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ORIGINAL RESEARCH

LianChuang ZhiXue Liquid Enema Modulates the Macrophage Polarization of Ulcerative Colitis via Inhibiting the Jagged-1/Notch1 Signaling Pathway

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Objective: LianChuang ZhiXue Liquid (LCZXL) enema, one of the classic Chinese medicine prescriptions, in which herbal decoction enema acts directly on the intestines, is clinically effective in patients with ulcerative colitis (UC). However, its specific molecular mechanism has not been clarified to explore the underlying macular mechanism of LCZXL enema effect on UC, based on Jagged-1/Notch1 signaling pathway and the macrophage polarization.

Methods: After modeling of UC mice, the experiment was conducted in two parts: Experiment I: Control, Model, Mesalazine, Low dose of LCZXL (LCZXL-L), High dose of LCZXL (LCZXL-H); Weight and Disease Activity Index (DAI) scores were recorded on days 1, 3, 5, 7, 9, 11, and 13. On day 14, the colon was taken off. Then the colon length was measured and the damage index (CMDI) score of colon mucosal was evaluated. Zonula occludens-1 (Zo-1) Immunohistochemistry (IHC) and Hematoxylin-Eosin (HE) staining were performed to visualize the colon injury. ELISA was used to detect cytokines content in serum. The M1 and M2 markers and Jagged-1/Notch1 signaling pathway-related genes/proteins were quantified by IF double staining, PCR, WB, and flow cytometry. Experiment II: Control, Model, Recombinant mouse Jagged-1 protein/ Fc Chimera Active (Jagged-1/Fc), LCZXL, Jagged-1/Fc +LCZXL. The M1 and M2 markers and Jagged-1 proteins were quantified by WB, and flow cytometry.

Results: Our results indicated that LCZXL could reduce the colon injury of UC mice effectively, which expressed DAI and CMDI score reduction, and inhibited colon structure damage. DSS induced a significant up-regulation of CD86, iNOS, TNF-α, IL-1β, and IL-6, and a down-regulation of Arg1, CD206, IL-4, IL-10 and increase of Jagged-1, Notch1, and Notch2. LCZXL enema treatment inhibiting the increase of UC modeling-induced CD86, TNF-α, IL-1β, and IL-6 and increased CD206, Arg1, IL-4, and IL-10 expression level. Notch signaling pathway activator Jagged-1/Fc aggravated M1 macrophage polarization and activated the Jagged-1/Notch1 signaling pathway. LCZXL treatment reversed this situation stop the activation of the Jagged-1/Notch1 signaling pathway.

Conclusion: Our study proved that LCZXL Enema could inhibit the M1 Macrophage Polarization and promote M2 macrophage polarization of ulcerative colitis via regulating the Jagged-1/Notch1 signaling pathway.

Keywords: LCZXL enema, M2 macrophage polarization, ulcerative colitis, Jagged-1/Notch1 signaling pathway

Introduction

Ulcerative Colitis (UC) is an inflammatory bowel disease that primarily affects the colon and rectum. Symptoms include fever, diarrhea, abdominal pain, and bloody stools.[1] UC has been reported to increase the risk of colon cancer and lead to depression and anxiety disorders.[2,3] Globally, UC is more prevalent in developed countries, especially in North America and Europe.[4] It has no specific age or gender preference and is mainly related to lifestyle and dietary habits. Currently, there is no definitive cure for UC. The primary goal of treatment is to control symptoms and prevent complications. Therefore, regular medical monitoring and exploration of new therapeutic agents are essential to control UC.

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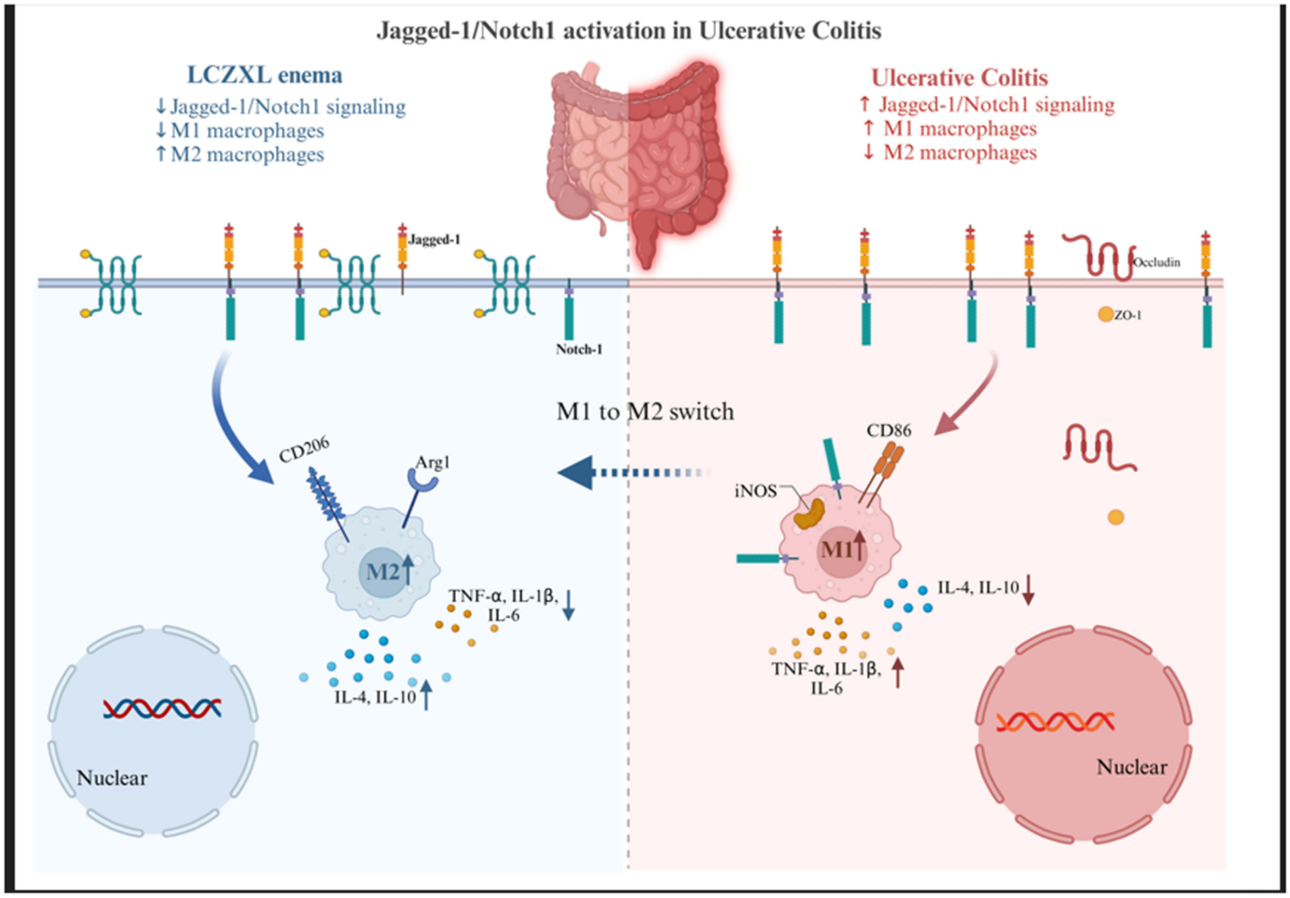
Graphical Abstract



