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Total Glucosides of Paeony Attenuates Ulcerative Colitis via Inhibiting TLR4/NF-κB Signaling Pathway

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The therapeutic effects and mechanisms of action of total glucosides of paeony (TGP) in treating ulcerative colitis remain to be clarified. Mouse model of ulcerative colitis was treated with TGP and the indexes including scores of disease activity index, gross morphologic damage and histological damage, and inflammatory and oxidative stress markers were determined. Patients with ulcerative colitis received TGP capsule therapy and the indexes including efficacy of colonoscopy and histology, scores of Ulcerative Colitis Activity Index (UCAI) and Short Inflammatory Bowel Disease Questionnaire (SIBDQ), and inflammatory parameters were assessed. The expressions of toll-like receptor 4 (TLR4) and nuclear factor-kappa B (NF-κB) were measured in colonic tissues of mice and patients. TGP treatment significantly increased weight, decreased scores of disease activity index, gross morphologic damage and histological damage, and reduced the levels of tumor necrosis factor-α, interleukin-1β, malondialdehyde and myeloperoxidase in mouse model. Patients treated with TGP capsule had significantly higher relief rates of diarrhea, abdominal pain, and bloody purulent stool, decreased UCAI and increased SIBDQ scores, and lower levels of erythrocyte sedimentation rate, C-reactive protein and $CD4^+/CD8^+$ T-cell ratio than those patients with routine therapy. The overall response rate of TGP capsule was significantly higher than that of routine therapy. TGP treatment significantly suppressed the expressions of TLR4 and NF-κB in colonic tissues of both mouse model and patients with UC. TGP shows a good therapeutic effect on ulcerative colitis in animals and human patients, and the underlying mechanisms may be related to the inhibition of TLR4/NF-κB signaling by TGP.

Keywords: inflammation; nuclear factor-kappa B; toll-like receptor; total glucosides of paeony; ulcerative colitis
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Introduction

Ulcerative colitis (UC) is a non-specific intestinal disease characterized by colonic mucosal-submucosal inflammation, beginning from the rectum and extending proximally to the colon. The clinical symptoms of UC are diverse, including diarrhea, abdominal pain, bloody stool, fecal urgency, and tenesmus (Feuerstein et al. 2019). UC not only reduces the quality of life of patients, but also increases the risk of colorectal cancer (Kim et al. 2021; Linson and Hanauer 2021). The pathogenesis of UC is not yet fully elucidated, and many factors are thought to be implicated in the occurrence and development of this disease, e.g., inflammation (Mandlik et al. 2021; Mohamed et al. 2021; Oladele et al. 2021), oxidative stress (Mandlik et al. 2021; Mohamed et al. 2021; Oladele et al. 2021), immune dysfunction (Xue et al. 2021), genetic variation (Yoganathan et al. 2021), intestinal microbiota (Tong et al. 2021), and apoptosis (Mohamed et al. 2021). The main therapeutic drugs for UC are salicylic acids, immunosuppressants and corticosteroids, but their clinical cure rates are usually unsatisfactory, and it is easy to relapse after

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drug withdrawal. In addition, the adverse reactions caused by these drugs could not be ignored, e.g., leucopenia caused by 5-aminosalicylic acid (Qiu et al. 2015; Santos et al. 2022), myelosuppression caused by immunosuppressant (Zudeh et al. 2021; Prabha and Khare 2022), and osteoporosis caused by corticosteroid (Laurent et al. 2022; Migliorini et al. 2022). Some rare and severe adverse reactions have also been reported, e.g., acute pancreatitis caused by 5-aminosalicylic acid (Kim et al. 2007; Correia et al. 2021) and fatal rhinocerebral mucormycosis caused by corticosteroid and azathioprine (Najafi et al. 2019). Due to these concerns, there have been increasing attempts to use Chinese patent drugs as alternatives to the routine therapeutic drugs for UC therapy in recent years, and usually they are more acceptable to patients in China.

Paeonia lactiflora Pall, a famous traditional Chinese herbal medicine, has a long history of effective and safe use in treating autoimmune diseases such as rheumatoid arthritis. *Paeonia lactiflora* Pall is generally used as a sovereign drug in combination with other traditional Chinese medicines (respectively called minister drug, assistant drug and courier drug) to form a compound recipe - Paeonia decoction. The formula of Paeonia decoction recorded in the ancient Chinese medical book "Su Wen Bing Ji Qi Yi Bao Ming Ji" is as follows: Paeonia lactiflora 30 g, Angelica sinensis 15 g, Coptis chinensis Franch 15 g, Areca catechu L. 6 g, Aucklandia lappa Decne. 6 g, Glycyrrhiza uralensis Fisch. 6 g, Rheum palmatum L. 9 g, Scutellaria baicalensis Georgi 15 g, and Cinnamomum wilsonii Gamble 7.5 g. Paeonia decoction is commonly used to treat bacillary dysentery, amoebic dysentery, allergic colitis, acute enteritis, etc. The adverse effects of Paeonia decoction were rarely observed in clinical practice, with occasional adverse reactions such as rash and slight gastrointestinal discomfort. Generally, special treatment is not required, and the adverse reactions will soon disappear by themselves (Chen 2014; Luo et al. 2022).

Total glucosides of paeony (TGP) is a group of biologically active natural compounds isolated from the roots of *Paeonia lactiflora* Pall. Approximately 15 compounds have been identified in TGP, among them paeoniflorin is the most common constituent (> 90%), and the other constituents include abbiflorin, hydroxyl-paeoniflorin, paeonin, benzoyl-paeoniflorin and so on. As a mixture of a group of monomer molecules, TGP has been reported to have a wide range of pharmacological activities such as anti-inflammation (Chen et al. 2019; Li et al. 2019a; Li et al. 2019b; Chen et al. 2020), anti-oxidation (Su et al. 2010; Luo et al. 2013) and anti-apoptosis (Chen et al. 2020; Li et al. 2020b; Shen et al. 2020). TGP capsule was the first anti-inflammatory and immune-regulatory drug approved for the therapy of rheumatoid arthritis in China (National Medicine Permission Number: H20055058). Except for rheumatoid arthritis, some studies have shown that TGP was effective for treating several other autoimmune diseases such as Sjögren's syndrome (Li et al. 2013; Zhou et al. 2016b; Li et al. 2020a; Liu et al. 2020), psoriasis (Chen et al. 2016; Yu et al. 2017), inflammatory bowel disease (Lin et al. 2017; Cao et al. 2021) and oral lichen planus (Zhou et al. 2016a). Therefore, we hypothesized that TGP could also be effective in treating UC which is currently considered as an autoimmune disease.

