

# Protective effect of *Gloeostereum incarnatum* on ulcerative colitis via modulation of Nrf2/NF- $\kappa$ B signaling in C57BL/6 mice

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**Abstract.** Chronic non-specific inflammatory cell infiltration of the colon is generally considered to be the cause of ulcerative colitis (UC). *Gloeostereum incarnatum* (GI), a fungus rich in amino acids and fatty acids, exhibits a variety of biological functions. In the present study, GI was identified to contain 15 fatty acids, 17 amino acids and 11 metallic elements. The protective effect of GI against UC was investigated in C57BL/6 mice with UC induced by free drinking 3.5% dextran sulfate sodium (DSS). After a 21-day oral administration, GI prevented weight loss, enhancement of the disease activity index and colonic pathological alterations in mice with UC. GI reduced the levels of pro-inflammatory factors including interleukin (IL)-1 $\beta$ , IL-2, IL-6 and IL-12, tumor necrosis factor  $\alpha$  and  $\beta$ , interferon  $\alpha$  and  $\gamma$ , and pro-oxidative factors including reactive oxygen species and nitric oxide. In addition, it enhanced the levels of immunological factors including immunoglobulin (Ig)A, IgM and IgG, and antioxidative factors including superoxide dismutase and catalase in the serum and/or colon tissues. GI enhanced the expression levels of nuclear factor erythroid 2-related factor 2 (Nrf2) and its downstream proteins and suppressed the phosphorylation of NF- $\kappa$ B signaling in colon tissues. Together, GI was shown to alleviate

the physiological and pathological state of DSS-induced UC in mice via its antioxidant and anti-inflammatory functions, which may be associated with its modulation of the activation of Nrf2/NF- $\kappa$ B signaling.

## Introduction

Inflammatory bowel disease is a chronic, repetitive and non-specific gastrointestinal inflammatory disease comprising two conditions: Ulcerative colitis (UC) and Crohn's disease (1). As a chronic intestinal disease, UC produces inflammatory reactions and immune response at the colonic mucosa (2), accompanied by weight loss, diarrhea and bloody stools (3). Long-term severe colitis is known to cause colorectal cancer (4).

The pathogenesis of UC involves tissue damage in the colon and deterioration of the digestive and absorptive functions (5). Inflammation of the colon is due to an imbalance between the expression of pro-inflammatory and anti-inflammatory factors, and the migration of neutrophils to the damaged site, leading to the accumulation of inflammatory factors (6). Interleukin (IL)-6, closely associated with the differentiation and maturation of B cells, promotes the function of pro-inflammatory cells and inhibits the regulation of immune-related cells including T cells (7), both of which increase the levels of pro-inflammatory factors including IL-1 and IL-17 (8). Therefore, blocking the expression of pro-inflammatory cytokines may be a method for the potential treatment of UC (9). NF- $\kappa$ B inhibitor  $\alpha$  is involved in the regulation of the development of early inflammation (10). The inflammatory response leads to the aggregation of peroxides and the imbalance of oxidation and antioxidation in the colon, particularly the over-accumulation of reactive oxygen species (ROS), causes oxidative damage (11). Antioxidant enzymes, including superoxide dismutase (SOD), are the first line of defense against oxidative stress, helping to remove peroxides from the body, but generally tend to promote oxidation in UC (12). During this process, the nuclear factor erythroid-2-related factor 2 (Nrf2) recognizes the transcription-enhancing sequence associated with antioxidation and repairs bodily damage by enhancing the expression of antioxidant enzymes (13).

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Dextran sulfate sodium (DSS) affects the normal replication of DNA in cells, causes the aggregation of inflammatory factors and destruction of the intestinal microenvironment via the overproduction of ROS and eventually leads to the onset of UC (14). DSS was used to induce the occurrence of UC in the present study. Sulfasalazine (SASP), which reduces the infiltration of inflammatory monocytes, has been used to treat UC in clinics (15) and was applied as a positive control agent in this study.

*Gloeostereum incarnatum* (GI) is an edible and medicinal fungus, commonly cultivated in north-central China and Hokkaido, Japan (16). The anti-inflammatory activities of GI extracts were found to reduce nitric oxide (NO) production in lipopolysaccharide (LPS)-induced RAW264.7 cells and delay melanin synthesis in B16/F10 cells (17). In our previous study, GI was confirmed to show immunomodulatory function in a cyclophosphamide monohydrate-induced mouse model by increasing immune-related factors including immunoglobulin (Ig) (7). Due to its anti-microbial activities, GI shows beneficial effects on gastrointestinal diseases including gastric ulcer and enteritis (18). However, thus far, there has been no systematic research on the protective effect of GI against UC.

In the present study, based on the detection of the main components of GI, its anti-inflammation and antioxidative properties were successfully confirmed in C57BL/6 mice with UC induced via oral administration of 3.5% DSS. The data provide experimental evidence for the applicability of GI for protection against UC.

## Materials and methods

**Detection of GI components.** GI was purchased from TengHui Agriculture, identified by Professor Yu Li (Jilin Agricultural University, China) and pulverized by a pulverizer (XL-06A, Guangzhou Xulang Machinery Equipment Co., Ltd.).

**Detection of main components.** In GI powder, the contents of total sugar, reducing sugar, mannitol, total ash, total flavonoids, total triterpenoids, crude fat and crude fiber were detected using phenol-sulfuric acid assay (19), 3,5-dinitrosalicylic acid colorimetry (20), iodometry (21), regression analysis (22), the alumina colorimetric method (23), vanillin-glacial acetic acid-perchloric acid colorimetry (24), petroleum ether extraction (25) and the Ankom filter bag method (26), respectively.

**Detection of minerals.** Inductively coupled plasma optical emission spectrometry was used to detect the contents of mercury (Hg), lead (Pb), selenium (Se), arsenic (As), cadmium (Cd), zinc (Zn), iron (Fe), manganese (Mn), chromium (Cr), calcium (Ca), copper (Cu), sodium (Na) and potassium (K) in GI (27).