In UC, macrophages in the intestinal mucosa are key players during the inflammatory process. They are typically polarized into M1 and M2 phenotypes. The imbalance in macrophage polarization is observed in UC.[5] The predominance of M1 macrophages leads to the production of pro-inflammatory cytokines and exacerbates inflammation. On the other hand, M2 macrophages, which are supposed to counteract this inflammation, are often insufficient or less effective in patients with UC. This imbalance contributes largely to the chronic inflammation that characterizes UC.[6] Recently, studies have explored the potential of using phytochemicals to modulate macrophage polarization in UC, suggesting that herbal compounds can effectively modulate macrophage responses.[7,8] Therefore, we believe that controlling M2-type macrophage polarization can reduce the inflammatory response, alleviating UC symptoms and preventing disease progression, which is emerged as a potential strategy for the treatment of UC.

The Notch signaling pathway is a highly conserved signaling pathway that plays a key role in a variety of physiological processes, including cell proliferation, apoptosis, differentiation, and fate determination, as well as being critical for the maintenance of tissue homeostasis. In addition, it is one of the major signaling pathways for cell-to-cell communication. And is also a critical regulator of mucosal inflammation and intestinal epithelial cell fate determination,[9] which was highly conserved in multicellular organisms and can regulate T cell development and differentiation. Notch1 signaling pathway could increase Reactive oxygen species (ROS) production, enhance the phagocytic and bactericidal capabilities of Mø, and upregulate NF-κB to enhance M1 polarization and pro-inflammatory response.[10] Activation of the Notch signaling pathway requires the binding of Notch receptors (Notch1-4) and ligands (Jagged-1, Jagged-2) in

neighboring cells.[11] Among them, Notch1 is a highly conserved type I transmembrane glycoprotein, and as one of the most prominent receptors of the Notch signaling pathway, Jagged-1 is most highly expressed in human cells.[12]

The Jagged-1/Notch1 signaling pathway plays an important role in various biological processes, which can activate pro-inflammatory cytokines promotion of inflammation. Studies showed that down-regulation of the Jagged-1/Notch1 signaling pathway facilitated the polarization of M1-type macrophages, thereby inhibiting the development of gastric cancer.[13] In previous UC studies, to inhibit the Notch1 pathway expression can decrease the inflammatory factors IL-6 and TNF-α. Jagged-1 (the Notch signaling with an exogenous Notch ligand) used in combination with treatment can reactivate the Notch1 pathway, with reductions in the expression of the tight junction proteins occludin, ZO-1 and the increases in the levels of the inflammatory factors IL-6 and TNF-α.[14] These findings highlight the dual role of the Jagged-1/Notch1 signaling pathway as both a promoter and inhibitor of inflammation. Understanding these dynamics is critical for the development of targeted therapies that can modulate this pathway to treat UC.

Currently, complementary and alternative agents are increasingly utilized for treating IBD because of their potential efficacy, and they account for approximately 21% of IBD patients now.[15] Chinese medicine enema are particularly beneficial for patients with ulcerative colitis,[16] as they deliver the medication directly to the inflamed area in the rectum and lower colon, providing rapid relief and minimizing systemic side effects. The LCZXL enema consists of Puhuang, Baiji Fen, Chishizhi, and Qingdai. Chishizhi and Baiji stop bleeding, regenerating tissue to heal wound, and nourish the lungs and yin. Puhuang promotes blood circulation for removing blood stasis, and alleviates pain. Qingdai clears heat, detoxification, cooling the blood and reducing swelling. Evidence suggested that crocus sativus reduced the inflammatory response in the gastrointestinal tract and therefore improved inflammatory bowel diseases such as chronic colitis.[17,18] Kuiyangling enema (including Baiji, Qingdai and Puhuang) can reduce the level of intestinal NETs and inhibit the TNF and oxidative phosphorylation signaling pathways to alleviate the symptoms of ulcerative colitis.[19] Our study will explore the underlying mechanism of LCZXL enema treating UC based on Jagged-1/Notch1 signaling pathway and the macrophage polarization.