In this study, we examined whether TGP attenuates UC symptoms in a UC mouse model induced by oxazolone, and in patients with mild to moderate UC. Moreover, we explored the underlying mechanisms by which TGP improved the symptoms of UC from the perspective of inhibition of inflammatory signaling pathway.

Materials and Methods

Induction of UC mouse model

After one week of adaptive feeding, BALB/C male mice (8 weeks old) were randomly divided into five groups (n = 10 in each group): normal control group, UC model group, salicylazosulfapyridine (SASP) treatment group, low-dose TGP treatment group (TGP-L), medium-dose TGP treatment group (TGP-M), and high-dose TGP treatment group (TGP-H). UC model was induced as follows: the abdomen was shaved to expose a 2 cm × 2 cm hairless area, and 0.2 mL of 3% oxazolone (Sigma, St. Louis, MO, USA; w/v, dissolved in absolute ethanol) was dripped onto the hairless skin for sensitization for two consecutive days. Five days later, the mice were anesthetized, and a silica gel tube with a diameter of about 2 mm was inserted into the intestine from the anus for about 4 cm, and 0.2 mL of 3% oxazolone (w/v, dissolved in 50% ethanol) was injected slowly. After injection, the mice were hanging upside down for 30 s to prevent reflux of oxazolone solution. TGP-L, TGP-M and TGP-H groups were intragastrically treated with 60, 120 and 240 mg/kg/day TGP (Lihua Pharmaceutical, Ningbo, China; dissolved in 5% carboxymethyl cellulose sodium), respectively, one hour before oxazolone enema, and continued at the same time point in the next three days. SASP group was intragastrically treated with 240 mg/kg/day SASP (Sinopharm Chemical Reagent, Shanghai, China; dissolved in 5% carboxymethyl cellulose sodium). Mice in normal control and UC model groups were treated with an equal volume of 5% carboxymethyl cellulose sodium. Finally, mice were anesthetized with intraperitoneal injection of ketamine/xylazine solution and sacrificed, and histological, immunological as well as molecular indexes were determined. The animal experiment protocol was reviewed and approved by the Ethics Committee of the Affiliated Hospital of Chengdu University. The tenets of the Declaration of Helsinki were adhered to in all procedures reported in the article.

Human patients

A total of 50 consecutive and unrelated Chinese adult patients with chronic active UC at the Department of Gastroenterology, Affiliated Hospital of Chengdu University (Chengdu, China) were enrolled in the study between

January 2019 and December 2020. The baseline demographic and clinical characteristics of the patients are shown in Table 1. The inclusion criteria were as follows: 1) patients diagnosed based on the consensus on diagnosis and treatment of inflammatory bowel disease established by the Inflammatory Bowel Disease Group of Gastroenterology Branch of Chinese Medical Association (2018), 2) patients with mild-to-moderate chronic active UC, and 3) patients who did not use immunosuppressants or hormones in recent one month. The exclusion criteria were as follows: 1) patients with severe heart, liver or kidney disease, hematological disease, or endocrinological disorder, 2) pregnant or lactating woman, 3) patients who were allergic to salicylic acid preparation, 4) patients who did not follow the doctors' advice or cooperate with the therapy, and 5) patients whose clinical symptoms worsened during therapy, and thereby comprehensive therapy was used. Signed informed consent form was provided by each of the patients or their guardians prior to participation in the study. Fifty patients were randomly divided into two groups according to their wishes: routine therapy group and TGP capsule therapy group. Patients in the routine therapy group were treated with 1.0 g of mesalazine orally, three times a day. Patients in the TGP capsule therapy group were treated with 1.0 g of mesalazine plus 0.6 g of TGP orally, three times a day. The therapy lasted for 8 weeks. All patients were told to avoid drinking alcohol and eating spicy food during the therapy period. The study protocol with human patients was reviewed and approved by the Ethics Committee of the Affiliated Hospital of Chengdu University. The tenets of the Declaration of Helsinki were adhered to in all procedures reported in the article.

Assessment of disease activity index in mice

Disease activity index was employed to assess the progression of UC in mice. Weight loss, diarrhea, stool shape and stool bleeding were monitored daily for calculation of disease activity index score during the experiment period. Disease activity index score was calculated as the average value of weight-loss score, stool-shape score and stool-bleeding score. Weight-loss score was given 0 for no loss, 1 point for 1%-5% loss, 2 points for 6%-10% loss, 3 points for 10%-20% loss, and 4 points for over 20% loss. Stool-shape score was given 0 for normal stool, 2 points for loose stool, and 4 points for diarrhea. Stool-bleeding score was given 0 for no bleeding, 1 point for extremely light bleeding, 2 points for mild bleeding, 3 points for obvious bleeding, and 4 points for massive hemorrhage.

Assessment of gross morphologic damage of the colon in mice

The colon was removed and opened longitudinally. Gross morphologic damage score was measured by a blinded observer based on the following scoring system: 0 for normal colon, 1 point for mucosal congestion, 2 points for ulcer area < 25%, 3 points for 25%-50% ulcer area, and 4 points for ulcer area > 50%.

Assessment of histological damage of the colon in mice

About 0.5-cm colonic fragment was cut off (1 cm above the anus), cleaned with normal saline, fixed with 4% paraformaldehyde, dehydrated, and embedded in paraffin. The paraffin-embedded tissues were cut into 4 μm sections, stained with hematoxylin-eosin, and observed under a light microscope by two pathologists who were unaware of the experimental treatments. Histological damage score was calculated according to the presence of ulcer, edema and bleeding, degree and depth of inflammatory cell infiltration, formation of fibrosis and pseudomembrane, crypt disappearance, and mucin reduction (Han et al. 2001). The

Table 1. Baseline characteristics of the study patients.

