Allergologia et immunopathologia
Sociedad Española de Inmunología Clínica, Alergología y Asma Pediátrica
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ORIGINAL ARTICLE

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Morroniside alleviates lipopolysaccharide-induced inflammatory and oxidative stress in inflammatory bowel disease by inhibiting NLRP3 and NF-κB signaling pathways

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Received 18 May 2022; Accepted 4 July 2022
Available online 1 November 2022

KEYWORDS
inflammatory and oxidative stress;
Iinflammatory bowel disease (IBD);
Morroniside;
NLRP3

Abstract
Objective: To investigate the effects of morroniside on inflammatory and oxidative stress in lipopolysaccharide (LPS)-induced inflammatory bowel disease (IBD) cell model.
Methods: NCM460 cells were treated with 2-, 5-, or 10-μg/mL LPS for 24 h to develop an IBD cell model. MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) colorimetric assay was performed to uncover the role of morroniside on the viability of LPS-treated NCM460 cells. Flow cytometry and immunoblot assays were performed to confirm the effects of morroniside on the apoptosis of LPS-treated NCM460 cells. Quantitative polymerase chain reaction and enzyme-linked-immunosorbent serologic assays were performed to confirm the effects of morroniside on inflammatory and oxidative stress by measuring the levels of tumor necrosis factor-α, interleukin-1β, IL-6, superoxide dismutase, malondialdehyde, total antioxidant capacity, and myeloperoxidase. In addition, immunoblot and immunofluorescence assays were performed to detect the effects of morroniside on NLRP3 and NF-κB pathways.
Results: Monosine attenuated LPS-induced injury of NCM460 cells. Monosine reduced LPS-induced inflammation in NCM460 cells. In addition, morroniside reduced LPS-induced oxidative stress in NCM460 cells. Mechanically, morroniside suppressed NLRP3 and NF-κB pathways, and alleviated LPS-induced inflammatory and oxidative stress in IBD.
Conclusion: Morroniside could serve as a promising drug for treating IBD.

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https://doi.org/10.15586/aei.v50i6.674

Introduction

Inflammatory bowel disease (IBD) is a noninfectious, chronic, and recurrent inflammation of the gastrointestinal tract.[1,2] Common symptoms of IBD include diarrhea, rectal bleeding, abdominal pain, fatigue, and weight loss.[3,4] It is important to diagnose IBD. In addition to colonoscopy, other procedures, such as upper endoscopy and sigmoidoscopy, can help the diagnosis of IBD, including common blood test that identifies inflammation.[2] Treatment of IBD mainly relies on anti-inflammatory drugs, immunosuppressants, and antibiotics, but many problems prevail with drug resistance.[5] Effective therapeutic drugs are to be developed to ameliorate symptoms.

Morroniside is an important iridoid glycoside extracted from *Cornus officinalis*.[6] Various pharmacological studies have shown that Morroniside is effective in the treatment of Alzheimer's disease, nerve protection, prevention of diabetic liver damage, kidney damage and other aspects.[7,8] Morroniside has a protective effect on human umbilical vein endothelial cells (HUVECs) in diabetic vascular disease, and strongly enhances the proliferation of endothelial progenitor cells and improves the microvascular function after cerebral ischemia.[9] However, the potential effects of morroniside on IBD remain unknown.

Oligomerization domain-like receptor family pyrin domain-containing 3 (NLRP3) mediates the production of pro-inflammatory cytokines.[10] NLRP3 is a speck-like protein associated with apoptosis, including the recruitment and activation of procaspase-1 and caspase-1.[11] Caspase-1 is required for the conversion of pro-IL-1β to its mature active form interleukin-1β (IL-1β), which is activated in macrophages by the lipopolysaccharide (LPS)-stimulated activation of NLRP3 inflammasome.[11] After activation, NLRP3 binds to the ASC connector and subsequently induces pro-caspase-1 translocation and activation.[12] In addition, the NF-κB signaling pathway is known critical in regulating the progression of inflammation by regulating the expressions of pro-inflammatory and pro-survival genes to control immune response and cell survival.[13]

An IBD cell model was developed by the treatment of 2-, 5-, or 10-μg/mL LPS (Sigma-Aldrich, St. Louis, USA) for 24 h, and results revealed that morroniside alleviated LPS-induced inflammatory and oxidative stress in IBD via inhibiting NLRP3 and NF-κB pathways. It is therefore believed that morroniside could act as a potential drug for IBD.