Methods and Materials

Preparation of LCZXL

LCZXL was provided by Xi'an Hospital of Traditional Chinese Medicine in China. The botanical drug compositions of LCZXL included the following: Puhuang (*Pollen Typhae*) 10 g, Baiji Fen (*Bletilla Striata (Thunb.Ex A.Murray)Rchb. F)*.10 g, Chishizhi (*Halloysitum Rubrum*) 10 g, and Qingdai (*Indigo Naturalis*) 10 g. The soaking of botanical drug was performed in a 6 times volume of ddH2O (w/v) for 0.5 h and subsequently boiled it twice, followed by combining two boiled solutions. The concentration of the aqueous extract solution of the drug was adjusted to 4 g/mL of enema by concentrating the filtrate by spinning it in a water bath at 65 °C (The plant names could refer to Lab of Systems Pharmacology www.tcmsp-e.com). According to the clinically recommended daily dose of 70 kg for adults and the conversion formula of mouse and human body surface area, the coefficient of mice was 9.1, and the dose of mice in the LCZXL-L group and LCZXL-H group was 1.8 g/kg and 3.6 g/kg.

C57BL/6 Mice UC Modeling and Drug Treatment

A total of 60 C57BL/6 mice (aged 6–8 weeks, weight 18–22 g) were purchased from Cavens Laboratory Animal Co. (Changzhou, China), adaptively feeding with 12 h light and 12 h dark for 7 days. Based on our previous research and literature, the Control group was given pure water for drinking, while the other groups were given 3% DSS for free drinking for 7 days, followed by 1% DSS for free drinking for 7 days.[20] Separating cages for each group, drug treatment was carried out on day 4. Experiment I (n=6): Control, Model, Mesalazine, LCZXL-L, LCZXL-H; Experiment II (n=6): Control, Model, Jagged-1/Fc, LCZXL, Jagged-1/Fc + LCZXL. Animal handling procedures and drug dosage were shown in Figure 1. The drug was administered as follows: feeding for DSS (265152-M, Sigma), enema for LCZXL, and intraperitoneal injection for Notch signaling pathway activator 1 mg/kg Jagged-1 mouse recombinant protein (ab109346,

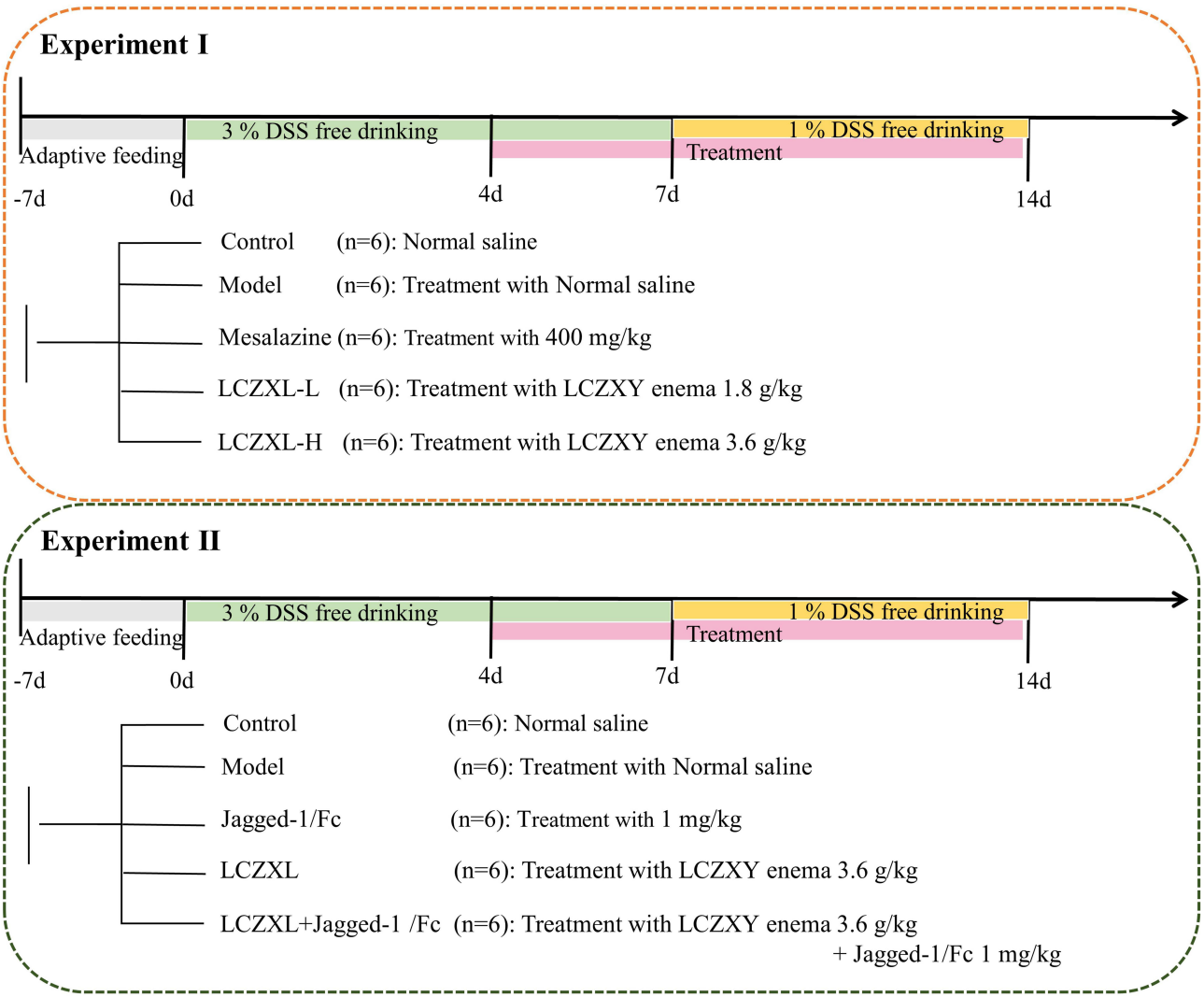


Figure 1 The methods of UC modeling and drug treatment, experiment I and experiment II.

Abcam). The animal experiment was executed with the agreement from the Experimental Animal Ethics Committee, Shaanxi University of Chinese Medicine (Approval SUCMDL20240307048).

