehicle (50% ethanol) alone. Fag and SASP were orally administrated once a day for 3 days following TNBS treatment. Mice were sacrificed 24 h following the final administration of test agents.

Macroscopic scoring and histological analysis of colitis. Mice were inspected and weighed daily. Following the induction of colitis, animals were sacrificed and colons were harvested and gently washed with ice-cold PBS. Colonic tissue was then stored at -80˚C for further experiments.

For histological analysis, colon tissues were fixed in 4% paraformaldehyde at room temperature for 24 h, dehydrated in a graded series of ethanol, embedded in paraffin and finally cut into 4-μm thick sections. The samples were stained with hematoxylin and eosin (room temperature, hematoxylin for 5 min and eosin for 2 min) in accordance with the standard procedures for histological evaluation (28). Histological scores were calculated by a blinded investigator on a scale from 0-9 based on the following criteria for inflammation: i) Erythema, ii) hemorrhage, iii) edema, iv) stricture formation, v) ulceration, vi) fecal blood, vii) presence of mucus, viii) diarrhea and ix) adhesions, with 1 point awarded for each parameter observed using a light microscope at magnification, x20. A maximum score of 9 indicated severe colitis with an overall diffuse pattern of chronic changes.

Colonic myeloperoxidase (MPO) activity. MPO activity is an indicator of neutrophil infiltration into the inflamed colon (n=6) (26,27). MPO activity was assessed using an MPO activity kit (cat. no. A044; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Briefly, colons were homogenized

in 10 mM PBS containing 0.5% hexadecyltrimethylammonium bromide (pH 7.0), and centrifuged at 4˚C and 12,000 x g for 10 min. The supernatant (50 μl) was added to a reaction mixture containing 0.1 mM H_2O_2 and 1.6 mM tetramethylbenzidine and incubated at 37˚C. Absorbance was measured at 650 nm. Myeloperoxidase activity was defined as the quantity degrading 1 μmol/ml of peroxide at 37˚C and expressed as U/mg.

Immunohistochemical evaluation. F4/80 positive inflammatory cell infiltration analysis was performed on paraffin-embedded colon tissue sections. The sections were deparaffinized, rehydrated with xylene and graded ethanol solutions, and washed with PBS. After blocking with 5% bovine serum albumin (Sigma-Aldrich; Merck KGaA) for 1 h at 37˚C, the sections were incubated with primary antibodies against F4/80 (cat. no. 14-4801-81; eBioscience; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at a dilution of 1:50 for 2 h at 37˚C, washed with PBS and subsequently co-incubated with biotinylated secondary antibody (cat. no. KS010; Nanjing Jiancheng Bioengineering Institute) for 30 min at room temperature at a dilution of 1:2,000. Following washing with PBS (pH 7.4), tissue sections were incubated at 37˚C with a horseradish peroxidase (HRP)-streptavidin complex (cat. no. KS001; Nanjing Jiancheng Bioengineering Institute) to detect secondary antibody for 30 min. Sections were stained with DAB at room temperature for 5 min and mounted according to standard protocols and observed under a light microscope at magnification, x20.

Determination of cytokine and LPS in plasma. The mouse plasma concentrations (n=6) of IL-1β (cat. no. 432601), IL-6 (cat. no. 431304), and TNF-α (cat. no. 430904) were determined using commercially available ELISA kits (BioLegend, Inc., San Diego, CA, USA), and the lower limit of quantification was 7.8 pg/ml for each cytokine. LPS (n=6) was measured using a Limulus Amebocyte Lysate assay kit (Xiamen Bioendo Technology, Co., Ltd., Xiamen, China) according to the manufacturer's protocol.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from colonic tissue (n=5) using TRIzol (Thermo Fisher Scientific, Inc.). A Takara PrimeScript First Strand cDNA Synthesis kit (Takara Bio, Inc., Otsu, Japan) was employed for the RT reactions with a temperature protocol as follows: 37˚C for 15 min, 85˚C for 15 sec and 4˚C for 10 min. qPCR analysis was performed in an ABI StepOnePlus Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with a temperature protocol as follows: 95˚C for 3 min; 95˚C for 30 sec, 58˚C for 30 sec and 72˚C for 30 sec (35 cycles). The sequences of primers used in this experiment were as follows: IL-1β forward, 5'-CTGTGTCTTTCCCGTGGACC-3' and reverse, 5'-CAGCTCATATGGGTCCGACA-3'; IL-6 forward, 5'-CCAGAAACCGCTATGAAGTTCCT-3' and reverse, 5'-CACCAGCATCAGTCCCAAGA-3'; TNF-α forward, 5'-ATCCGCGACGTGGAACTG-3' and reverse, 5'-CAGCTCATATGGGTCCGACA-3'; and β-actin forward, 5'-TCTGGCACCACACCTTCTA-3' and reverse, 5'-AGGCATACAGGGACAGCAC-3'. The SYBR-Green PCR mix kit (Takara Bio, Inc.) was used to quantify gene expression. Reactions were performed in a total volume of 20 μl following the manufacturer's protocol. Three replicates were performed for each qPCR run. The mRNA concentrations of all target genes were normalized to that of the β-actin in each sample using the $2^{-\Delta\Delta Cq}$ method (29).