Materials and Methods

Cell culture and treatment

The NCM460 cells were bought from ATCC (Virginia, United States), and maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and incubated at 37°C in a 5% CO_2 incubator. NCM460 cells were treated with 2-, 5-, or 10-μg/mL LPS (Sigma-Aldrich) for 24 h. Morroniside (Sigma-Aldrich) was administrated into NCM460 cells at a concentration of 10-, 30-, 60-, 100-, or 200-umol/L for 24 h.

Immunoblot assay

Cell samples were lysed by RIPA lysis buffer (Solarbio, Beijing, China), and total proteins were extracted from cells in the presence of protease inhibitors. Proteins from NCM460 cells were isolated for extracting proteins and separated by 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), sequentially transferred onto polyvinylidene fluoride (PVDF) membranes, blocked with 5% bovine serum albumin (BSA) for 2 h. PVDF membranes were subsequently treated with primary antibodies, including Bax (1:1000, ab32503; Abcam, Cambridge, MA, USA), Bcl-2 (1:1000, ab32124; Abcam), NLRP3 (1:1000, ab263899; Abcam), p65 (1:1000, ab32536; Abcam), p-p65 (1:1000, ab183559; Abcam), IκBα (1:1000, #9242; Cell Signaling Technology (CST), Beverly, MA, USA), p-IκBα (1:1000, #2859; CST), and β-actin (1:3000, #3700; CST) at room temperature for 1.5 h. Subsequently, the membranes were treated with horseradish peroxidase (HRP)-conjugated antibodies for 1 h. Signals were detected using enhanced luminol-based chemiluminescent (ECL) kit.

Quantitative Polymerase Chain Reaction (qPCR) assays

Trizol (Invitrogen, Carlsbad, CA, USA) was used to extract RNA from NCM460 cells. Then, RNA was reverse-transcribed by a type of M-MLV reverse transcriptase (Promega, Madison, WI, USA). Total messenger RNA (mRNA) was reverse-transcribed using complementary DNA (cDNA)-specific synthesis system. qPCR was conducted through SYBR Ex type of Taq kit (Takara, Dalian, China). Primers for reference genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and target genes were synthesized by Sangon Biotech (Shanghai, China) and shown as follows: GAPDH: forward: 5′-GGCACCGTCAAGGCTGAGAAC-3′, reverse: 5′-GGTGGCAGTGATGGCATGGAC-3′; tumor necrosis factor-α (TNF-α): forward: 5′-ATGAGCACTGAAAGCATGATC-3′, reverse: 5′-TCACAGGGCAATGATCCCAAAGTAGACCTGCCC-3′; interleukin-6 (IL-6): forward: 5′-GGCCCTTGCTTTCTCTTCG-3′, reverse: 5′-ATAATAAAGTTTTGATTATGT-3′; and IL-1β: forward: 5′-ATGGCAGAAGTACCTAAGCTC-3′; reverse: 5′- TTAGG AAGACACAAATTGCATGGTGAACTCAGT-3′.

MTT colorimetric assay

NCM460 cells were seeded and maintained into 96-well plates with a density of 1000 cells. Thereafter, 10-μL MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) solution (5 mg/mL) was added to cells and incubated for additional 4 h at 37°C. Then, the insoluble formazan crystals were dissolved in 150-μL dimethyl sulfoxide (DMSO). Subsequently, the absorbance of each well was measured on a microplate spectrophotometer at 570-nm wavelength.