Identification of the LCZXL Components Using UHPLC-MS/MS

The decoction of LCZXL were centrifuged at 12000 rpm (RCF = 13,800 (×g), R= 8.6 cm) for 15 min at 4 °C. 300 μL of the supernatant liquids was taken, mixed with 1000 μL of extraction solution (MeOH:ACN:H2O, 2:2:1 (v/v/v)) which contained deuterated internal standards. Then the samples were centrifuged at 12000 rpm (RCF =13800 (×g), R = 8.6 cm) for 15 min at 4 °C; The supernatant was filtrated by a microporous filter membrane (0.22 μm) and used for LC-MS/MS detection. LC-MS/MS analysis was conducted on an UHPLC system (Vanquish, Thermo Fisher Scientific), which was with a Waters UPLC BEH C18 column (2.6 μm 2.1*100 mm).

General Condition Observation

In the experiment, after establishing a model of ulcerative colitis (UC) in mice, their body weight and disease activity index (DAI) scores[21] were recorded on days 1, 3, 5, 7, 9, 11, and 13. On the 14th day of the experiment, mice were anesthetized with 1% pentobarbital sodium and euthanized by cervical dislocation. Subsequently, the cecum and a portion of the colon were removed from the mice, and the length of the colon was measured. The colon was then

dissected to assess the extent of mucosal damage, and a Colon Mucosal Damage Index (CMDI) score[22] was assigned on the basis of the observed damage.

Hematoxylin and Eosin (HE) and Immunohistochemistry (IHC) Staining

The colon was fixed in 4% paraformaldehyde for 24 h, followed by PBS washing, dehydration, and paraffin embedding. The paraffin blocks were then sectioned into 5 μm slices. For HE staining, we used an HE staining kit (Beyotime, China). For IHC, the slices were treated with 3% hydrogen peroxide to block endogenous peroxidases. Subsequently, 3% bovine serum (GC305010, Servicebio) was added and incubated for 20 min. The Zo-1 (1:100, GB111402, Servicebio) antibody was incubated with the slices overnight at 4 °C. After PBS washing, the secondary antibody (1:100, GB23303, Servicebio) was added, and the slices were incubated at 37 °C for 30 min. Tissue staining was visualized by using DAB, followed by counterstaining with hematoxylin. The expression of Zo-1 in the colon was analyzed by using the Halo 101-WL-HALO-1 data imaging analysis system (Indica Labs, USA). Image acquisition was performed by using the BA210 Digital trinocular microscope (Meiji Techno Co., Ltd., USA).

Immunofluorescence Double Staining

The colon slices were prepared as IHC, followed by an incubation of primary antibodies including Jagged-1 (1:100, ET1702-63, HUAbio, China), iNOS (1:100, GB11119, Servicebio, China), and CD206 (1:100, GB113497, Servicebio), and an incubation of the secondary antibody (1:100, GB23303, Servicebio). The antigen was subjected to antigen retrieval once again, which was then incubated overnight with an F4/80 (1:100, 111602, BioLegend) antibody. The slices were washed by using PBS and subsequently incubated with DAPI for 10 min. The DAPI marked the cell nuclei with a blue color, while F4/80-positive expression was indicated in green. Additionally, expressions of INOS, Jagged-1, and CD206 were visualized in red.

Flow Cytometry for M1 and M2 Macrophages Detection

Flow cytometric analysis was conducted by following an established procedure. The colon tissue was finely chopped and then incubated in RPMI-1640 medium (R4130, Sigma, USA), which was enriched with specific supplements, followed by centrifugation to isolate the cells. For the flow cytometry process, colon macrophages were stained by using fluorescently labeled antibodies targeting CD86 (Ab239075, Abcam, UK), CD206 (GB113497, Servicebio), and F4/80 (111602, BioLegend, USA). The flow cytometric analysis was performed by using a Cytoflex system (located in Brea, CA, USA), and the data acquired from the stained cells were processed and interpreted by using Kaluza software (Beckman Coulter, USA).

qPCR Assay

Colon tissue of each group was collected to extract total RNA by Tissue Total RNA Kit (19221ES50, YEASEN). After total RNA was transformed to cDNA, a TB Green ™ Premix Ex Taq™ II (RR820A, Takara) kit was used to perform qPCR assay by real-time fluorescence quantification (QuantStudio TM3, ThermoFisher, USA). All primers in this study were listed in Table 1, which were synthesized by Sangon Biotech (Shanghai, China), using the Quant Studio TM Design

Table 1 Primers in This Study Were Used for qPCR Assay

Gene	Forward Primer	Reverse Primer
β-actin	ctacctcatgaagatcctgacc	cacagcttctctttgatgtcac
Arg1	catatctgccaaagacatcgtg	gacatcaaagctcaggtgaatc
CD86	gcagcacggacttgaacaaccagact	agcctttgtaaatgggcacggcagat
CD206	cctatgaaaattgggcttacgg	ctgacaaatccagttgttgagg
iNOS	tcactcagccaagccctcacctact	gcctccaatctctgcctatccgtctc
Jagged-1	aaaaatcaggacacacaactcg	ctgtttatttgtccagttcggg
Notch1	gtgctggaagtattttagcgac	gtccttgcagtactggtcatac
Notch2	catcaacaaccagtgtgatgag	tttgtcatacttgcacgtcttg

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& Analysis SE Software (Thermo, USA) to analyze the CT values. Genes mRNA expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. A procedure: 95 °C for 30s, 45 circle of 95 °C for 5s, 55 °C for 30s, and 72 °C for 30s was run.

ELISA

Enzyme-linked immunosorbent assay (ELISA) kits including TNF-α (ZC-39024), IL-1β (ZC-37974), IL-6 (ZC-37988), IL-4 (ZC-37986), and IL-10 (ZC-37962) were obtained from ZIO Co. (Shanghai, China). Mice serum of each group was employed to perform ELISA based on the manufacturer's instructions. A microplate reader (SpectraMAX Plus384, MEGU Molecular Instruments Co.) was used to obtain the OD value at 450 nm.