Cell culture and treatment. Raw264.7 cells (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) were treated with 0, 5, 10, 20 or 50 μg/ml Fag for 30 min followed by incubation with 0.5 μg/ml LPS for 24 h. Cells with no treatment were set as the control group, and the cells only treated with LPS were set as model group. Dexamethasone (Dex; 1 μM) was selected as the positive drug and the treatment time was the same as the Fag treatment. The inhibitory effects of Fag on TNF-α and IL-6 production were measured using the aforementioned ELISA kits in supernatants collected from cells of 3 independent experiments run in triplicate.

Western blotting. Raw264.7 cells were pretreated with 0, 10, 20, or 50 μg/ml Fag for 30 min. LPS was added to a final concentration of 0.5/ml and incubated at 37˚C for 4 h. Cells were harvested and nuclear and cytoplasmic extracts were prepared using Nuclear and Cytoplasmic Extraction Reagents (Beyotime Institute of Biotechnology, Haimen, China) with protease inhibitor cocktail (Sangon Biotech Co., Ltd., Shanghai, China). All protein samples were quantified using a BCA assay kit (Beyotime Institute of Biotechnology). The protein samples (50 μg) were separated by 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membrane was blocked with 5% bovine serum albumin for 1 h at 37˚C, followed by incubation with antibodies against phosphorylated-IκBα (BS1190, 1:500), NF-κB (BS70527, 1:500), cyclooxygenase 2 (Cox-2) (BS1076, 1:500), inducible nitric oxide synthase (iNOS) (BS1267, 1:1,000), β-actin (AP0060, 1:2,000) or Lamin A (BS1446, 1:2,000) (all from Bioworld Technology, Inc., St. Louis Park, MN, USA) at 4˚C overnight. Membranes were subsequently incubated at 37˚C for 1 h with an HRP-conjugated secondary antibody (BS13278, 1:10,000; Bioworld Technology, Inc.). Antibody signals were detected using an enhanced chemiluminescence kit (Thermo Fisher Scientific, Inc.) and images were captured using a ChemiDoc XRS+ system (Bio-Rad Laboratories, Inc.).

Data analysis. Data are expressed as the mean ± standard deviation. Statistical analysis was performed using a two-tailed Student's t-test and one-way analysis of variance (with Tukey's post hoc test). $P<0.05$ was considered to indicate a statistically significant difference.

Clinical analysis. A total of 60 patients were recruited from Haian Hospital of Traidtional Chinese Medicine (Haian, China) from May 2013 to July 2015 [37.8±7.8 years old; 32 (53.3%) male; 28 (46.7%) female; 48 (80.0%) with stomachache; 58 (96.7%) with diarrhea; 46 (76.6%) with mucous bloody stool] were randomly divided into the experimental and control groups (n=30 in each). Patients in the experimental group were administered with 5 jinqiaomaipian tablets (0.33 g Fag/tablet; Nantong Jinghua Pharmaceutical Co., Ltd., Nantong, China)

3 times daily for 2 months. Patients in the control group were administered with 1 g SASP 4 times per day for the first month and twice daily for the second month. Glucocorticoid and 5-aminosalicylic acid analogues were avoided throughout the study period. The clinical study was approved by the Ethics Committee of Haian Hospital of Traditional Chinese Medicine, and all patients provided informed consent.

According to the Chinese Consensus on Diagnosis and Treatment Standard of Inflammatory Bowel Disease (2007), curative effects were grouped into three levels: Complete remission, effective and invalid. The total efficiency was calculated by the following formula: Total efficiency (%) = (total number of complete remissions/total number of cases) x 100.

Results

Effective components in Fag extracts. Fag extracts were analyzed using an established sensitive HPLC method within 70 min and the components were well separated (Fig. 1). The effective compounds, including protocatechuic acid, procyanidin B_1, catechin, procyanidin B_2 and epicatechin, had retention times of 9.46, 25.84, 32.19, 44.87 and 53.17 min, respectively (Fig. 1). Furthermore, quantification was also performed using HPLC. The results revealed that there was 6.98 mg protocatechuic acid, 4.07 mg procyanidin B_1, 2.64 mg catechin, 8.43 mg procyanidin B_2 and 17.84 mg epicatechin per g of the crude 50% ethanol extracts.