**Detection of fatty acids.** An oil sample of GI was extracted using a chloroform and methanol solution (2:1) and treated by alkaline hydrolysis to prepare the corresponding fatty acid methyl esters. Fatty acids were detected using gas chromatography-mass spectrometry (QP2010, Shimadzu Corporation) based on retention times (28).

**Detection of amino acids.** The protein in GI was hydrolyzed to single amino acid residues by hydrochloric acid hydrolysis

and the contents of different amino acids were analyzed using an automatic amino acid analyzer (L-8900, Hitachi, Ltd.) (29).

**Establishment of UC model mice and agent administration.** A total of 48 male C57BL/6 mice [22-25 g; 8-10 weeks old; SPF grade, SCXK (LIAO) 2015-001], purchased from Liaoning Changsheng Biotechnology Co., Ltd., were housed in an exhaust ventilation cage system with a temperature of 22±2°C and humidity of 50±10%, with *ad libitum* access to food and water and a 12-h light/dark cycle. All animal experiments were approved by the Experimental Animal Ethics Committee of Jilin University [approval no. SYXK(JI)2014-0013]. The study was conducted using the Laboratory Guidelines for Animal Care (30,31) and the Guide for the Care and Use of Laboratory Animals (eighth edition) (32).

After 7 days of adaptive feeding, all mice with the exception of the control mice drank freely available clean water containing 3.5% DSS (S14048, Shanghai Yuanye Biological Technology Co., Ltd.) every day for 7 days. From the 8th to the 28th day, the mice drank 3.5% DSS on 1 day out of every 3 days. At the 7th day, the mice were randomly divided into 6 groups: The control group (n=8) that orally received double distilled (D.D.) water, the model group (n=8) that orally received D.D. water, the positive control group (n=8) that orally received 0.6 g/kg of SASP dissolved in D.D. water and the GI-treated groups that orally received 1.0 g/kg (n=8), 2.0 g/kg (n=8) and 4.0 g/kg (n=8) of GI suspended in D.D. water for the remaining 21 days. The doses of GI and SASP were selected based on previous studies (7,33) and our preliminary experiments (data not shown). On the 28th day, blood samples were collected from the orbital venous plexus of the mice and the mice were then euthanized by intraperitoneal injection of 100 mg/kg sodium pentobarbital (Xiya Reagent Co., Ltd.); mortality was characterized by cessation of heartbeat (34). Tissues including the colon, liver, spleen and kidney of each mouse were collected for further detection. The length of the colon and the organ index of the liver, spleen and kidney were calculated. The following formula was used to calculate the organ index (35): Organ index (%) = mean organ weight/mean body weight x 100.

During the entire experimental period, the activity, physiological status and body weight were monitored daily. The status of the UC mice was scored using the disease activity index (DAI) according to the degree of weight loss, fecal viscosity and bleeding (36).

**Detection of biochemical factors in the serum and colon of UC mice.** Colon tissue was homogenized in D.D. water contained 1.0% 50-mM phenylmethanesulfonyl fluoride (PMSF; Sigma-Aldrich; Merck KGaA) and 1.0% protease inhibitor cocktail (Sigma-Aldrich; Merck KGaA) using a homogenizer [S-18KS, Leopard scientific instruments (Beijing) Co., Ltd.]. After centrifuging the homogenate at 1,000 x g for 10 min at 4°C, the supernatant was collected, and the protein content was determined using a BCA protein assay kit (EMD Millipore). The levels of IL-1β (E20180501A), IL-2 (E20180501A), IL-6 (E20180501A), IL-12 (E20180501A), tumor necrosis factor (TNF)-α (E20180501A), TNF-β (E20180501A), interferon (IFN)-α (E20180501A) and IFN-γ (E20180501A) in the colon and IgA (E20180401A), IgM

(E20180401A), IgG (E20170601A), SOD (E20180401A), catalase (CAT) (E20180401A), ROS (E20180401A) and NO (E20180401A) in the colon and serum were measured using ELISA kits purchased from Shanghai Yuanye Biological Technology Co., Ltd.

**Histopathological examination of organs in the UC mice.** The tissues were fixed in 4% paraformaldehyde at 37°C for 24 h and dehydrated in an ascending series of ethanol, embedded in paraffin and then cut into 5–8- $\mu$ m-thick sections by a microtome (Leica Microsystems GmbH). The samples were dewaxed with xylene at 37°C and rehydrated in a descending series of ethanol. The samples were stained with 0.45% hematoxylin for 10 min and 0.5% eosin for 3 min, both at 37°C, and then observed and images captured using a light microscope (magnification, x400; Olympus Corporation).

**Western blot analysis of colon tissue.** The proteins were extracted from colon tissue as detailed in our previous study (37). Total protein was quantified using a bicinchoninic acid protein assay kit (EMD Millipore) and 40  $\mu$ g protein/well was separated by 12% SDS-PAGE and transferred to polyvinylidene fluoride membranes (EMD Millipore) using the same method as that used in our previous study (37). The membranes were blocked with 5% BSA (Sigma-Aldrich; Merck KGaA) at 4°C for 4 h and then incubated overnight with primary antibodies against nuclear factor erythroid 2-related factor 2 (Nrf2; 1:2,000; cat. no. ab89443), CAT (1:2,000; cat. no. ab16731), heme oxygenase-1 (HO-1; 1:2,000; cat. no. ab137749), SOD-1 (1:2,000; cat. no. ab16831), SOD-2 (1:5,000; cat. no. ab13533), phosphorylated (p)-NF- $\kappa$ B (1:2,000; cat. no. ab86299), total (T)-NF- $\kappa$ B (1:1,000; cat. no. ab7970), p-inhibitor of NF- $\kappa$ B kinase  $\alpha$ + $\beta$  (IKK $\alpha$ + $\beta$ ; 1:1,000; cat. no. ab195907), T-IKK $\alpha$ + $\beta$  (1:1,000; cat. no. ab178870), p-inhibitor of NF- $\kappa$ B (I $\kappa$ B $\alpha$ ; 1:500; cat. no. ab12135), T-I $\kappa$ B $\alpha$  (1:1,000; cat. no. ab32518) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:1,000; cat. no. ab8245; all from Abcam) at 4°C. The antibodies were diluted according to the ratio given in the manufacturer's protocols. Following washing with a Tris-buffered saline solution containing 0.05% Tween 20, the membranes were incubated with goat anti-rabbit (1:5,000; cat. no. IH-0011) or goat anti-mouse secondary antibody (1:5,000; cat. no. IH-0031; both Beijing Dingguo Changsheng Biotechnology Co Ltd.) at 4°C for 4 h. Electrochemiluminescence detection kits (EMD Millipore) and a Gel Imaging System (UVP, LLC) were used to detect changes in the protein content. The optical densities of bands were measured using ImageJ 1.48u software (National Institutes of Health).