Western Blotting Assay

Colon samples were cracked on ice by PIRA lysis solution and tissue grinding instrument. The supernatant was collected and the protein content was detected by a BCA kit (P0009, Beyotime, China). Total protein was denatured by following 95 °C for 15 min before SDS-PAGE electrophoresis. PVDF membranes were diverted with protein bands and incubated with primary antibodies, including Jagged-1 (1:1000, DF8269, Affbiotech), Notch1 (1:1000, A19090, Abclonal), Notch2 (1:1000, BS-21664R, Bioss), and β-actin (1:50000, AC026, Abclonal) at 4°C overnight. After 3 times of washes, the second antibody (1:5000, S0001, Affbiotech) was used to incubate with the membranes for 2 h at room temperature. ECL solution and Fluorescence Image Analysis System Software V2.0 (5200 Multi, Tanon, China) were used to expose the bands. The integrated optical density (IOD) was used to represent the target protein.

Data Statistics and Analysis

The GraphPad 8.0 program was utilized to analyze data. Data from various groups were presented in the form of mean values with standard deviation (SD) included. When comparing two normally distributed groups, the Student's *t*-test was applied to evaluate mean differences. Additionally, the Kolmogorov–Smirnov test was employed to conduct analyses between two groups. For cases which more than two groups were involved, the one-way ANOVA test was used, followed by Bonferroni post hoc adjustment to assess mean variations. Any outcome with a P-value less than 0.05 was deemed to hold statistical significance.

Results

Identification of Components in LCZXL

UHPLC-MS/MS was used to identify the components in LCZXL. The total ion current chromatograms (TIC) obtained in positive and negative mode were shown in (Figure 2A, B). Table 2 identified and labeled the representative components of each botanical drug in LCZXL. Galactose, 3.5-dimethoxy-4 and Loganic acid belong to Baiji; 16-Hydroxypalmitic acid and 14-(hydroxymethyl) belong to Chishizhi; Fructose, Kaempferol-3-O-galactoside and Trigonelline belong to Puhuang; Indican, Guanine, 4-Aminobutyric Acid and L-Uridine belong to Qingdai. The active ingredients of LCZXL were found to include mainly carbohydrates, alkaloids, hydroxy fatty acids, terpenoids, Shikimic acid and phenylpropanoids.

LCZXL Enema Treatment Alleviates UC Development Effectively

Phenotypic changes were the basis for studying the effects of LCZXL enema on UC mice. As Figure 3A and B have shown, UC modeling caused a significant shortage of colon length. LCZXL-H alleviated this status significantly. Besides, the model group showed the highest CMDI score among all groups, LCZXL, and mesalazine treatment down-regulated it obviously (Figure 3C). It was worth noting that the mice's body weight were gradually lost (Figure 3D) and the DAI score was increased (Figure 3E). However, both LCZXL and mesalazine treatment reversed it to a certain extent. Thus, we can confirm that LCZXL enema treatment was beneficial with UC mice, and the underlying mechanism needed to be studied.