Fag ameliorates the symptoms of TNBS-induced colitis in mice. It has previously been demonstrated that TNBS induces colitis in mice (30). Following treatment with TNBS in 50% ethanol, the mice were inappetent, exhausted and emaciated, and had diarrhea with bloody and purulent stool, indicating that severe inflammation in the colon, or colitis. Weight loss and shortening of the colon were detected in the TNBS-treated mice (Fig. 2). Treatment with SASP ameliorated TNBS-induced weight loss and colon shortening when comparing with the TNBS treated mice (P<0.01). Fag was demonstrated to significantly ameliorate weight loss and colon shortening compared with the TNBS group in a dose-dependent manner (0.57 g/kg, P<0.05; 1.14 and 2.28 g/kg, P<0.01; Fig. 2). Although it was not as effective as SASP, treatment with Fag extracts improved colon health in mice with TNBS-induced colitis.

Histopathology. Histopathological analysis was utilized to evaluate the severity of colonic inflammation and ulceration. There was clear evidence of mucosal damage in the TNBS-treated mice, characterized by crypt abscesses, neutrophils, mononuclear infiltrating glandular epithelium and epithelial hyperplasia (Fig. 3A). Following treatment with Fag extract or SASP, mouse colons exhibited intact colonic architecture with no apparent ulceration, indicating less inflammatory cell infiltration compared with the TNBS group (Fig. 3A). Histopathological scoring was used to quantify the colon damage, and a marked increase was observed in the TNBS group compared with the control group (P<0.01; Fig. 3B). Treatment with Fag extract induced a dose-dependent decrease in histopathological scores (P<0.05 for the 0.57 g/kg group and P<0.01 for the 1.14 and 2.28 g/kg groups), which was also observed in the SASP group (P<0.01; Fig. 3B).

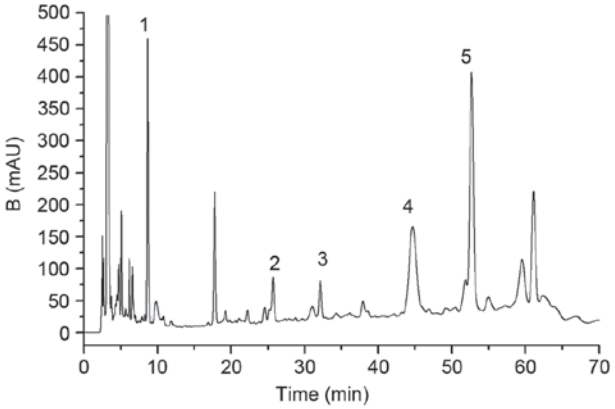

Figure 1. High performance liquid chromatography chromatogram of 50% ethanol extract solution of *Fagopyrum cymosum* (Trev.) Meisn. B, intensity; t, retention time; 1, protocatechuic acid; 2, procyanidin B_1; 3, catechin; 4, procyanidin B_2; 5, epicatechin.

Immunohistochemistry. To further evaluate the protective effects of Fag on TNBS-induced colitis, immunohistochemical analysis of F4/80 was employed. An increased number of F4/80 positive inflammatory cells was observed in TNBS-treated mice mucosa compared with the control group, whereas treatment with Fag or SASP were observed to inhibit this increase (Fig. 4A). MPO activity was also evaluated, and was demonstrated to be significantly increased in the TNBS group compared with the control group (14.4 fold; P<0.01; Fig. 4B). The MPO activity increase was significantly ameliorated following SASP treatment or treatment with 1.14 or 2.28 g/kg Fag (P<0.01; Fig. 4B).

Fag decreases proinflammatory cytokine and LPS levels. The levels of IL-1β, IL-6 and TNF-α mRNA were significantly increased in the TNBS group compared with the control group (P<0.01; Fig. 5). Following treatment with SASP, the mRNA expressions of IL-1β, IL-6 and TNF-α were significantly decreased compared with the TNBS-treated group (all P<0.01; Fig. 5). Fag also significantly attenuated the expression of IL-1β, IL-6 and TNF-α compared with the TNBS group in a dose-dependent manner (P<0.05; Fig. 5).

LPS is the major component of the outer membrane of Gram-negative bacteria, and it has been reported that LPS is upregulated in patients with IBD due to gut leakage and microbiota dysbiosis (31). Following treatment with TNBS for 3 days, the level of LPS in peripheral plasma was significantly increased in the TNBS group compared with the control group (P<0.01; Fig. 6A). Treatment with Fag significantly decreased the LPS level in a dose-dependent manner (1.14 g/kg, P<0.05; 2.28 g/kg, P<0.01; Fig. 6A). Furthermore, SASP treatment significantly ameliorated the TNBS-induced increase in LPS (P<0.01; Fig. 6A). Plasma levels of IL-1β, IL-6, and TNF-α were significantly increased in the TNBS group compared with the control (P<0.01; Fig. 6B-D), and these increases were significantly ameliorated by treatment with SASP (P<0.01) or Fag in a dose-dependent manner (1.14 g/kg, P<0.05; 2.28 g/kg, P<0.01; Fig. 6B-D).