Flow Cytometry (FCM) assay

1×10^6 NCM460 cells were re-suspended. Then, cells were fixed with 70% ethyl alcohol for 24 h at −20°C,

and incubated with 50-μg/mL propidium iodide (PI) and annexin V-FITC at 37°C for 10 min to indicate cell apoptotic percentage.

Enzyme Linked-immunosorbent Serologic Assay (ELISA)

NCM460 cell culture supernatant was obtained for measuring TNF-α, IL-1β, IL-6, superoxide dismutase (SOD), malondialdehyde (MDA), total antioxidant capacity (T-AOC), and myeloperoxidase (MPO) concentrations according to the manufacturer's instructions (Dakewei, Beijing, China).

Statistical analysis

All data were presented as mean ± SD. Statistical significance was determined by Student's *t*-test between two groups. Statistical analysis was performed using GraphPad. $P < 0.05$ was considered as statistically significant.

Results

Morroniside treatment alleviates LPS-induced injury of NCM460 cells

In order to uncover the possible effects of morroniside on the progression of IBD, a cell model of IBD was developed by treating NCM460 cells with 2-, 5-, or 10-ug/mL LPS for 24 h. The structural formula of morroniside is shown in Figure 1A. LPS treatment significantly decreased NCM460 cell viability, indicating that the cell model of IBD was successfully constructed (Figure 1B). Then, morroniside was administrated into NCM460 cells at a concentration of 10-, 30-, 60-, 100-, or 200-umol/L for 24 h. However, no significant difference in cell viability was observed between control and morroniside treatment groups using MTT assays (Figure 1C). Interestingly, morroniside treatment alleviated LPS-induced cell injury in a concentration-dependent manner (Figure 1D), and effects of morroniside on LPS-induced NCM460 cells were detected. Immunoblot assay showed that morroniside treatment rescued alteration of Bax and Bcl-2 expressions caused by LPS treatment in NCM460 cells (Figure 1E). FCM assay revealed that morroniside treatment suppressed the apoptosis of NCM460 cells caused by LPS