Variables	Routine therapy group (n = 25)	TGP therapy group (n = 25)	*P* value
Age, years	40.1 ± 2.55	38.2 ± 2.75	0.89
Male/female	16/9	15/10	0.77
Disease course, years	3.1 ± 0.41	2.7 ± 0.49	0.67
Appendectomy, n (%)	4 (16.00)	5 (20.00)	0.71
Clinical type			
Chronic relapse, n (%)	19 (76.00)	17 (68.00)	0.52
Initial onset, n (%)	1 (4.00)	1 (4.00)	1.00
Lesion range			
Rectosigmoid colon, n (%)	11 (44.00)	9 (36.00)	0.56
Left hemicolon, n (%)	8 (32.00)	8 (32.00)	1.00
Total colon, n (%)	6 (24.00)	8 (32.00)	0.52
Severity grading			
Mild, n (%)	8 (32.00)	7 (28.00)	0.75
Moderate, n (%)	17 (68.00)	18 (72.00)	0.75

Data are presented as mean ± standard error of the mean, or n (%).

scores of histological damage range from 0 to 25 points, with 0 representing normal colon and 25 points representing the most severe damage.

Measurement of inflammatory and oxidative stress indexes in mice

Serum levels of tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β) as well as colonic contents of malondialdehyde and myeloperoxidase were measured to examine the anti-inflammatory and anti-oxidative properties of TGP. The serum levels of TNF-α and IL-1β were measured by using ELISA kits (Jiangsu Jingmei Biotechnology, Yancheng, China) according to manufacturer's protocols, and both of them were expressed as ng/L. Colonic tissues were homogenized in cool normal saline, and the centrifuged supernatants were collected for assays of malondialdehyde and myeloperoxidase. The concentrations of total proteins in supernatants were measured by using BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Concentrations of malondialdehyde and myeloperoxidase in supernatants were measured by using malondialdehyde detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and myeloperoxidase activity detection kit (Nanjing Jiancheng Bioengineering Institute), respectively, according to manufacturer's protocols. Malondialdehyde content in colonic tissues was expressed as nmol/mg protein, and myeloperoxidase activity in colonic tissues was expressed as U/g tissue.

Clinical, colonoscopic and histologic evaluation in patients with UC

Indexes including relief rates of diarrhea, abdominal pain and bloody purulent stool, efficacy of colonoscopy and histology, and scores of Ulcerative Colitis Activity Index (UCAI) and Short Inflammatory Bowel Disease Questionnaire (SIBDQ) were determined to evaluate the efficacy of TGP capsule on UC in patients. Relief rates of diarrhea, abdominal pain and bloody purulent stool were calculated by dividing the numbers of patients with diarrhea relief, abdominal pain relief and bloody purulent stool relief, respectively, by the total number of patients. UCAI score was assessed by using Sutherland's scoring system (Sutherland et al. 1987), in which four variables (stool frequency, rectal bleeding, mucosal appearance and physician's rating of disease activity) were examined. Each variable has a range of values from 0 to 3 points, with 0 point representing normal and 3 points representing the most severe symptoms. The UCAI score ranges from 0 to 12 points. Efficacy of colonoscopy was assessed by using Baron's grading system (Baron et al. 1964), and efficacy of histology was assessed by using Truelove-Richards's grading system (Truelove and Richards 1956). SIBDQ was originally developed by Guyatt et al. (1989) to assess the quality of life of patients with inflammatory bowel disease. Later, Jowett et al. (2001) reported that it was a reliable index to assess the quality of life of patients with UC. SIBDQ uses 10 questions to find out how patients have been feeling during the last two weeks. The SIBDQ scores range from 10 points (worst feeling) to 70 points (best feeling).

Measurement of serum inflammatory indexes in patients with UC

Inflammatory indexes such as erythrocyte sedimentation rate, C-reactive protein, α1-acid glycoprotein, $CD3^+$ T cell, $CD4^+$ T cell, $CD8^+$ T cell and $CD4^+/CD8^+$ T-cell ratio were determined to examine the anti-inflammatory properties of TGP in UC patients. Erythrocyte sedimentation rate was measured by using an ESR-30 fully automatic dynamic analyzer (Shanghai Xunda Medical Instrument, Shanghai, China). Serum C-reactive protein was measured by using a Human C-reactive protein ELISA Kit (Solarbio Science & Technology, Beijing, China). Serum α1-acid glycoprotein was measured by using turbidimetric inhibition immunoassay in an automatic chemistry analyzer (Roche Molecular Systems, Bassel, Switzerland). Peripheral blood T-cell subsets including $CD3^+$ T cells, $CD4^+$ T cells and $CD8^+$ T cells were blocked with 5% human serum (Sigma) prior to staining with antibodies against cell surface antigens. Flow cytometry was performed by using Cell Lab Quanta SC flow cytometry (Beckman Coulter, Breya, CA, USA). $CD4^+/CD8^+$ T-cell ratios were calculated.

Determination of TLR4 and NF-κB expressions in colonic tissues of mice and human patients with UC

The expressions of TLR4 and NF-κB p65 in colonic tissues of mice and patients with UC were examined to investigate the possible mechanisms underlying the therapeutic effects of TGP on UC. Immunohistochemistry, western blot and quantitative real-time PCR were employed to determine the expressions of toll-like receptor 4 (TLR4) and nuclear factor-kappa B (NF-κB) p65 in colonic tissues. The colon tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 4 μm for immunohistochemistry staining. After incubation with xylene and descending concentrations of ethanol, antigens were retrieved by citrate buffer for 15 min at 100℃. Endogenous peroxidases were removed in 3% H_2O_2 at room temperature followed by blocking with 5% goat serum at 37℃. Subsequently, sections were incubated with rabbit anti-mouse or anti-human TLR4 and NF-κB p65 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in an optimum concentration overnight and then incubated with horseradish peroxidase-conjugated secondary antibody at 37℃. Finally, the sections were counterstained with hematoxylin, dehydrated in ascending concentrations of ethanol, cleared in xylene, and mounted using Harleco synthetic resin. Images were acquired with a microscope (Olympus Corporation, Tokyo, Japan). Total proteins were extracted from colonic tissues for western blot experiments. Proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide gel, and transferred onto a polyvinylidene fluoride

membrane (Millipore, Boston, MA, USA). The membranes were incubated with the primary antibodies against TLR4 and NF-κB p65 overnight at 4°C after blocking with 5% non-fat milk in Tris-buffered saline with 0.1% Tween® 20 detergent (TBST) buffer at room temperature. The TLR4 and NF-κB p65 proteins were detected by using ECL reagents after incubation with the secondary antibody at room temperature. For quantitative real-time PCR analysis, total RNAs were extracted from colonic tissues by using TRIzol reagent according to the manufacturer's protocol. Reverse transcription was carried out by RevertAid First Strand cDNA synthesis kit (Thermo Scientific Fermentas, Waltham, MA, USA). The mRNA levels of TLR4 and NF-κB p65 were quantified with SYBR Green Realtime PCR Master Mix (Toyobo, Osaka, Japan) and an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Waltham, MA, USA). GAPDH was used as a reference control. The relative gene expression levels were calculated by the $2^{-\Delta\Delta Cq}$ method.