Fag reduces proinflammatory cytokines in LPS-stimulated macrophages. LPS levels may be used as a marker of

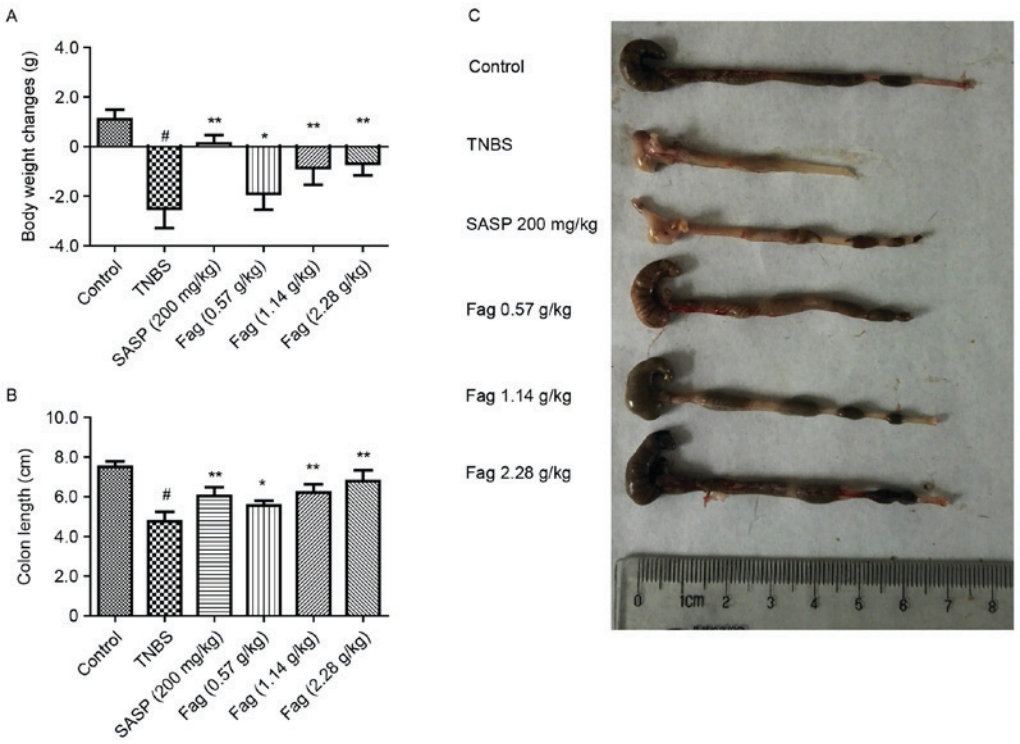

Figure 2. Fag ameliorates colitis induced by TNBS in mice. (A) Body weight change, (B) colonic length and (C) colonic appearance in mice following treatment. n=8 for control mice and n=10 for other treatment groups. #P<0.01 vs. control group; *P<0.05 and **P<0.01, vs. TNBS group. Fag, *Fagopyrum cymosum* (Trev.) Meisn; TNBS, 2,4,6-trinitrobenzenesulfonic acid; SASP, salicylazosulfapyridine.