**Statistical analysis.** Data are expressed as the mean  $\pm$  standard error of the mean. One-way analysis of variance (ANOVA) and subsequent post-hoc multiple comparisons (Tukey's test) were performed using SPSS 16.0 software (SPSS, Inc.).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Evaluation of GI components.** GI contained 16.6% of total sugar, 16.8% of crude fiber, 2.1% of mannitol, 13.9% of total ash, 6.3% of reducing sugar, 0.394% of total flavonoids,

5.328x10<sup>-3</sup>% of total triterpenes and 2.8% of crude fat (Table I). The presence of 35 fatty acids was detected, with linoleic acid (1.626%), oleic acid (0.236%) and hexadecenoic acid (0.229%) being the most abundant; by contrast, the fatty acids caprylic acid, undecanoic acid, translinoleic acid and docosadienoic acid were not detected (Table I). GI contained 17 amino acids and 11 minerals, with no harmful elements (Table I).

**Protective effect of GI against UC.** In the model mice with UC, DSS free-drinking caused marked body weight loss ( $P < 0.05$ ; Table II) and the worsening of the DAI ( $P < 0.001$ ; Fig. 1A), both of which were reversed after 21-day GI administration ( $P < 0.05$ ; Table II and Fig. 1A). Compared with the model mice, GI treatment in the UC mice prevented the increase of the spleen and liver indexes ( $P < 0.05$ ; Table II) and increased the kidney indexes ( $P < 0.05$ ; Table II); however, SASP demonstrated no significant effects on the liver indexes (Table II). Inflammatory cell infiltration and ulceration of the colon can lead to the shortening of the colon, which was noted in the model mice (Fig. 1B). SASP and GI notably prevented this pathological reduction of the colon length (Fig. 1B). In the colons of the model mice with UC, a large number of exfoliated lymphocytes were identified; meanwhile, the goblet cells were reduced and the crypt cells were damaged, which were all significantly alleviated following SASP and GI treatment (Fig. 1C and D). Furthermore, inflammatory cell infiltration in the spleen (Fig. 1E), interstitial edema in the liver (Fig. 1F) and eosinophilic renal tubular epithelial cells in the kidney (Fig. 1G) were all observed in the DSS-only-treated mice (the model mice). These pathological alterations were all alleviated following SASP and GI administration (Fig. 1E–G).

**Anti-inflammatory effects of GI.** Inflammation of the mucosal surface of the colon leads to the development of UC (38). The free-drinking of DSS in the present study increased the levels of pro-inflammatory factors including IL-1 $\beta$ , IL-2, IL-6, IL-12, TNF- $\alpha$ , TNF- $\beta$ , IFN- $\alpha$  and IFN- $\gamma$  ( $P < 0.05$ ; Table III). GI and SASP demonstrated strong anti-inflammatory effects, as evidenced by the regulation of inflammatory cytokines ( $P < 0.05$ ; Table III). Compared with the model mice, 21-day GI administration in the UC mice resulted in >19.3% ( $P < 0.05$ ), >31.7% ( $P < 0.05$ ), >31.5% ( $P < 0.05$ ), >30.8% ( $P < 0.05$ ), >23.4% ( $P < 0.05$ ), >30.2% ( $P < 0.05$ ), >28.1% ( $P < 0.05$ ) and >33.5% ( $P < 0.05$ ) reduction in IL-1 $\beta$ , IL-2, IL-6, IL-12, TNF- $\alpha$ , TNF- $\beta$ , IFN- $\alpha$  and IFN- $\gamma$  levels, respectively, in the colon (Table III).

The immune response is involved in the pathogenesis of UC (39). Compared with the control mice, the UC mice (the model group) demonstrated lower levels of IgA, IgG and IgM in the colon and serum ( $P < 0.05$ ; Fig. 2). GI enhanced the levels of IgA ( $P < 0.05$ ) (Fig. 2A) and IgG ( $P < 0.05$ ; Fig. 2C) in the colon and serum and the levels of IgM ( $P < 0.001$ ) (Fig. 2B) in the serum of the UC mice, but did not enhance the levels of IgM in the colon (Fig. 2B).

**Effect of GI on oxidative factors.** Oxidative stress destroys the cellular macromolecules of the colon, which is the key to the pathogenesis of UC (40). Compared with the control mice, the UC mice demonstrated lower levels of SOD and

Table I. Composition of *Gloeostereum incarnatum*.

A, Main components	
Compounds	Contents, %
Total sugar	16.6
Total ash	13.9
Total triterpenes (x10 <sup>-3</sup> )	5.328
Crude fiber	16.8
Reducing sugar	6.3
Crude fat	2.8
Mannitol	2.1
Total flavonoids	0.394
B, Fatty acids	
Compounds	Contents, %
Caprylic acid (C8:0)	ND
Capric acid (C10:0)	ND
Undecanoic acid (C11:0)	ND
Lauric acid (C12:0)	0.015
Tridecanoic acid (C13:0)	ND
Myristic acid (C14:0)	0.003
Myristoleic acid (C14:1)	ND
Pentadecanoic acid (C15:0)	0.028
Pentadecenoic acid (C15:1)	ND
Hexadecanoic acid (C16:0)	0.229
Palmitoleic acid (C16:1)	0.021
Heptadecanoic acid (C17:0)	0.005
Margaroleic acid (C17:1)	0.004
Stearic acid (C18:0)	0.014
Elaidic acid (C18:1n9t)	ND
Oleic acid (C18:1n9c)	0.236
Translinoleic acid (C18:2n6t)	ND
Linolic acid (C18:2n6c)	1.626
$\alpha$ -Linolenic acid (C18:3n3)	ND
$\gamma$ -Linolenic acid (C18:3n6)	ND
Arachidic acid (C20:0)	ND
Cis-11-Eicosenoic acid C20:1n9)	0.067
Docosanoic acid (C22:0)	ND
Erucic acid (C22:1n9)	ND
Docosadienoic acid (C22:2n6)	ND
Docosahexaenoic acid (C22:6n3)	ND
Tricosanoic acid (C23:0)	0.005
Tetracosanoic acid (C24:0)	0.008
Nervonic acid (C24:1n9)	0.016
Dihomo- $\gamma$ -linolenic acid (C20:3n6)	ND
Arachidonic acid (C20:4n6)	ND
Eicosapentaenoic acid (C20:5n3)	ND
Heneicosanoic acid (C21:0)	ND
Eicosadienoic acid (C20:2)	0.005
Eicosatrienoic acid (C20:3n3)	ND