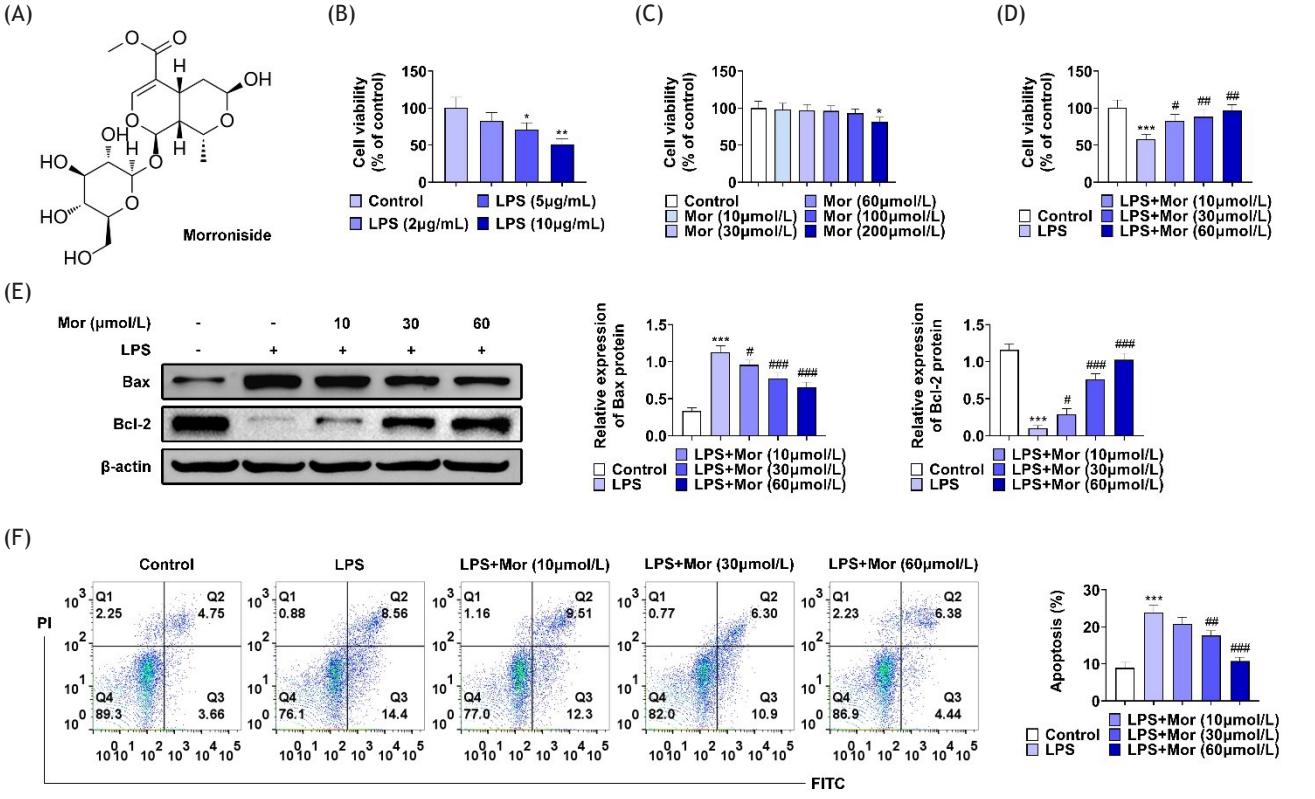

Figure 1 Morroniside treatment alleviates LPS-induced injury in NCM460 cells. (A) The structural formula of morroniside. (B) MTT assay showed the viability of NCM460 cells after LPS treatment at a concentration of 2, 5, or 10 ug/mL for 24 h. (C) MTT assay showed NCM460 cells viability after morroniside treatment at a concentration of 10, 30, 60, 100, or 200 umol/L for 24 h. (D). MTT assay showed NCM460 cells viability with indicated treatment. (E) Immunoblot assay showed the expressions of Bax and Bcl-2 in NCM460 cells with indicated treatment. (F) FCM assay showed the apoptosis of NCM460 cells with the indicated treatment. Results were presented as mean ± SEM. LPS vs. control, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; LPS + morroniside vs. LPS, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$.