Statistical analysis

Data are expressed as means ± standard error of the mean from triplicate experiments. SPSS 17.0 (SPSS Inc., Chicago, IL, USA) was used for significance of difference analysis ($P < 0.05$). Differences among groups were analyzed by Chi-square test for categorical variables, and two-tailed *t* test for continuous variables.

Results

TGP improved the symptoms of oxazolone-induced UC in mice

Fig. 1A shows the effect of TGP on body weight. Body weight of UC model group decreased sharply after oxazolone enema, but it was effectively rescued upon TGP-M, TGP-H or SASP treatment. TGP-M, TGP-H and SASP groups displayed a higher body weight than UC model group at days 2, 3 and 4 after treatment ($P < 0.05$ for all). Fig.1B displays the effect of TGP on disease activity index score. Disease activity index score of UC model group increased gradually after oxazolone enema, and reached its maximum value at day 3. TGP-M, TGP-H and SASP groups had lower disease activity index scores than UC model group at days 2, 3 and 4 after treatment ($P < 0.05$ for all). Fig. 1C demonstrates the effect of TGP on gross mor-

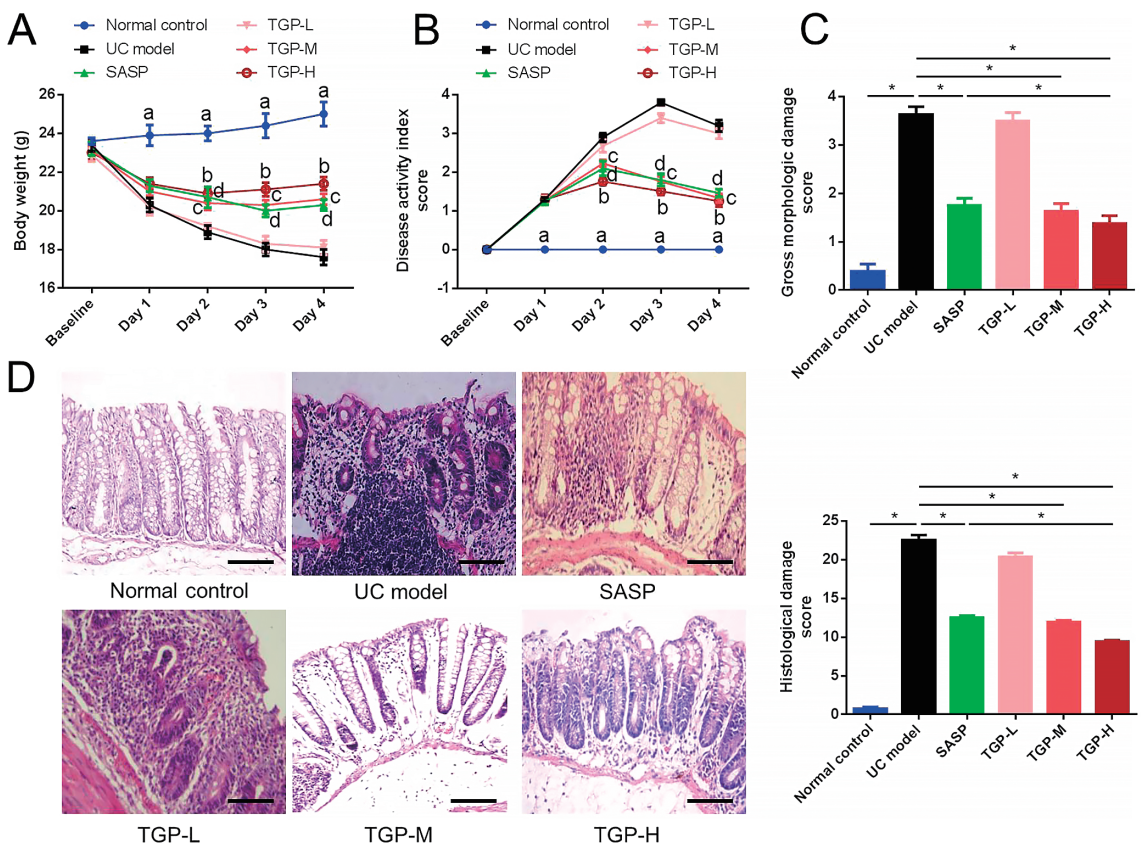

Fig. 1. Effects of total glucosides of paeony (TGP) on ulcerative colitis (UC) symptoms in mice.
(A, B) Weight loss (A) and disease activity index scores (B) after treatment in each group (n = 10, $^{a,b,c,d}P < 0.05$ compared with that of UC model group at the same time point). (C, D) Gross morphologic damage scores (C) and representative hematoxylin-eosin staining images of colonic tissues as well as histological damage scores of colonic tissues (D) after treatment in each group (n = 10, $^{*}P < 0.05$).
SASP, salicylazosulfapyridine; TGP-L, low-dose TGP treatment; TGP-M, medium-dose TGP treatment; TGP-H, high-dose TGP treatment. Scale bars, 100 μm. Data are represented as mean ± standard error of the mean.

phologic damage score. Colons in UC model group were dilated to varying degrees, and this group had higher gross morphologic damage score than normal control group ($P < 0.05$). Mice in TGP-M, TGP-H and SASP groups had basically regular colons, and their gross morphologic damage scores were lower than those in UC model group ($P < 0.05$ for all). Fig. 1D shows the effect of TGP on histological damage score. Mice in UC model group had damaged mucosal epithelium and deformed glands, and higher histological damage scores than those in normal control group ($P < 0.05$). TGP-M, TGP-H and SASP groups had lower histological damage scores than UC model group ($P < 0.05$ for all). TGP-H group displayed intact mucosal epithelium and orderly arranged glands, and had lower histological damage score than SASP group ($P < 0.05$). The gross morphological and histological features of TGP-L group were roughly the same as those of UC model group.