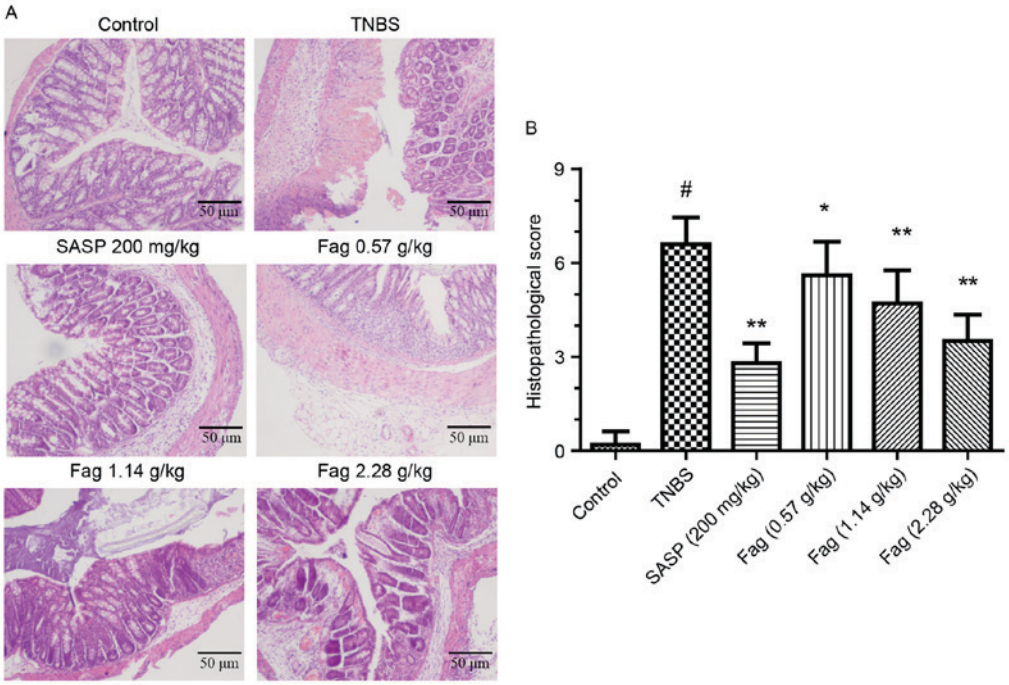

Figure 3. Fag treatment protects against TNBS-induced colon damage in mice. (A) Representative colonic tissue stained with hematoxylin and eosin. (B) Histopathological score of the colon. n=8 for control mice and n=10 for other treatment groups. #P<0.01 vs. control group; *P<0.05 and **P<0.01, vs. TNBS group. Fag, *Fagopyrum cymosum* (Trev.) Meisn; TNBS, 2,4,6-trinitrobenzenesulfonic acid; SASP, salicylazosulfapyridine.

inflammation *in vivo*, and LPS stimulates the inflammatory response of macrophages *in vitro* (31). Raw264.7 cells were used to verify the *in vitro* anti-inflammatory effects of Fag. LPS stimulation significantly increased the production of TNF-α and IL-6 in RAW264.7 cells (P<0.01), and Fag treatment significantly ameliorated these LPS-induced increases (P<0.01; Fig. 7) in a dose-dependent manner. iNOS and Cox-2 are two injury-induced enzymes in macrophages. Western blotting results demonstrated that LPS-induced overexpression of iNOS and Cox-2 was ameliorated by Fag treatment *in vitro* (Fig. 8).v

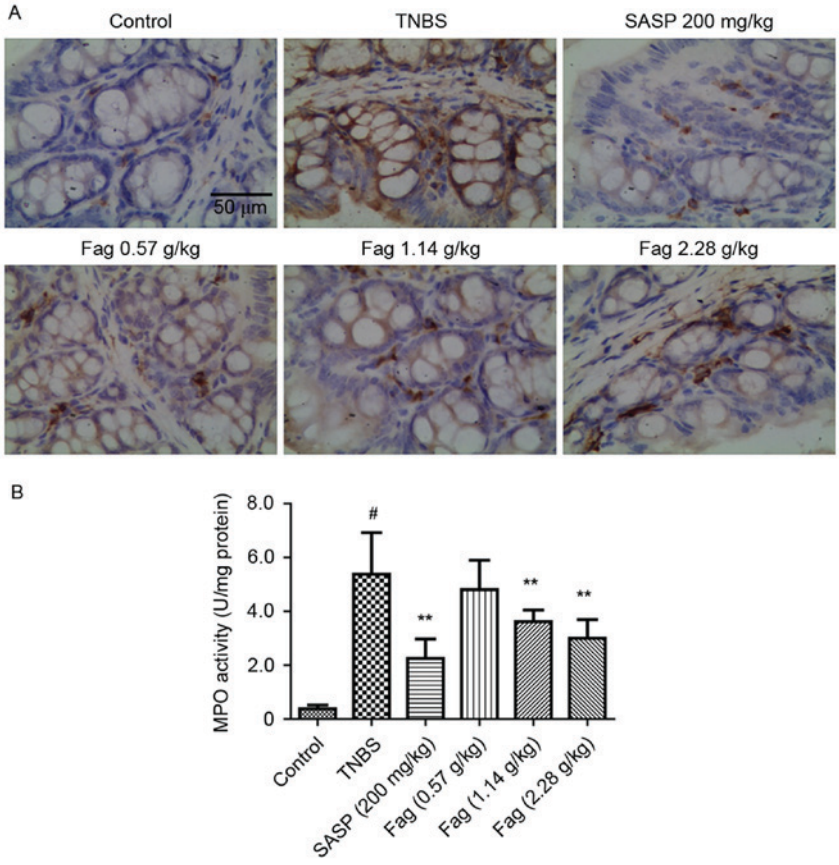

Figure 4. Fag attenuates inflammatory cell infiltration in TNBS-induced colitis mice. (A) Immunohistochemical analysis of F4/80-positive inflammatory cells. (B) Colonic myeloperoxidase activity in each group. n=6. $^{\#}$P<0.01 vs. control group and **P<0.01, vs. TNBS group. Fag, *Fagopyrum cymosum* (Trev.) Meisn; TNBS, 2,4,6-trinitrobenzenesulfonic acid; SASP, salicylazosulfapyridine.