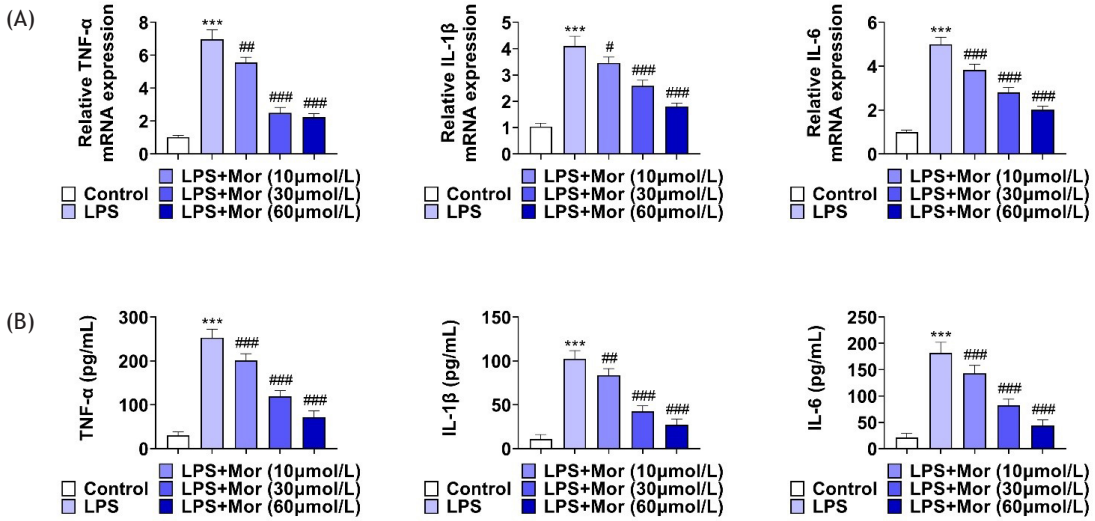

Figure 2 Morroniside treatment alleviates LPS-induced NCM460 cells inflammatory stress. (A) qPCR assay showed the mRNA levels of TNF-α, IL-1β, and IL-6 in NCM460 cells with indicated treatment. (B) ELISA showed the levels of TNF-α, IL-1β, and IL-6 in NCM460 cells with indicated treatment. Results were presented as mean ± SEM, LPS vs. control, ***P < 0.001, LPS + morroniside vs. LPS + control, #P < 0.05, ##P < 0.01, and ###P < 0.001.

treatment (Figure 1F). In summary, morroniside treatment alleviated LPS-induced NCM460 cell injury.

Morroniside treatment alleviates LPS-induced NCM460 cell inflammation

Subsequently, effects of morroniside on the inflammation of LPS-induced NCM460 cells were detected. qPCR assays showed the mRNA levels of TNF-α, IL-1β, and IL-6 in NCM460 cells (Figure 2A). LPS treatment dramatically increased the mRNA levels of TNF-α, IL-1β, and IL-6 in NCM460 cells whereas morroniside treatment decreased these mRNA levels in LPS-treated NCM460 cells (Figure 2A). ELISA confirmed that the levels of TNF-α, IL-1β, and IL-6 were upregulated in LPS-treated NCM460 cells, whereas morroniside treatment dramatically decreased these levels in LPS-treated NCM460 cells (Figure 2B). Taken together, morroniside treatment inhibited the LPS-induced inflammation in NCM460 cells.

Morroniside treatment alleviates LPS-induced oxidative stress in NCM460 cells

In order to examine the anti-oxidative stress effects of morroniside on LPS-treated NCM460 cells, levels of SOD, MDA, T-AOC, and MPO were discovered, which would reflect oxidative stress levels. The stress levels of SOD and T-AOC were significantly decreased whereas that of MDA and MPO were markedly enhanced in LPS-treated NCM460 cells (Figure 3). Morroniside treatment restored antioxidant capacity dramatically, as exhibited by enhancement of SOD and T-AOC levels, and reduction of MDA and MPO levels (Figure 3). These data established that morroniside treatment alleviated LPS-induced oxidative stress in NCM460 cells.

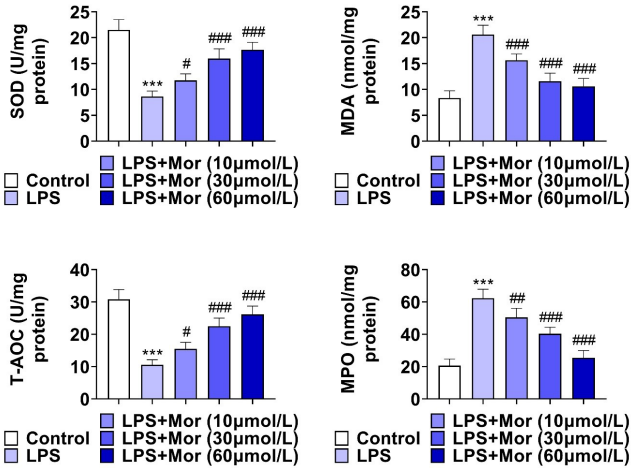

Figure 3 Morroniside treatment alleviates LPS-induced NCM460 cell inflammatory stress. Levels of SOD, MDA, T-AOC, and MPO were detected by ELISA. Results were presented as mean ± SEM. LPS vs. control, ***P < 0.001, LPS + morroniside vs. LPS + control, #P < 0.05, ##P < 0.01, and ###P < 0.001.