TGP suppressed pro-inflammatory cytokines and oxidative stress factors in UC mice

As shown in Fig. 2, UC model group had higher levels of TNF-α, IL-1β, malondialdehyde and myeloperoxidase than normal control group ($P < 0.05$ for all). High doses of TGP or SASP treatment decreased the levels of TNF-α, IL-1β, malondialdehyde and myeloperoxidase as compared to UC model group ($P < 0.05$ for all). Mice in TGP-M group had significantly lower levels IL-1β, malondialdehyde and myeloperoxidase ($P < 0.05$ for all), but insignificantly reduced levels of TNF-α ($P > 0.05$) than those in UC model group. The levels of TNF-α, IL-1β, malondialdehyde and myeloperoxidase in TGP-L group were slightly lower than those in UC model group, but the differences were all not statistically significant ($P > 0.05$).

TGP improved the clinical symptoms and quality of life in patients with UC

As shown in Fig. 3, patients with TGP capsule therapy had higher relief rates of diarrhea at week 8 (Fig. 3A), abdominal pain at weeks 4 and 8 (Fig. 3B), and bloody purulent stool at week 8 (Fig. 3C) than those patients with routine therapy ($P < 0.05$ for all). UCAI scores decreased significantly in both routine and TGP capsule therapy groups, but the decrease was greater in TGP capsule therapy group (Fig. 3D). Evaluation of colonoscopy showed that the overall response rate of TGP therapy group was higher than that of routine therapy ($P < 0.05$) (Fig. 3E). Evaluation

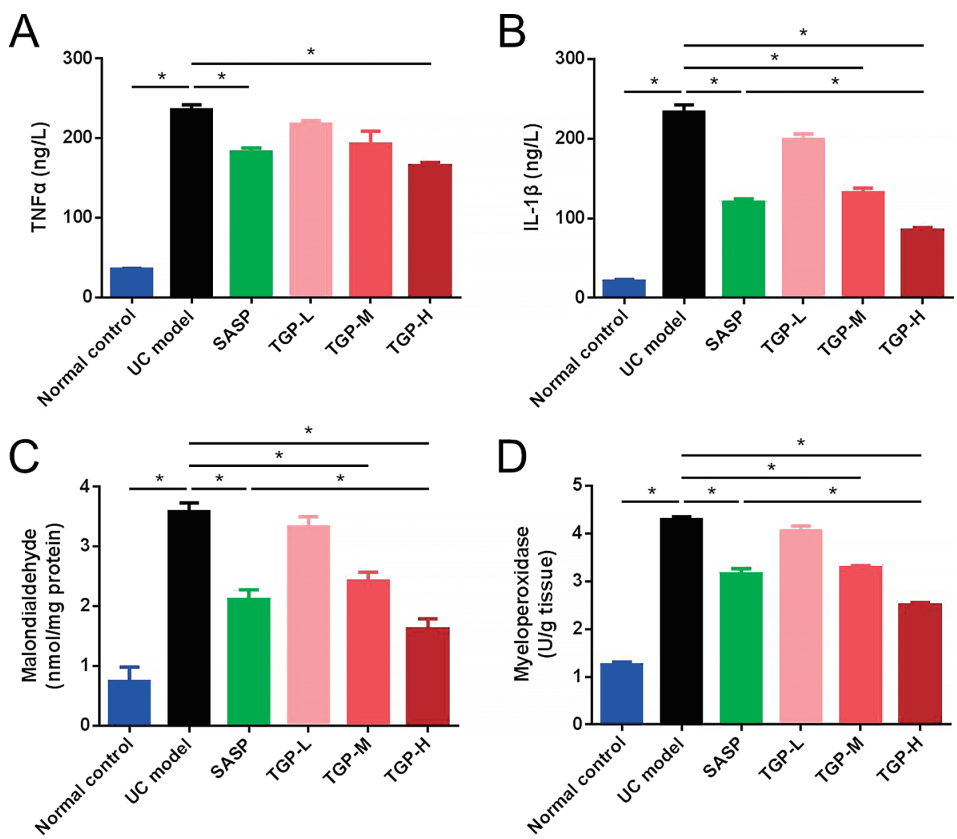

Fig. 2. Effects of total glucosides of paeony (TGP) on inflammatory and oxidative stress indexes in mice.
(A-D) Serum levels of TNF-α (A) and IL-1β (B), and colonic contents of malondialdehyde (C) and myeloperoxidase (D) after treatment in each group (n = 10, $^{*}P < 0.05$).
SASP, salicylazosulfapyridine; TGP-L, low-dose TGP treatment; TGP-M, medium-dose TGP treatment; TGP-H, high-dose TGP treatment; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β. Data are presented as mean ± standard error of the mean.