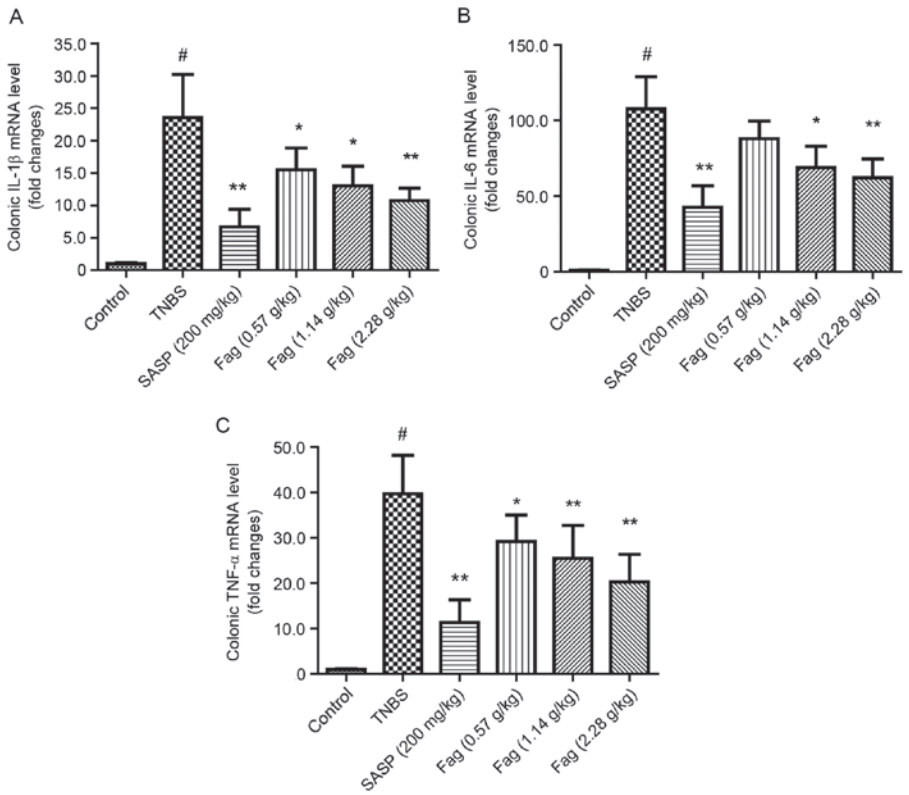

Figure 5. Fag reduces (A) IL-1β, (B) IL-6 and (C) TNF-α mRNA expression in the colon of TNBS-induced colitis mice. n=5. $^{\#}$P<0.01 vs. control group; *P<0.05 and **P<0.01, vs. TNBS group. Fag, *Fagopyrum cymosum* (Trev.) Meisn; IL, interleukin; TNF, tumor necrosis factor; TNBS, 2,4,6-trinitrobenzenesulfonic acid; SASP, salicylazosulfapyridine.

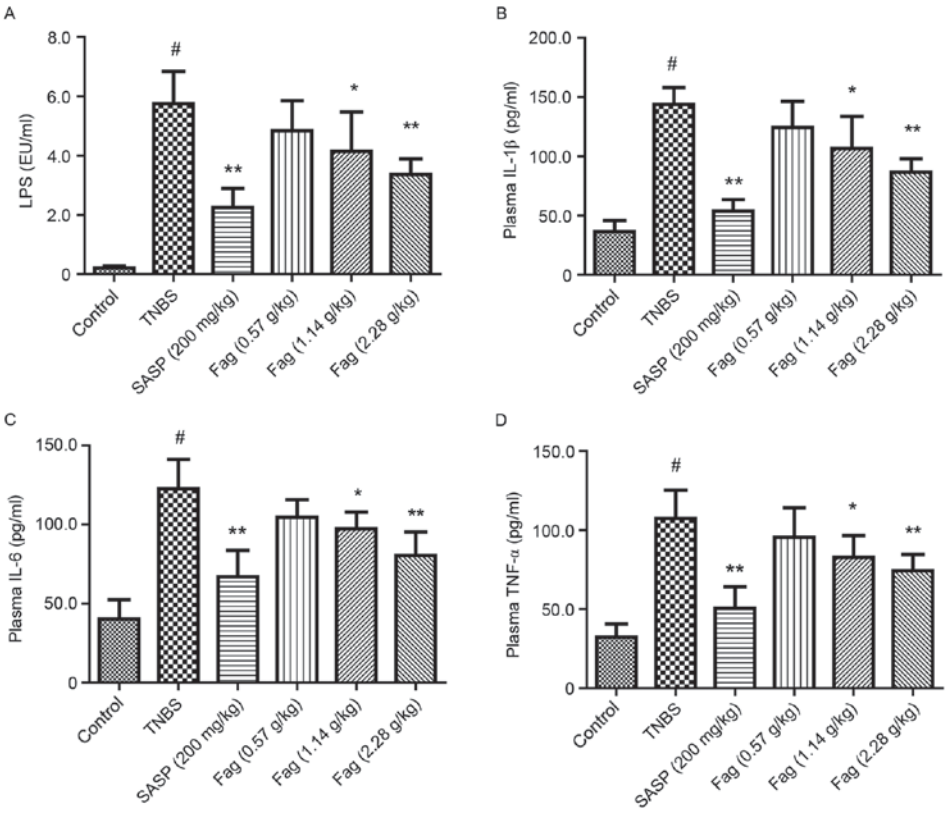

Figure 6. Fag decreases the plasma levels of (A) LPS, (B) IL-1β, (C) IL-6 and (D) TNF-α in TNBS-induced colitis mice. n=6. #P<0.01 vs. control group; *P<0.05 and **P<0.01, vs. TNBS group. Fag, *Fagopyrum cymosum* (Trev.) Meisn; LPS, lipopolysaccharide; IL, interleukin; TNF, tumor necrosis factor; TNBS, 2,4,6-trinitrobenzenesulfonic acid; SASP, salicylazosulfapyridine.