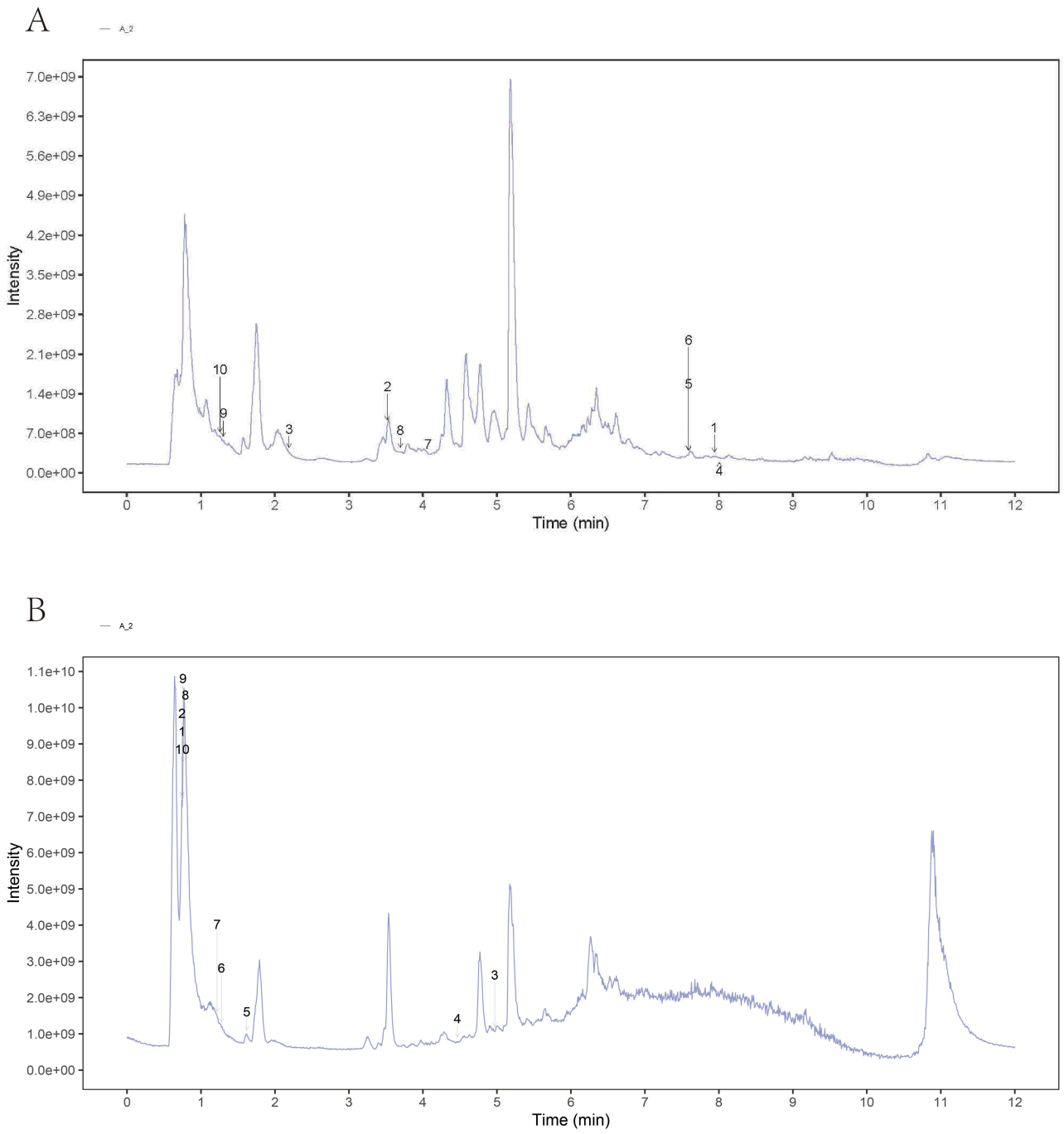

Figure 2 Identification of Components in LCZXL using UHPLC-MS/MS. (**A**) Negative ion mode and (**B**) Positive ion mode.

LCZXL Enema Treatment Strengthens the Intestinal Barrier of UC Mice by Controlling the Release of Inflammatory Cytokines

Pathological observation was essential for judging the severity of UC. The colon of UC mice expressed serious epithelium loss, infiltration of inflammatory cells, depletion of goblet cells, and crypt damage (Figure 4A). Compared with the model group, mesalazine, LCZXL-L, and LCZXL-H groups re-established a part of goblet cells, and alleviated crypt damage. Especially in the LCZXL-H group, the inflammatory cell infiltration was reduced significantly and epithelium was recovered. Zo-1 was a marker protein of intestinal barrier function, which had a high expression in normal colon. Figure 4B and C showed that UC induced a Zo-1 decrease expression, and LCZXL reversed it.

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Table 2 Chemical Characterization of the Formula

No.	RT(min)	Name	Formula	m/z
1	44.7	Galactose	C6H12O6	203.0521
2	44.7	Fructose	C6H12O6	203.0521
3	44.7	Tagatose	C6H12O6	203.0521
4	45.4	4-Aminobutyric Acid	C4H9NO2	104.0703
5	47.3	Trigonelline	C7H7NO2	138.0545
6	73.1	Guanine	C5H5N5O	152.0562
7	75.4	Adenine	C5H5N5	134.047
8	76.6	Hypoxanthine	C5H4N4O	137.0454
9	78.1	L-Uridine	C9H12N2O6	243.0619
10	97.1	Matrine	C15H24N2O	249.1955
11	131.4	Xanthosine	C10H12N4O6	283.0684
12	211	3,5-dimethoxy-4-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl) tetrahydropyran-2-yl]oxy-benzoic acid	C15H20O10	359.0982
13	221.7	Loganic acid	C16H24O10	375.1296
14	244.2	Indican	C14H17NO6	294.0982
15	268	Rotundine	C21H25NO4	356.1845
16	298.3	Kaempferol-3-O-galactoside	C21H20O11	449.1065
17	455.5	14-(hydroxymethyl)-5,9-dimethyl-tetracyclo[11.2.1.01,10.04,9]hexadecane-5-carboxylic acid	C20H32O3	319.2272
18	455.5	(5S,9R)-14-(hydroxymethyl)-5,9-dimethyl-tetracyclo[11.2.1.01,10.04,9] hexadecane-5-carboxylic acid	C20H32O3	319.2272
19	476.4	IS2		190.2579
20	480.4	16-Hydroxypalmitic acid	C16H32O3	271.2273

Furthermore, Figure 4D expressed that LCZXL-H treatment rescued an increase of TNF-α, IL-1β, and IL-6 expression levels in the serum of UC mice. Interestingly, IL-4 and IL-10 did have no significant differences between the model and control group. However, LCZXL-H and mesalazine treatment significantly up-regulated the IL-4 and IL-10 expression of

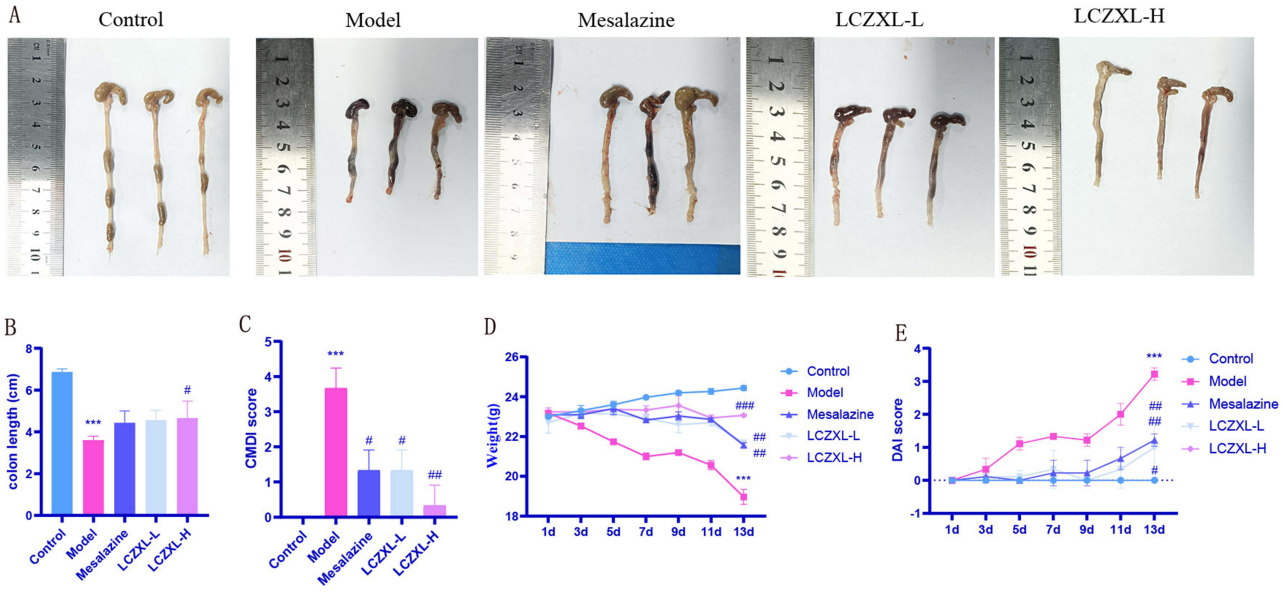

Figure 3 LCZXL enema treatment alleviating UC development effectively. (**A**) The measurement of colon length. (**B**) The colon length count. (**C**) CMDI score. (**D**) weight of mice. (**E**) DAI score. ***P < 0.001, compared with control group. #P < 0.05, ##P < 0.01 and ###P < 0.001, compared with model group. mean±SD.