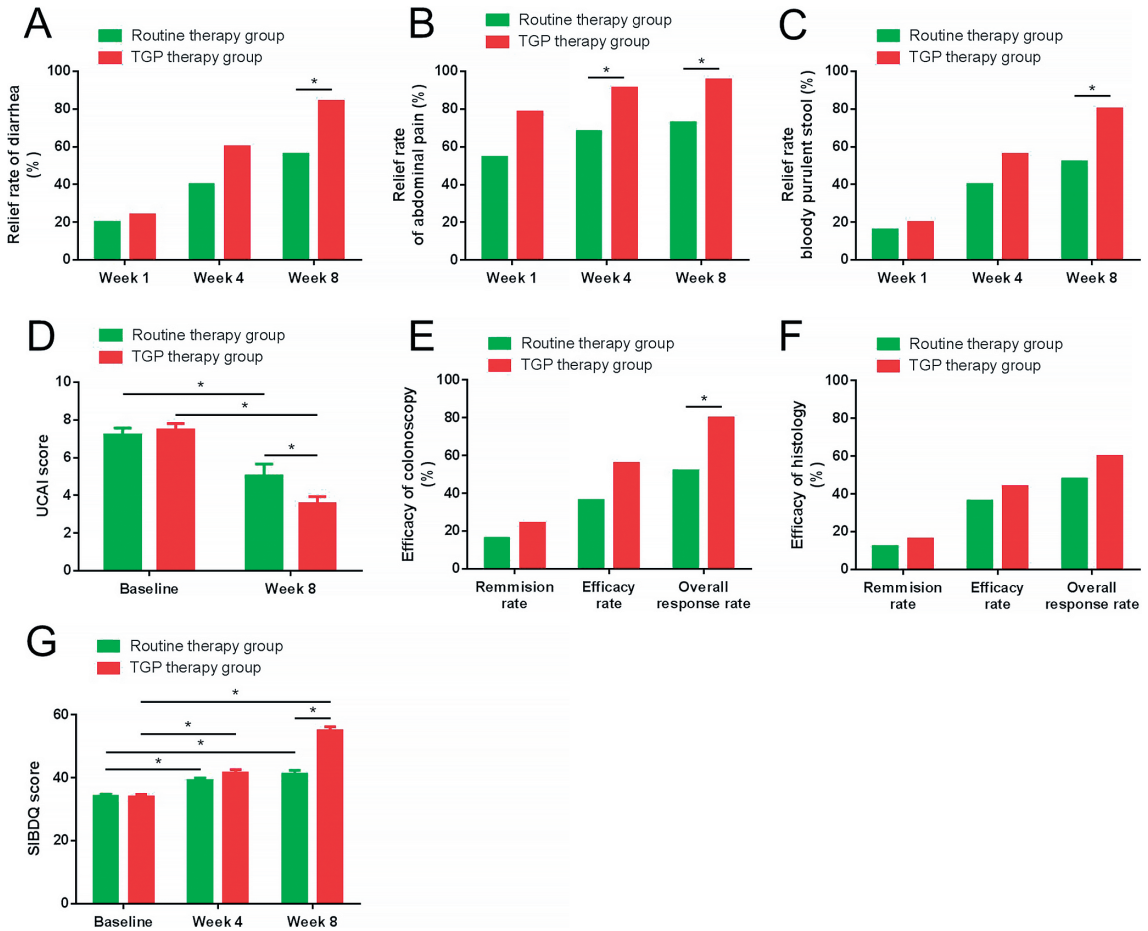

Fig. 3. Effects of total glucosides of paeony (TGP) on clinical symptoms in patients with ulcerative colitis (UC). (A-F) Relief rates of diarrhea (A), abdominal pain (B) and bloody purulent stool (C), UCAI scores (D), efficacy of colonoscopy (E) and histology (F) of the colon, and SIBDQ scores (G) after therapy in each group (n = 25, $^{*}P < 0.05$). TGP, total glucosides of paeony; UCAI, ulcerative colitis activity index; SIBDQ, Short Inflammatory Bowel Disease Questionnaire. Data are presented as mean ± standard error of the mean.

of histology indicated that TGP therapy group had higher rates of remission, efficacy and overall response than routine therapy group, but the differences were not statistically significant (Fig. 3F). SIBDQ scores increased orderly with time during the period of therapy in both groups, but TGP capsule therapy group had a faster increase and higher SIBDQ score than routine therapy group at week 8 ($P < 0.05$) (Fig. 3G).

TGP reduced inflammatory parameters in patients with UC

The levels of erythrocyte sedimentation rate (Fig. 4A), C-reactive protein (Fig. 4B) and α1-acid glycoprotein (Fig. 4C) decreased orderly and all the three parameters reached a significant level at week 8 in TGP capsule therapy group ($P < 0.05$ for all), but only C-reactive protein (Fig. 4B) ($P < 0.05$) experienced a significant decrease at week 8 in routine therapy group. $CD4^+$ T cell (Fig. 4D) and $CD4^+/CD8^+$ T-cell ratio (Fig. 4E) experienced a significant decrease in both groups at week 8 after therapy. There were no significant differences in the levels of $CD8^+$ T cell (Fig. 4F) and $CD3^+$ T cell (Fig. 4G) in both groups after therapy.

TGP downregulated the expressions of TLR4 and NF-κB in colonic tissues of mice and patients with UC

Mice in UC model group displayed increased expressions of TLR4 and NF-κB p65 in colonic tissues than normal control group, as indicated by higher levels of TLR4 and NF-κB p65 proteins (Fig. 5A, B) and mRNAs (Fig. 5C) in UC mouse model group. However, TGP-M, TGP-H or SASP treatment decreased the expressions of TLR4 and NF-κB p65 as compared to UC mouse model group (Fig. 5B, C) ($P < 0.05$ for all). TGP-L group had lower expressions of TLR4 and NF-κB p65 than UC mouse model group, but it did not reach a statistically significant level (Fig. 5B, C). The mice treated with high dose of TGP had significantly decreased expressions of TLR4 and NF-κB p65 in colonic tissues than those mice treated with SASP (Fig. 5B, C). As for the expressions of TLR4 and NF-κB p65 in colonic tissues of human patients with UC, both of them were effectively inhibited after eight weeks of treatment with TGP capsule (Fig. 5D).

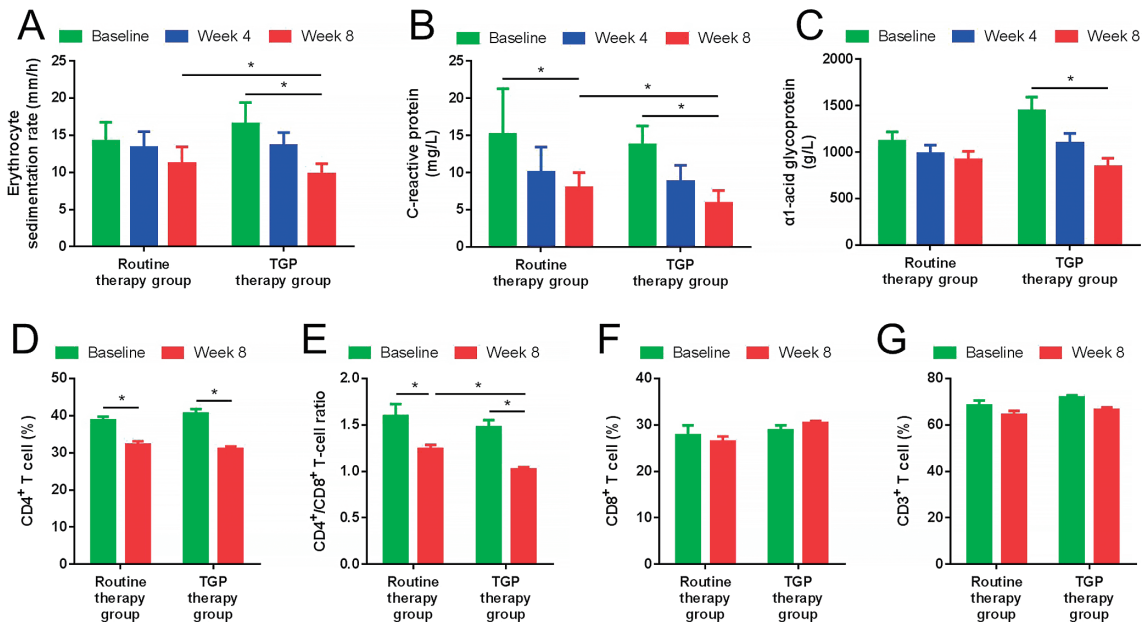

Fig. 4. Effects of total glucosides of paeony (TGP) on inflammatory parameters in patients with ulcerative colitis (UC). (A-G) Levels of erythrocyte sedimentation rate (A), C-reactive protein (B), α1-acid glycoprotein (C), $CD4^+$ T (D), $CD4^+/CD8^+$ T-cell ratio (E), $CD8^+$ T (F), and $CD3^+$ T (G) after therapy in each group (n = 25, $^*P < 0.05$). Data are presented as mean ± standard error of the mean.