Morroniside treatment suppressed NLRP3 and NF-κB pathways in LPS-treated NCM460 cells

Previous studies have demonstrated that morroniside regulated NLRP3 and NF-κB pathways; hence, expressions of NLRP3- and NF-κB-related proteins were discovered. LPS treatment significantly increased NLRP3, p-p65-p65, and p-IκBα-IκBα expressions in NCM460 cells, suggesting the activation of NLRP3 and NF-κB pathways whereas morroniside treatment decreased the expressions of NLRP3, p-p65-p65, and p-IκBα-IκBα in LPS-treated NCM460 cells (Figure 4A). Immunofluorescence assay showed that p65

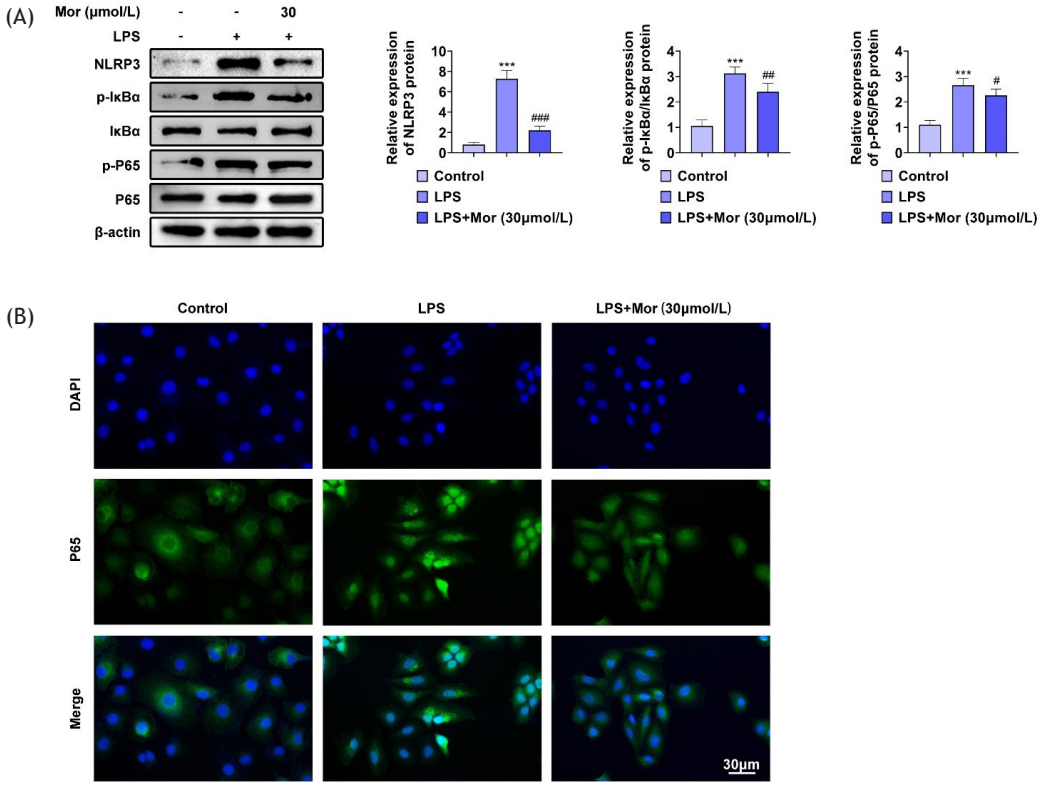

Figure 4 Morroniside treatment suppresses NLRP3 and NF-κB pathways in LPS-induced NCM460 cells. (A) Immunoblot assay showed the expressions of NLRP3, p65, and IκBα, and the phosphorylation levels of p65, and Iκ-Bα in NCM460 cells with indicated treatment. (B). Immunofluorescence assay showed the location of p65 in NCM460 cells with indicated treatment. Scale bar, 30 μm. Results were presented as mean ± SEM. LPS vs. control, ***$P < 0.001$, LPS + morroniside vs. LPS + control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$.

was located in the nucleus of LPS-treated NCM460 cells, suggesting the activation of NF-κB pathway (Figure 4B). Importantly, the expression of p65 was located in the cytoplasm of LPS-induced NCM460 cells after morroniside treatment (Figure 4B). Therefore, morroniside treatment suppressed NLRP3 and NF-κB pathways in LPS-induced NCM460 cells.