Table I. Continued.

C, Amino acids	
Compounds	Contents, %
Aspartic acid	0.728
L-Threonine	0.405
Serine	0.471
Glutamic acid	1.012
Glycine	0.400
Alanine	0.463
Cystine	0.266
Valine	0.406
Methionine	0.096
Isoleucine	0.343
Leucine	0.616
Tyrosine	0.248
Phenylalanine	0.335
Lysine	0.221
Histidine	0.188
Arginine	0.510
Proline	0.377
D, Minerals	
Compounds	Contents, mg/kg
Mercury	ND
Lead	0.11493
Selenium	0.03450
Arsenic	0.12343
Cadmium	ND
Zinc	44.64
Iron	105.9
Manganese	6.135
Chromium	3.15357
Calcium	601.5
Copper	9.230
Sodium	345.8
Potassium	48,605
ND, not detected.	

CAT and higher levels of ROS and NO in the serum and colon tissues ( $P < 0.05$ ; Table IV). In the serum, GI resulted in  $>14.8\%$  ( $P < 0.01$ ) and  $>17.4\%$  ( $P < 0.05$ ) increase in SOD and CAT levels and  $>12.9\%$  ( $P < 0.05$ ) reduction in NO levels, but no effect on ROS levels (Table IV). In the colon tissues, GI failed to affect CAT levels, but resulted in  $>36.3\%$  ( $P < 0.05$ ) increase in SOD levels and  $>25.8\%$  ( $P < 0.05$ ) and  $>45.2\%$  ( $P < 0.05$ ) reductions in ROS and NO levels respectively (Table IV). SASP significantly prevented the pathological alterations in the levels of anti- and pro-oxidative factors in the colon tissues ( $P < 0.05$ ; Table IV).

Table II. Effect of GI on body weight and organ indexes.

Variable	3.5% dextran sulfate sodium					
	Control	Model	Sulfasalazine, 0.6 g/kg	GI, g/kg		
				1.0	2.0	4.0
<b>Body weight, g</b>						
Day 1	22.8±0.2	22.6±0.4	22.3±0.4	22.5±0.3	22.6±0.4	22.6±0.3
Day 7	22.4±0.3	20.3±0.3 <sup>a</sup>	20.2±0.3	20.5±0.4	20.3±0.3	20.5±0.3
Day 14	24.4±0.3	20.6±0.8 <sup>b</sup>	21.2±0.5	21.4±0.7	21.6±0.3	21.2±0.7
Day 21	25.4±0.4	20.8±0.8 <sup>b</sup>	22.1±0.4	22.3±0.5	22.9±0.3 <sup>d</sup>	22.0±0.5
Day 28	24.3±0.4	21.2±0.5 <sup>b</sup>	22.1±0.3	22.7±0.5 <sup>d</sup>	23.4±0.3 <sup>d</sup>	21.5±0.3
<b>Organ index, %</b>						
Spleen	0.25±0.05	0.56±0.10 <sup>c</sup>	0.45±0.05 <sup>d</sup>	0.49±0.14	0.44±0.10 <sup>d</sup>	0.38±0.11 <sup>e</sup>
Liver	3.72±0.31	4.34±0.24 <sup>a</sup>	4.32±0.13	3.95±0.44 <sup>d</sup>	3.79±0.40 <sup>d</sup>	3.58±0.27 <sup>e</sup>
Kidney	1.16±0.05	1.03±0.05 <sup>a</sup>	1.13±0.06 <sup>d</sup>	1.10±0.10	1.11±0.05 <sup>d</sup>	1.01±0.07

Data were analyzed using a one-way ANOVA and are expressed as mean ± SEM (n=8). <sup>a</sup>P<0.05, <sup>b</sup>P<0.01 and <sup>c</sup>P<0.001 vs. control mice, <sup>d</sup>P<0.05 and <sup>e</sup>P<0.01 vs. DSS-induced UC mice. GI, *Gloeostereum incarnatum*.

GI regulates the Nrf2/NF- $\kappa$ B signaling in the colon of UC mice. Compared with the model mice, the UC mice administered with GI demonstrated lower expression levels, in the colon, of Nrf2 and its downstream proteins, including CAT, HO-1, SOD-1 and SOD-2, and higher phosphorylated activities of NF- $\kappa$ B, IKK $\alpha$ / $\beta$  and I $\kappa$ B $\alpha$ , all of which were prevented by GI administration (P<0.05; Fig. 3).

## Discussion

The commonly used anti-colitis drugs mainly include non-steroidal drugs, which exhibit side effects including intestinal ulcers (41,42). Recently, edible mushrooms have attracted researchers' attention due to their diverse pharmacological effects and minimal adverse effects. For example, *Ganoderma lucidum* alleviates intestinal damage induced by indomethacin in mice (43) and *Hericium erinaceus* improves testicular damage via its antioxidative effects and regulation of the GPR41/43 receptor in UC rats and Caco-2 cells (44). Based on the immunomodulatory and anti-inflammatory effects of GI reported previously (7), the present study successfully confirmed its protective effect against UC in C57BL/6 mice.