Occurrence of adverse reactions after therapy in patients with UC

There were three cases of adverse reactions in TGP capsule therapy group with an incidence of 12%, and five cases of adverse reactions in routine therapy group with an incidence of 20%. The adverse reactions in TGP capsule therapy group were mild nausea and diarrhea, which subsequently disappeared without special treatment. The adverse reactions in routine therapy group were epigastric pain, anorexia, nausea and leucopenia, which were tolerated by most patients, but a few patients were not relieved of epigastric pain until the acid suppression treatment.

Discussion

The present study demonstrates that TGP has a good therapeutic effect on UC in animal model and human patients with UC. TGP treatment significantly decreased the scores of disease activity index, gross morphologic damage and histological damage in UC mouse model, and improved the symptoms of UC such as diarrhea, abdominal pain and bloody purulent stool in human patients. To our knowledge, this is the first demonstration that TGP is effective in treating UC in animal model and human patients with UC, although several studies indicated that TGP was able to suppress the intestinal inflammation in animal models (Lin et al. 2017; Liu et al. 2020; Cao et al. 2021). In line with our findings, Cao et al. (2021) reported that TGP significantly reduced the scores of disease activity index and histological damage, and increased body weight after 10 days of treatment in a mouse model of colitis induced by dextran sulphate sodium. In a rat model of colitis induced by 2,4,6-trinitrobenzene sulfonic acid, TGP significantly improved the symptoms of colitis such as diarrhea and stool bleeding and increased body weight after 7 days of treatment (Lin et al. 2017). However, our study investigated the therapeutic effect of TGP on UC not only in mouse model but also in human patients with UC, which is much closer to the actual situation and can provide more information for clinical practice.

In the present study, it was found that TGP significantly inhibited the production of pro-inflammatory cytokines such as TNF-α and IL-1β and oxidative stress factors such as malondialdehyde and myeloperoxidase in UC mouse model, and that TGP capsule significantly decreased the inflammation-related indicators such as erythrocyte sedimentation rate, C-reactive protein, α1-acid glycoprotein, $CD4^+$ T cells and $CD4^+/CD8^+$ T-cell ratios in patients with UC, which lays the foundation for its efficacy in treating UC since both inflammation and oxidative stress are closely involved in the occurrence and development of UC (Liu et al. 2021; El-Tanbouly and Abdelrahman 2022). Anti-inflammatory and antioxidant compounds have also been shown to have therapeutic effects on UC (Abdelhamid et al. 2022; Eskandari et al. 2022). Abdelhamid et al. (2022) investigated the potential coloprotective role of carbocisteine in acetic acid-induced UC in rats, and found that carbocisteine had anti-inflammatory and antioxidant activities, and significantly improved the histologic and macroscopic features of colon tissues. Eskandari et al. (2022) explored the protective effects of mebendazole, an FDA-approved drug, against DSS-induced UC in a murine model, and observed that mebendazole significantly reduced oxidative stress markers, suppressed inflammatory cell infiltration and downregulated inflammatory genes in colon tissues,