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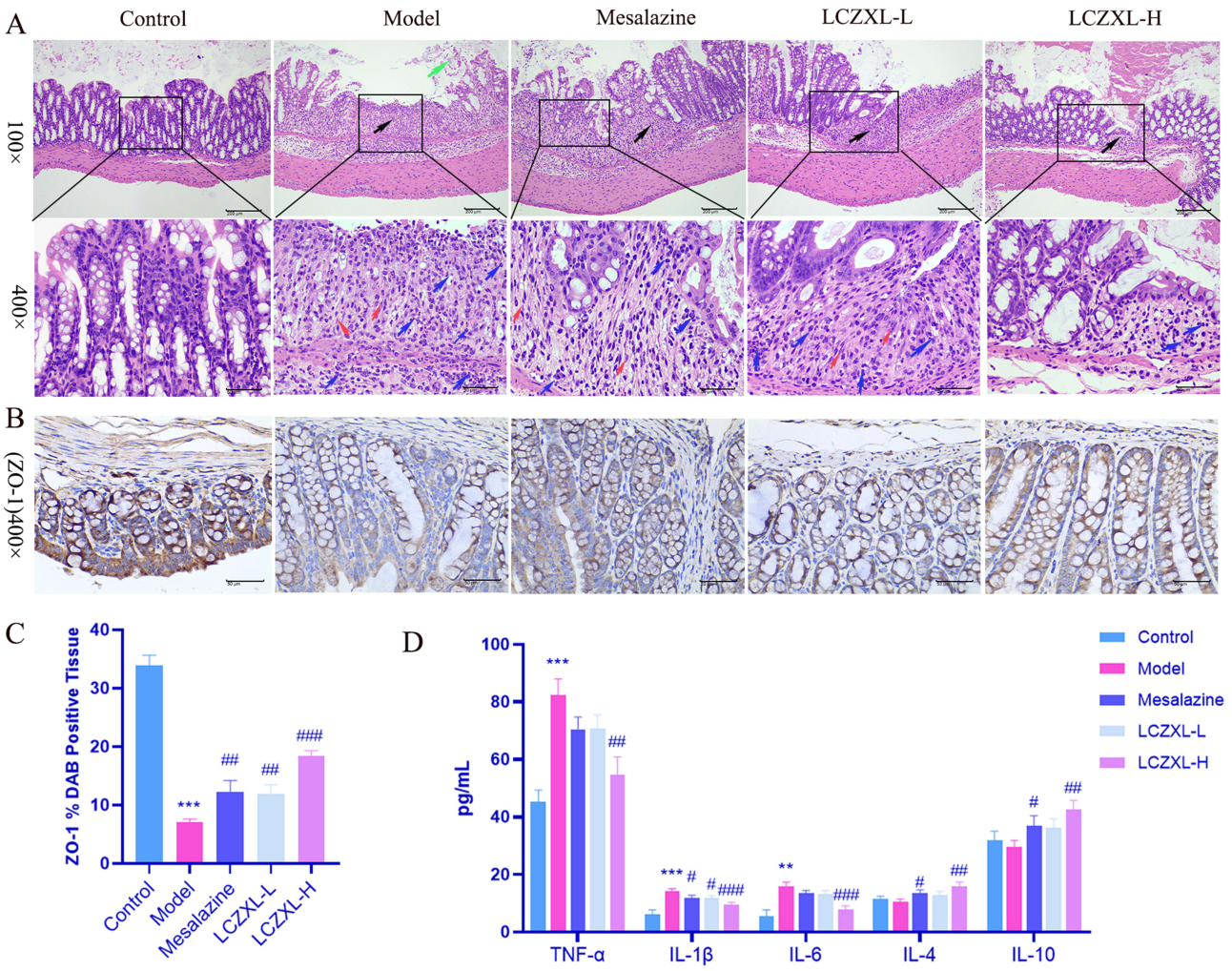

Figure 4 LCZXL enema treatment strengthening the intestinal barrier of UC mice by controlling the release of inflammatory cytokines. (**A**) HE staining of the colon (100× and 400×). The green arrows indicate Intestinal epithelial cell detachment, black arrows indicate colonic fibrosis, the blue arrows indicate neutrophil infiltration and the red arrows indicate fiber cells. (**B**) ZO-1 IHC of staining of the colon (400×). (**C**) ZO-1% DAB positive tissue count. (**D**) ELISA assay was performed to quantify the content of TNF-α, IL-1β, IL-6, IL-4, and IL-10 in serum. $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$, compared with model group. mean±SD.

UC mice. Based on these findings, we inferred that LCZXL may rescue UC by inhibiting pro-inflammatory and promote anti-inflammatory cytokines release.