GI is a medicinal and edible fungus with complex ingredients and multiple nutritional value components. Its natural and crude character not only supports its low toxicity with various pharmacological efficacy, but also helps to explain its non-dose dependent effects during the experiments in the present study, which may show anti-UC effects via multiple targets. Indeed, the non-dose dependent manner can be noted in the research associated with Traditional Chinese Medicine (37). GI contains 6.3% crude fiber, which is beneficial for intestinal peristalsis and eases constipation (45). Mixed fiber can markedly regulate blood sugar levels, in addition to inflammatory factors, and exhibits a beneficial regulatory effect on the intestinal microbiota (46). GI contains 0.394% flavonoids, which exert antioxidant effects by regulating the

body's oxygen free radical levels. In an LPS-damaged mice model, a flavonoid-rich fraction of *Ocimum gratissimum* leaves regulated oxidative stress and inflammation in the liver and brain by reducing the levels of TNF- $\alpha$  and malondialdehyde (47). The flavonoid apigenin can reduce the elevation of pro-inflammatory cytokines in the colon, reduce the density of eosinophils and transform M1 pro-inflammatory macrophages to the M2 anti-inflammatory phenotype in diet-induced obese mice (48). GI contains  $5.328\% \times 10^{-3}$  triterpenes, which trigger the anti-inflammatory response and reduce the expression of pro-inflammatory cytokines in a streptozotocin-induced diabetes model (49). Thus, GI shows a good nutritional foundation for its antioxidation and anti-inflammation activities.

UC is a process driven by T helper type 2 (Th-2)-like T-cells combined with the infiltration of lymphocytes and macrophages (50). DSS-induced damage to the colon tissue in mice causes the activation of macrophages, destruction of the composition of the lamina propria cells and aggregation of inflammatory factors, which eventually leads to imbalance in Th-1/2 cells (51). Cluster of differentiation 1a acts as an inflammatory mediator of UC, inducing T cell activation to elicit an immune response in the body (52). As a central factor in immune mediation, the elevated levels of secretory IgA in the colon help to prevent intestinal damage and restore mucosal barrier function (53). IgG exerts important protective and regulatory effects in the placental barrier (54) and IgM serves an important role in its own immune regulation (55). In the present study, GI increased the levels of Ig in the UC mice to stimulate an immune response.

Furthermore, TGF- $\beta$  serves a role in pro-inflammatory mediator production during the development of UC, which can regulate the levels of ILs, TNFs and IFNs (56). During the development of UC, signaling of the receptor-interacting protein kinase 3 can upregulate the expression of repair-associated cytokines, including cyclooxygenase 2 and IL-22 (57). As a pro-inflammatory factor, abnormal