Discussion

Inflammatory bowel disease signifies disorders involving chronic inflammation of the digestive tract, that is, a type of inflammatory disease that affects the rectum and colon.[1] The treatment mainly includes drug therapy and surgery. More effective drugs are to be developed to improve treatment effectiveness.[3] Inflammatory response caused by an abnormal immune system reaction in the intestinal mucosa influences the pathogenesis of IBD, and is believed to be caused by the interaction of multiple factors.[14] In the present study, a traditional Chinese medicine, morroniside, was found to be used as a potential treatment of IBD. An IBD model of LPS-induced intestinal epithelial cell injury was established, and the results revealed that morroniside alleviated LPS-induced inflammatory and oxidative stress. It is therefore believed that morroniside could serve as a promising drug for IBD.

MTT, FCM, and immunoblot assays revealed that morroniside reduced LPS-induced cell injury. Furthermore, qPCR assay and ELISA discovered that morroniside could alleviate LPS-induced inflammation. In addition, morroniside also alleviated the oxidative stress caused by induction of LPS in NCM460 cells. Collectively, morroniside could serve as a promising drug for IBD. The biological activities of morroniside have been revealed in multiple studies.[6,7] Morroniside could promote peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1-mediated cholesterol efflux in high glucose (HG)-induced tubular epithelial cells.[8] Morroniside could also protect OLN-93 cells against HO-induced injury via anti-oxidative stress and anti-apoptotic activities.[6] Similarly, the effects of morroniside on the oxidative stress of NCM460 cells were also determined. In addition, a study indicated that morroniside protected HT-22 cells from deprivation/reperfusion.[15] The neuroprotective role of morroniside in spinal cord injury has also been revealed.[16] These studies confirmed that morroniside could serve as a drug for inflammation and oxidative stress-related diseases.

Interestingly, a previous study established that morroniside attenuated apoptosis and pyroptosis of chondrocytes and ameliorated osteoarthritic development

via suppressing NF-κB pathway.[17] Another study demonstrated that the regulation of NF-κB pathway was involved in the inhibition of LPS-induced inflammation and oxidative reactions by morroniside.[18] The present study also found that morroniside suppressed the apoptosis of LPS-induced NCM460 cells. Furthermore, it was observed that morroniside could suppress NLRP3 and NF-κB pathways in NCM460 cells. In fact, the effects of NLRP3 and NF-κB pathways on the inflammation, apoptosis, and oxidative stress of NCM460 cells have been revealed widely. NLRP3 is a speck-like protein associated with apoptosis and plays an important role in regulating apoptosis and inflammation.[10] In addition, the NF-κB signaling pathway plays an important role in regulating the progression of inflammation by regulating the expressions of pro-inflammatory and pro-survival genes to control immune response and cell survival.[19,20] Morroniside could also suppress NLRP3 and NF-κB pathways to alleviate LPS-induced inflammatory and oxidative stress in IBD. Therefore, morroniside could serve as a promising drug for IBD treatment.

Conclusion

In summary, this study revealed that morroniside alleviated LPS-induced inflammatory and oxidative stress in IBD by suppressing NLRP3 and NF-κB pathways. Therefore, it could serve as a promising drug for treating IBD.

Competing Interests

The authors stated that there were no conflicts of interest to disclose.

Ethics Approval

This article does not contain any studies/experiments with human participants or animals performed by any of the authors.

Data Availability

The authors declare that all data supporting the findings of this study are available in the paper. Any raw data can be obtained from the corresponding author upon request.

Author Contributions

All authors contributed to the study's conception and design. Material preparation and experiments were performed by Shifen Zhang and Qiaohua Lai. Data collection and analysis was done by Liming Liu. The first draft of the manuscript was written by Yajie Yang and Juan Wang. All authors commented on the previous versions of the manuscript. All authors read and approved the final manuscript.

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