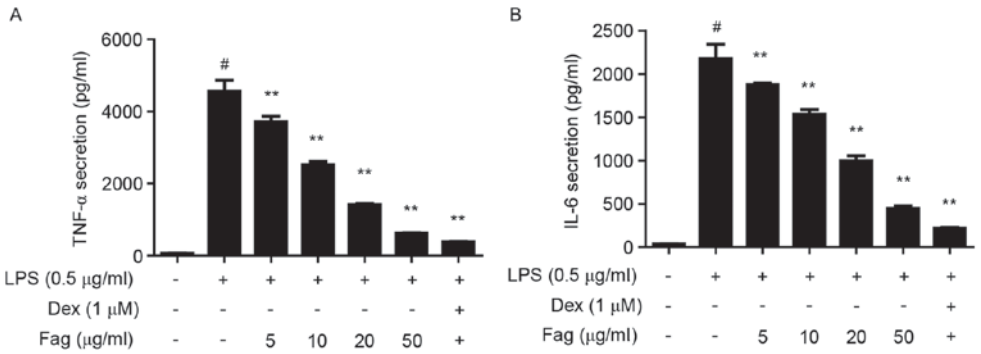

Figure 7. Fag inhibits the production of (A) TNF-α and (B) IL-6 in LPS-stimulated RAW264.7 cells. n=3. #P<0.01 vs. untreated RAW264.7 cells; **P<0.01 vs. LPS-stimulated RAW264.7 cells. Fag, *Fagopyrum cymosum* (Trev.) Meisn; TNF, tumor necrosis factor; IL, interleukin; LPS, lipopolysaccharide; Dex, dexamethasone.

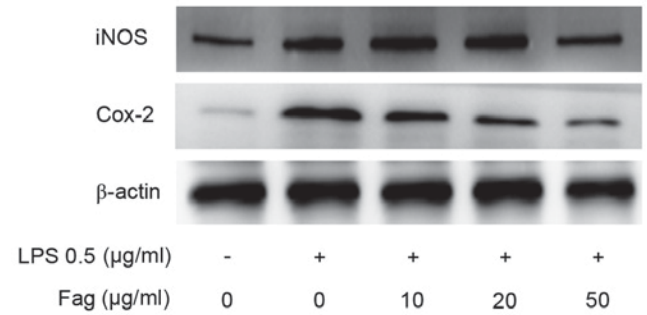

Figure 8. Fag inhibits the production of iNOS and Cox-2 in LPS-stimulated RAW264.7 cells. Fag, *Fagopyrum cymosum* (Trev.) Meisn; iNOS, inducible nitric oxide; Cox-2, cyclooxygenase-2; LPS, lipopolysaccharide.

Western blotting was used to investigate the effect of Fag on NF-κB activation signaling. The results revealed that Fag attenuated the LPS-induced nuclear translocation of p65 NF-κB in a dose-dependent manner (Fig. 9). Although the exact mechanism by which this occurs is not fully understood, the western blotting results indicate that Fag was able to inhibit IκBα phosphorylation in LPS-induced cells *in vitro* (Fig. 9).

Fag efficiently treatsv IBD with fewer side effects than SASP. The results of the clinical study demonstrate that jinqiao-maipian was able to ameliorate stomachache, diarrhea and mucous bloody stool with a total efficiency of ~80% (Table I).

Table I. Clinical study of jinqiaomaipian treatment for ulcerative colitis.

Group	Clinical manifestation	Total cases (n)	Complete remission (n)	Effective (n)	Invalid (n)	Total efficiency (%)
Experimental group	Stomachache	23	13	6	4	82.6
	Diarrhea	28	12	10	6	78.6
	Mucous bloody stool	24	11	8	5	79.2
	Colonoscopy	30	12	13	5	83.3
Control group	Stomachache	25	11	10	4	84.0
	Diarrhea	30	9	13	8	73.3
	Mucous bloody stool	22	10	8	4	81.8
	Colonoscopy	30	12	11	7	76.7

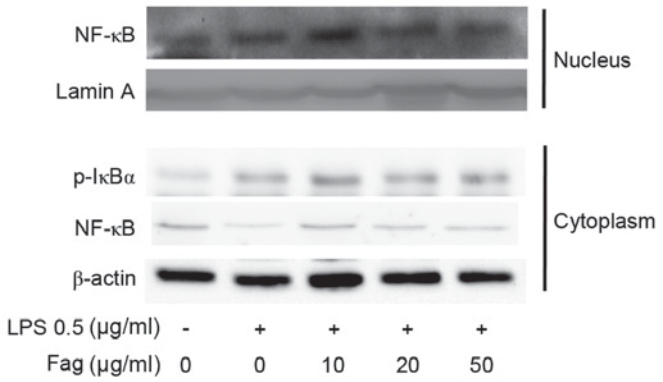

Figure 9. Fag inhibits the NF-κB p65 nuclear translocation and IκB phosphorylation in Raw264.7 cells. Fag, *Fagopyrum cymosum* (Trev.) Meisn; NF-κB, nuclear factor-κB; IκB, inhibitor of NF-κB; p, phosphorylated; LPS, lipopolysaccharide.