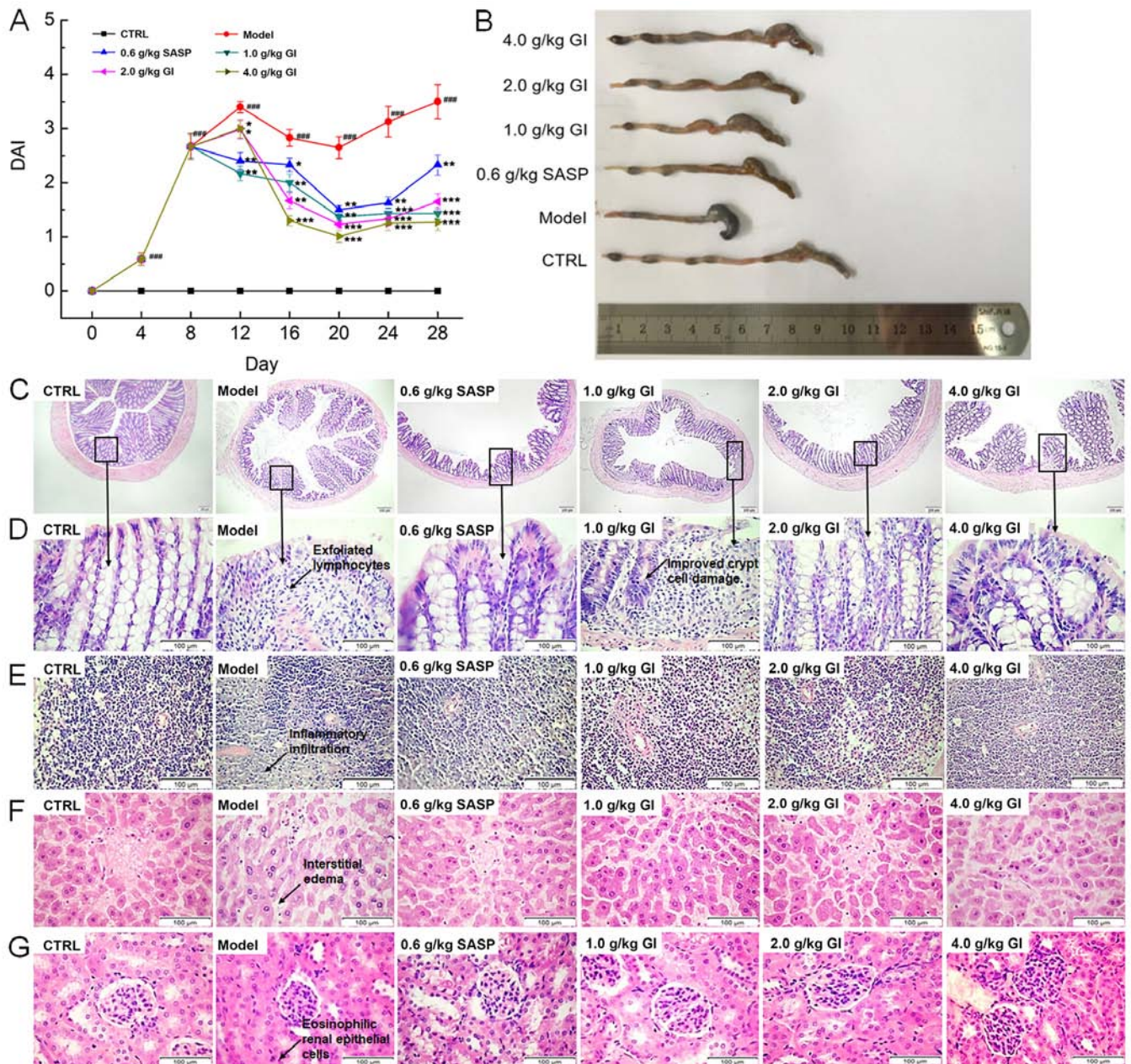


Figure 1. GI regulates the physiology and pathology of UC mice. The mice were continuously exposed to DSS (3.5% DSS dissolved in D.D. water) for 28 days, SASP (0.6 g/kg of SASP dissolved in D.D. water) and GI (1.0, 2.0 and 4.0 g/kg of GI suspended in D.D. water) were administered from the 7th day. (A) GI reduced the DAI index of UC mice and (B) ameliorated the shortening of colon length. Hematoxylin and eosin staining of (C) colon (scale bar, 100  $\mu$ m; magnification, x40), (D) colon, (E) spleen, (F) liver and (G) kidney tissues (scale bar, 100  $\mu$ m; magnification, x400) from C57BL/6 mice.  $^{***}P<0.001$  vs. control mice;  $^{*}P<0.05$ ,  $^{**}P<0.01$  and  $^{***}P<0.001$  vs. DSS-induced UC mice. GI, *Gloeostereum incarnatum*; UC, ulcerative colitis; DSS, dextran sulfate sodium; D.D., double distilled; SASP, sulfasalazine; DAI, disease activity index.

release of TNF- $\alpha$  recruits more macrophages and neutrophils, which in turn increases inflammatory damage and intestinal permeability (58). In the morphological observations of the current study, reduced goblet cells, crypt abscesses and inflammatory cell infiltration were noted in the colon tissue following DSS administration; these were alleviated following GI administration. The overexpression of IL-6 may aggravate the inflammatory cell infiltration at the injured site and recruit more pro-inflammatory factors (8), which further activate the inducible nitric oxide synthase and cyclooxygenase-2 pathways, in turn increasing neutrophil aggregation (59). In the present study,

the regulatory activity of GI on inflammatory factors may have been involved in its protective effect against UC in C57BL/6 mice.

As an upstream regulatory protein, NF- $\kappa$ B can also be activated by increased levels of pro-inflammatory factors (60). The IKK $\alpha$ / $\beta$  complex is an aggregate composed of a catalytic subunit (61) and is essential for the initiation of the NF- $\kappa$ B pathway (62). In general, I $\kappa$ B $\alpha$  forms a dimer with NF- $\kappa$ B in the cytoplasm. When the inflammatory factor stimulates the IKK $\alpha$ / $\beta$  complex, IKK $\alpha$ / $\beta$  phosphorylates I $\kappa$ B $\alpha$ , thereby separating it from NF- $\kappa$ B. NF- $\kappa$ B is thus activated and enters the nucleus to induce the expression of relevant genes to reduce the

Table III. Effect of GI on inflammatory factors of colon tissues of mice with ulcerative colitis.

Inflammatory factors	3.5% dextran sulfate sodium					
	Control	Model	SASP, 0.6 g/kg	GI, g/kg		
				1.0	2.0	4.0
IL-1 $\beta$ , pg/mgprot	45.7 $\pm$ 3.9	60.0 $\pm$ 3.5 <sup>a</sup>	44.1 $\pm$ 3.1 <sup>d</sup>	48.4 $\pm$ 1.4 <sup>c</sup>	42.2 $\pm$ 3.8 <sup>c</sup>	39.7 $\pm$ 3.7 <sup>d</sup>
IL-2, pg/mgprot	158.1 $\pm$ 9.4	219.1 $\pm$ 14.6 <sup>a</sup>	153.8 $\pm$ 11.9 <sup>c</sup>	130.2 $\pm$ 11.0 <sup>d</sup>	149.5 $\pm$ 16.4 <sup>c</sup>	128.4 $\pm$ 10.1 <sup>d</sup>
IL-6, pg/mgprot	61.6 $\pm$ 3.7	83.7 $\pm$ 6.3 <sup>a</sup>	54.5 $\pm$ 8.2 <sup>c</sup>	57.3 $\pm$ 1.4 <sup>c</sup>	62.1 $\pm$ 6.8	50.0 $\pm$ 4.8 <sup>d</sup>
IL-12, pg/mgprot	37.3 $\pm$ 2.6	57.2 $\pm$ 6.3 <sup>a</sup>	31.9 $\pm$ 3.9 <sup>d</sup>	30.5 $\pm$ 2.0 <sup>d</sup>	39.6 $\pm$ 2.0 <sup>c</sup>	27.8 $\pm$ 2.1 <sup>d</sup>
TNF- $\alpha$ , pg/mgprot	407.5 $\pm$ 21.9	489 $\pm$ 22.2 <sup>a</sup>	429.0 $\pm$ 28.9	374.5 $\pm$ 27.2 <sup>c</sup>	358.0 $\pm$ 43.1 <sup>c</sup>	337.5 $\pm$ 14.6 <sup>d</sup>
TNF- $\beta$ , pg/mgprot	78.6 $\pm$ 7.8	119.8 $\pm$ 11.5 <sup>b</sup>	62.9 $\pm$ 7.7 <sup>d</sup>	70.1 $\pm$ 4.1 <sup>d</sup>	83.6 $\pm$ 10.9 <sup>c</sup>	63.3 $\pm$ 4.4 <sup>d</sup>
IFN- $\alpha$ , pg/mgprot	19.7 $\pm$ 1.6	28.8 $\pm$ 2.6 <sup>b</sup>	16.3 $\pm$ 1.9 <sup>d</sup>	20.7 $\pm$ 1.2 <sup>c</sup>	20.2 $\pm$ 1.6 <sup>c</sup>	17.2 $\pm$ 1.8 <sup>d</sup>
IFN- $\gamma$ , pg/mgprot	72.4 $\pm$ 6.6	126.6 $\pm$ 14.8 <sup>b</sup>	72.9 $\pm$ 9.5 <sup>d</sup>	75.3 $\pm$ 5.4 <sup>d</sup>	84.2 $\pm$ 5.5 <sup>c</sup>	69.8 $\pm$ 7.0 <sup>c</sup>

Data were analyzed using a one-way ANOVA and are expressed as mean  $\pm$  SEM (n=8). <sup>a</sup>P<0.05 and <sup>b</sup>P<0.01 vs. control mice, <sup>c</sup>P<0.05 and <sup>d</sup>P<0.01 vs. DSS-induced UC mice. GI, *Gloeostereum incarnatum*; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon.