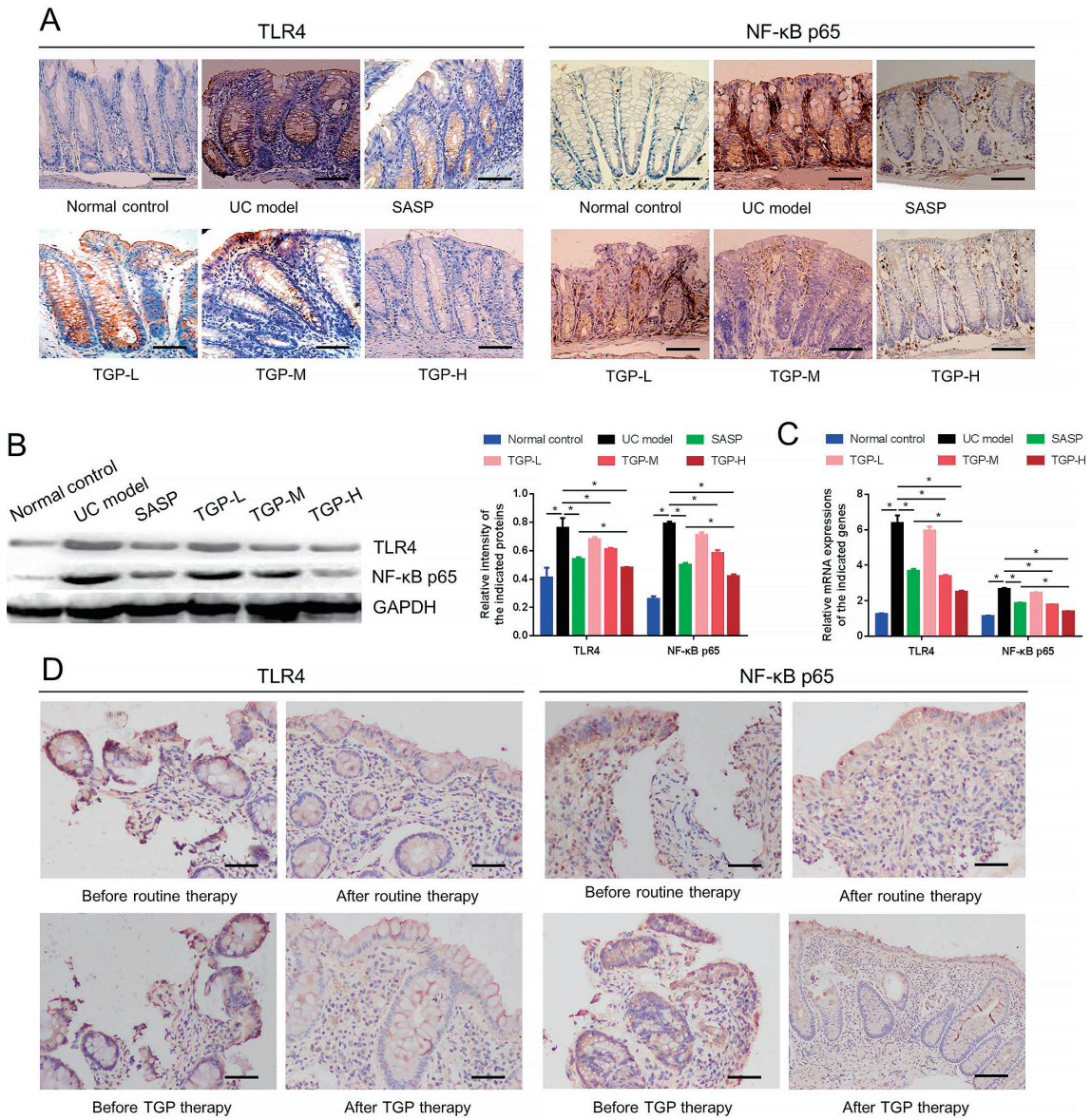

Fig. 5. Effects of total glucosides of paeony (TGP) on expressions of TLR4 and NF-κB in colonic tissues of mice and patients with ulcerative colitis (UC).
(A) Representative immunohistochemical staining of TLR4 and NF-κB p65 in colonic tissues of mice. (B) Representative western blot and relative image quantification (n = 10, $^{*}P < 0.05$). (C) mRNA expressions of TLR4 and NF-κB p65 in colonic tissues of mice (n = 10, $^{*}P < 0.05$). (D) Representative immunohistochemical staining of TLR4 and NF-κB p65 in colonic tissues of patients.
SASP, salicylazosulfapyridine; TGP-L, low-dose TGP treatment; TGP-M, medium-dose TGP treatment; TGP-H, high-dose TGP treatment; TLR4, toll-like receptor 4; NF-κB p65, nuclear factor-kappa B p65. Scale bars, 100 μm. Data are presented as mean ± standard error of the mean.

and improved colitis disease activity index as well as ameliorated the colon histopathological score.

Extensive efforts have been made to investigate the mechanisms underlying the protective role of TGP on UC and other inflammatory diseases. Several signal transduction pathways have been reported to be implicated in the anti-inflammatory properties of TGP. Among them, TLR/NF-κB was the most frequently reported one in mediating the anti-inflammatory effects of TGP. Xu et al. (2014) and Zhang et al. (2014) examined the effects of TGP on TLR/NF-κB pathway in the kidney of streptozotocin-induced diabetic rats, and found that the expressions of TLR2/TLR4 as well as the phosphorylation of NF-κB p65 were significantly inhibited by TGP, which in turn inhibited the production of TNF-α and IL-1β. In a cell model of oral lichen planus induced by LPS, TGP treatment decreased the phosphorylation of both IκBα and NF-κB p65, and reduced the production of IL-6 and TNF-α in a dose-dependent manner (Wang et al. 2016). Some other studies have also shown that NF-κB signaling was inhibited by TGP *in vitro*

and *in vivo*, and accordingly, the levels of pro-inflammatory factors such as TNF-α, IL-1β, IL-6, IL-8, IL-21, intercellular adhesion molecule-1 and/or $CD4^+/CD8^+$ T-cell ratio were downregulated (Wu et al. 2009; Zhang et al. 2009; Su et al. 2010; Zhang et al. 2017; Chen et al. 2019; Li et al. 2019a; Liu et al. 2020; Meng et al. 2021).

TGP capsule is a Chinese patent medicine approved by China Food and Drug Administration in 1998. There were a set of strict demands for new Chinese patent drug application such as drug efficacy evaluation and adverse reaction monitoring, and a number of certification steps including application, acceptance, data review, on-site inspection, and audit announcement. Each TGP capsule contains 300 mg TGP. Patients need to take two capsules each time and two times a day in the morning and evening after meals. Currently, there were few clinical studies being conducted on TGP capsule in the treatment of UC, but from the results of this study point of view, TGP had a significant therapeutic effect on mild to moderate UC. Patients with TGP capsule therapy had significantly higher relief rates of diarrhea, abdominal pain and bloody purulent stool, decreased UCAI and increased SIBDQ scores, and lower levels of erythrocyte sedimentation rate, C-reactive protein and $CD4^+$ T cell than those patients with routine treatment (mesalazine). Like Paeonia decoction, TGP capsule is safe in clinical use and its adverse reactions were rarely seen. Nausea and soft stool occasionally occurred, but they disappeared quickly without special treatment (Chen 2004; Wang et al. 2022). In the present clinical study, the adverse reactions caused by TGP capsule were very low, and there might even be no adverse reactions of TGP at all since the incidence of adverse reactions in TGP capsule therapy group was lower than that in routine therapy group (12% vs. 20%), but both groups used routine treatment drug mesalazine.

In this study, we also intended to compare the differences of curative effect between TGP capsule and steroid drugs in the treatment of severe patients with UC, but considering that there were many kinds of drugs with large doses being used for such patients, it was hard to directly compare the curative effect between TGP capsule and steroid drugs since too many confounding factors were present. Generally, steroid therapy is more effective than TGP capsule from the perspective of anti-inflammation, but steroid drugs also have greater adverse effects, such as gastrointestinal bleeding, osteoporosis and so on. Therefore, steroid drugs are suitable for short-term treatment of severe UC, while TGP capsule is suitable for long-term use in patients with mild to moderate UC.

In conclusion, the present study demonstrates that TGP is effective in treating UC in animal model and human patients with UC. TGP inhibited TLR4/NF-κB signaling pathway and suppressed the production of inflammatory and oxidative stress factors, which in turn improved the symptoms of UC. The findings of this study can provide an experimental basis for TGP to treat patients with UC in clinical practice.

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Author Contributions

Junying Xiang: methodology, diagnosis and evaluation of ulcerative colitis, original draft preparation. Renwei Hu: methodology, diagnosis and evaluation of ulcerative colitis, data processing and data analysis. Qunhua Li: methodology, colonoscopic evaluation, histological evaluation. Shujin Li, Youjin Zhang and Xue Wang: methodology, molecular and animal experiments. Yongyan Song: conceptualization, manuscript reviewing and editing, funding acquisition and supervision. All authors agreed to be accountable for all aspects of work ensuring integrity and accuracy.

Conflict of Interest

The authors declare no conflict of interest.

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