No significant difference was observed between the control and experimental groups, suggesting that Fag may be as effective as SASP for the treatment of IBD.

Of the 30 patients in the SASP group, 9 (30.0%) experienced side effects (3 nausea, 3 abdominal discomfort, 1 headache, and 2 leukopenia). However, only 1 out of 30 patients (3.3%) in the experimental group experienced nausea. The incidence of side effects was significantly lower in the experimental group ($P<0.01$; data not shown).

Discussion

Recent studies have demonstrated that IBD cannot be cured using medication, and treatment mainly focuses on increasing remission periods and improving patient quality of life (17,32). Novel pharmacological agents and natural medicines for treating IBD are urgently needed. TNBS-induced colitis in mice is a well-established animal model with an enhanced Th1/Th17 response (27). As the process is similar to the cluster of differentiation-related immune response (28), the TNBS-induced murine model has been widely used for screening IBD treatments and exploring the therapeutic effects of potential agents.

Fag is a traditional Chinese medicine used to treat bacterial dysentery, lung infection and rheumatism (22,24,26). It has previously been demonstrated that there are effective treatment components in the ethanol extract of Fag, including luteolin (22) and epicatechin (24). Fag has been reported to exert anti-inflammatory effects in Raw264.7 cells via inhibiting p38 and c-Jun N-terminal kinase phosphorylation (33) or inducing 67 kD alaminin receptor internalization (34). To the best of our knowledge, the present study is the first time that the anti-inflammatory effects of Fag extract on TNBS-induced colitis have been reported.

In the present study, the TNBS-induced mouse model was used to evaluate the protective effects of ethanol extract of Fag on colitis. The anti-inflammatory effects of Fag in LPS-induced RAW264.7 macrophages were also verified. The results demonstrated that oral administration of ethanol extract of Fag ameliorated TNBS-induced colitis in mice, reducing body weight loss and colon shortening. The staining results confirmed that Fag effectively decreased mucosal erosion, submucosal edema and disruption of crypts and villi, which are crucial for maintaining normal colonic function (25,35). These mucosal events significantly affect the structure and function of tight junctions, leading to a damaged intestinal barrier and immune activation (36). Subsequently, proinflammatory cytokines are overexpressed, triggering the signaling cascade of inflammation and leading to inflammatory cell infiltration in the inflamed mucosa (37). The results of the present study revealed that ethanol extract of Fag effectively reduced macrophage infiltration in the colon of TNBS-induced mice, with F4/80 and colonic MPO activity used as macrophage markers (35).

On the molecular level, the colonic levels of proinflammatory cytokines, including IL-1β, IL-6 and TNF-α, correlate with the degree of inflammation (35). These cytokines serve an important role in the pathogenesis of various inflammatory diseases (38,39). Colonic levels of IL-1β, IL-6 and TNF-α mRNA in TNBS-induced colitis mice were markedly reduced following treatment with Fag. Previous studies have also reported that IL-6 and TNF-α are able to significantly decrease barrier function by influencing tight junction proteins, including claudin, occludin, and zonula occludens-1 (40,41). The inflamed colon may result in gut leakage and bacterial translocation (37). In the present study, an increased plasma LPS level was identified in TNBS-induced colitis mice, suggesting that bacteria were translocated to the circulation via the leaky gut (42). The administration of Fag significantly

decreased the plasma LPS level in TNBS-treated mice, as well as the levels of IL-1β, IL-6 and TNF-α. To further verify the anti-inflammatory effects of Fag, an LPS-stimulated murine macrophage cell line was used. The results revealed that Fag significantly inhibited LPS-induced secretion of TNF-α and IL-6. Further experiments demonstrated that Fag inhibited the expression of proinflammatory cytokines via inhibiting the phosphorylation of IκB.

Compared with the conventional treatment for IBD, SASP, the incidence of side effects was significantly decreased with Fag treatment, whereas the therapeutic efficiency was similar. The results of the present study provide evidence that Fag may be a suitable alternative herbal medicine for the treatment of IBD in clinical practice, although identifying the optimal dosage requires further investigation.

In conclusion, the present study demonstrates that the ethanol extract of Fag exerts protective effects in TNBS-induced colitis mice via its anti-inflammatory action. These results indicate that Fag may be an effective therapeutic treatment for IBD.

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