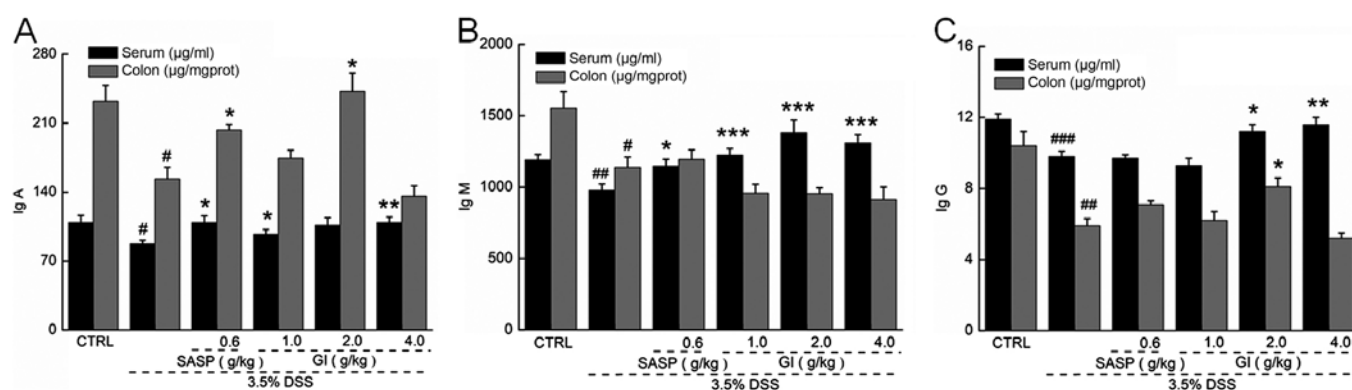


Figure 2. GI increases the levels of immune factors in serum and colon tissues of mice with UC. GI raised the levels of (A) IgA, (B) IgM and (C) IgG in serum and colon tissues. <sup>#</sup>P<0.05, <sup>##</sup>P<0.01 and <sup>###</sup>P<0.001 vs. control mice; <sup>\*</sup>P<0.05, <sup>\*\*</sup>P<0.01 and <sup>\*\*\*</sup>P<0.001 vs. DSS-induced UC mice. GI, *Gloeostereum incarnatum*; UC, ulcerative colitis; DSS, dextran sulfate sodium.

expression of pro-inflammatory cytokines (63). In the present study, GI markedly reduced the phosphorylated activation of NF- $\kappa$ B by suppressing the phosphorylated IKK $\alpha$ / $\beta$  and I $\kappa$ B $\alpha$ .

Inflammatory cells in the colon produce ROS and the accumulation of ROS damages proteins and nucleic acids, leading to oxidative stress (64). SOD and CAT are enzymatic antioxidants that catalyze the decomposition of oxides or peroxides to avoid oxidative damage (65). A previous study indicates that SOD reduces inflammation by enhancing the body's antioxidant function (66). SOD decomposes superoxide into hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and H<sub>2</sub>O<sub>2</sub> can be further decomposed into H<sub>2</sub>O by CAT to exert antioxidant effects (67). The abnormal levels of anti- and pro-oxidation factors caused by DSS in the present study were all restored by GI treatment. Nrf2 is an important starting element in antioxidant systems (68) that can counteract the damaging effect of peroxide by activating the *peroxiredoxin-1* gene (69). Under peroxidative conditions, Nrf2 dissociates from the dimer and transfers to the nucleus, binding to the antioxidant responsive element (70). Binding

to this promoter activates the expression of a cytoprotective enzyme, including HO-1, CAT, or SOD (71). Nrf2 upregulates the *GST-A4* gene expression via the mitogen-activated protein kinase pathway to protect against UC (69). Nrf2 and NF- $\kappa$ B are inseparable in the process of anti-inflammation and antioxidation. The activation of Nrf2 attenuates inflammatory signaling by inhibiting NF- $\kappa$ B entry into the nucleus, whereas NF- $\kappa$ B directly inhibits the Nrf2 pathway at the RNA level (72). The data from the present study suggested that Nrf2/NF- $\kappa$ B signaling is involved in GI-mediated protection against UC; however, more experiments are required to investigate the synergistic process between Nrf2 and NF- $\kappa$ B signaling.

There remains a limitation the present study. Although the constituents contained in GI, based on the present data, were detected, the active ingredients with the anti-UC effect were not isolated and purified; this will be further investigated.

In summary, GI was identified to alleviate the physiological and pathological state of DSS-induced UC in mice via its

Table IV. Effect of GI on oxidative factors in serum and colon tissues of mice with ulcerative colitis.

A, Serum						
Oxidative factors	CTRL	Model	SASP, 0.6 g/kg	DSS		
				GI, g/kg		
				1.0	2.0	4.0
SOD, U/ml	99.7±3.3	81.7±2.6 <sup>c</sup>	94.8±3.1 <sup>e</sup>	83.9±3.1	93.8±2.2 <sup>e</sup>	98.2±1.9 <sup>f</sup>
CAT, U/ml	26.8±0.9	21.9±1.1 <sup>b</sup>	28.1±1.6 <sup>e</sup>	24.7±1.7	29.5±1.7 <sup>e</sup>	25.7±0.5 <sup>d</sup>
ROS, U/ml	177.1±3.1	174.2±3.9	165.6±2.8	167.3±5.7	172.4±2.1	179.9±0.7
NO, μmol/l	14.0±0.6	17.8±0.8 <sup>b</sup>	14.3±0.6 <sup>d</sup>	15.3±0.8 <sup>d</sup>	15.5±0.8 <sup>d</sup>	15.1±0.4 <sup>d</sup>

B, Colon						
Oxidative factors	CTRL	Model	SASP, 0.6 g/kg	DSS		
				GI, g/kg		
				1.0	2.0	4.0
SOD, U/mgprot	152.0±9.6	104.5±9.1 <sup>b</sup>	145.0±11.2 <sup>d</sup>	113.8±3.7	119.7±8.6	142.4±4.3 <sup>d</sup>
CAT, U/mgprot	31.3±2.2	21.4±2.1 <sup>a</sup>	27.8±0.9 <sup>d</sup>	22.2±0.6	25.3±1.9	22.2±1.4
ROS, U/mgprot	310.7±23.9	418.2±33.7 <sup>a</sup>	308.5±22.7 <sup>d</sup>	292.2±19.3 <sup>d</sup>	310.3±13.2 <sup>d</sup>	284.1±13.0 <sup>d</sup>
NO, μmol/gprot	13.8±1.1	21.9±2.8 <sup>a</sup>	11.5±1.9 <sup>c</sup>	11.6±0.9 <sup>d</sup>	12.0±1.7 <sup>d</sup>	8.0±0.5 <sup>e</sup>

Data were analyzed using a one-way ANOVA and are expressed as the mean ± SEMs (n=8). <sup>a</sup>P<0.05, <sup>b</sup>P<0.01 and <sup>c</sup>P<0.001 vs. control mice, <sup>d</sup>P<0.05, <sup>e</sup>P<0.01 and <sup>f</sup>P<0.001 vs. DSS-induced UC mice. GI, *Gloeostereum incarnatum*; CTRL, control; DSS, dextran sulfate sodium.

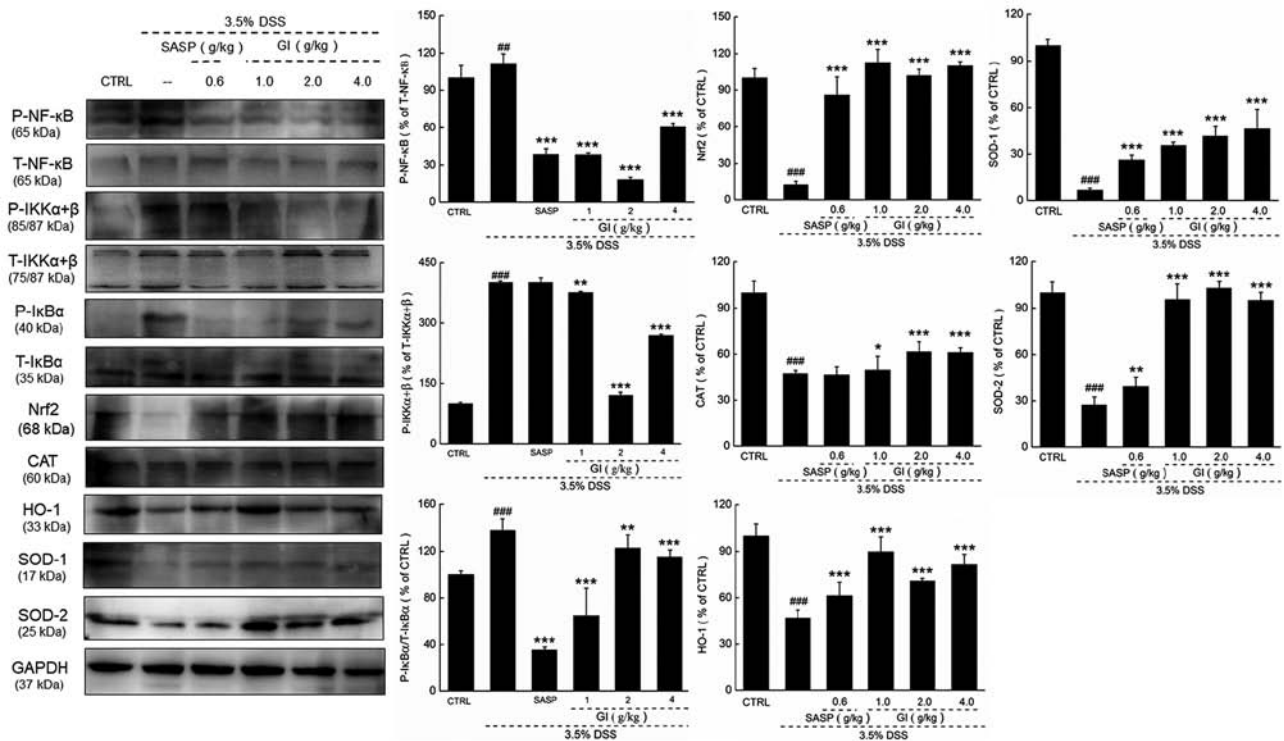


Figure 3. GI demonstrates antioxidant and anti-inflammatory functions via modulation the activation of Nrf2/NF-κB signaling. GI enhanced the expression levels of Nrf2, CAT, HO-1, SOD-1 and SOD-2, and reduced the phosphorylation level of NF-κB, IKKα+β and IκBα. The quantitative expression of each protein was normalized using GAPDH. ##P<0.01 and ###P<0.001 vs. control mice; \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001 vs. DSS-induced UC mice. GI, *Gloeostereum incarnatum*; SASP, sulfasalazine; P-, phosphorylated; T-, total; Nrf2, nuclear factor erythroid 2-related factor 2; CAT, catalase; HO-1, heme oxygenase-1; SOD, superoxide dismutase; IKK, inhibitor of NF-κB kinase; IκB, inhibitor of NF-κB; DSS, dextran sulfate sodium; CTRL, control.



antioxidant and anti-inflammatory functions, which may be associated with its modulation of the activation of Nrf2/NF- $\kappa$ B signaling.

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### Availability of data and materials

All data generated and analyzed during this study are included in this published article.

### Authors' contributions

DW and YL contributed to the conceptual design of the research. XiaL, XinL, YoZ, YaZ, SL and NZ performed the experiments. DW, YL, XiaL and XinL analyzed the data and wrote the manuscript. DW and YL helped perform the analysis with constructive discussions. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

The experimental animal protocol was approved by the Animal Ethics Committee of Jilin University [approval no. SYXK (JI) 2014-0013]. The present study was carried out under relevant guidelines and regulations.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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