LCZXL Enema Treatment Promotes Macrophages Polarized to M2 Type in UC Mice

The balance of M1/M2 macrophage polarization is a key controller of inflammation procession. Flow cytometry was performed to quantify M1 and M2 macrophages in the colon. Figure 5A and B showed that LCZXL enema treatment reversed the increase of UC modeling-induced CD86 and increased CD206 expression level. As Figure 5C and D has shown, the red fluorescence pointed out that CD206 and iNOS; Compared to the control group, iNOS mean fluorescence intensity was higher in the model group; Compared to the model group, CD206 mean fluorescence intensity was higher and iNOS was lower in the Mesalazine and LCZXL enema group. Moreover, a qPCR assay was performed to detect the iNOS, CD86, Arg1, and CD206 mRNA expression (Figure 5E and F). Importantly, the trend of iNOS and CD86 mRNA expression was consisted with CD86, and Arg1. CD206 mRNA expression was consisted with CD206 in flow cytometry assay. It suggested that LCZXL enema can inhibit M1 macrophage polarization thereby suppressing inflammation.

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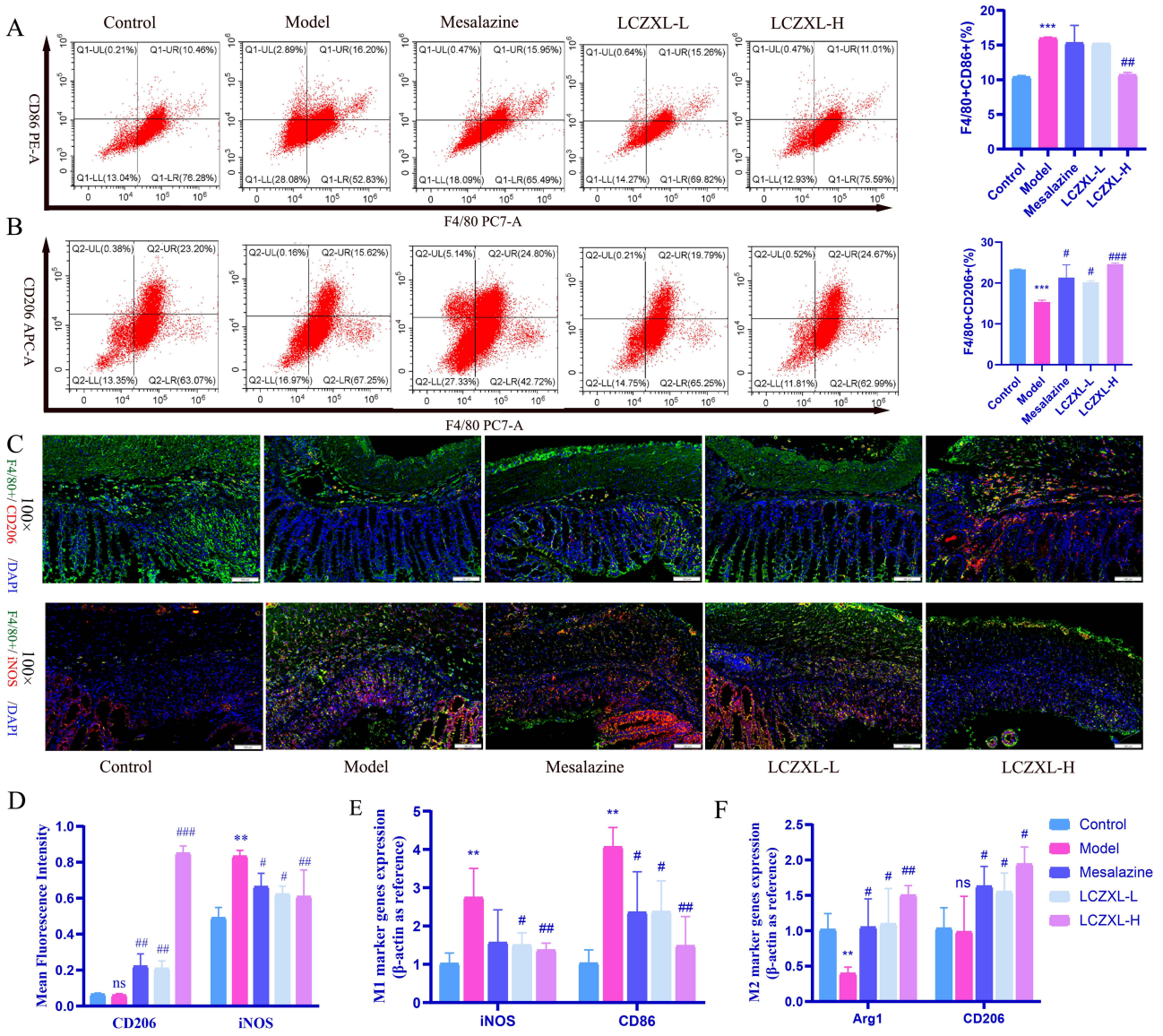

Figure 5 LCZXL enema treatment promoting macrophages polarized to M2 type in UC mice. Flow cytometry was used to measure the serum level of (**A**) CD86, and (**B**) CD206. (**C**) IF was used to detect the CD86 and CD206 expression in the colon (100×). (**D**) Mean fluorescence Intensity. The M1 marker gene (**E**) iNOS and CD86, and M2 marker gene (**F**) Arg1 and CD206 were quantified by qPCR assay. **P < 0.01 and ***P < 0.001, compared with control group. #P < 0.05, ##P < 0.01 and ####P < 0.001, compared with model group. mean±SD.

LCZXL Enema Treatment Inhibits the Jagged-1/Notch1 Signaling Pathway Activation in UC Mice

The Jagged-1/Notch1 signaling pathway may relate to macrophage polarization. We measured the mean fluorescence intensity in the colon by IF first, compared with control group, Jagged-1 showed the highest (P<0.001) in model group, and compared with model group LCZXL down-regulated effectively, LCZXL-H was better than LCZXL-L (Figure 6A and B). Furthermore, the mRNA and proteins of Jagged-1, Notch1, and Notch2 were quantified by qPCR (Figure 6C) and WB assay (Figure 6D and E). Results indicated that UC modeling caused an obvious increase of Jagged-1, Notch1, and Notch2, and LCZXL treatment inhibited these genes or proteins expression, suggesting that LCZXL could stop the activation of the Jagged-11/Notch1 signaling pathway.

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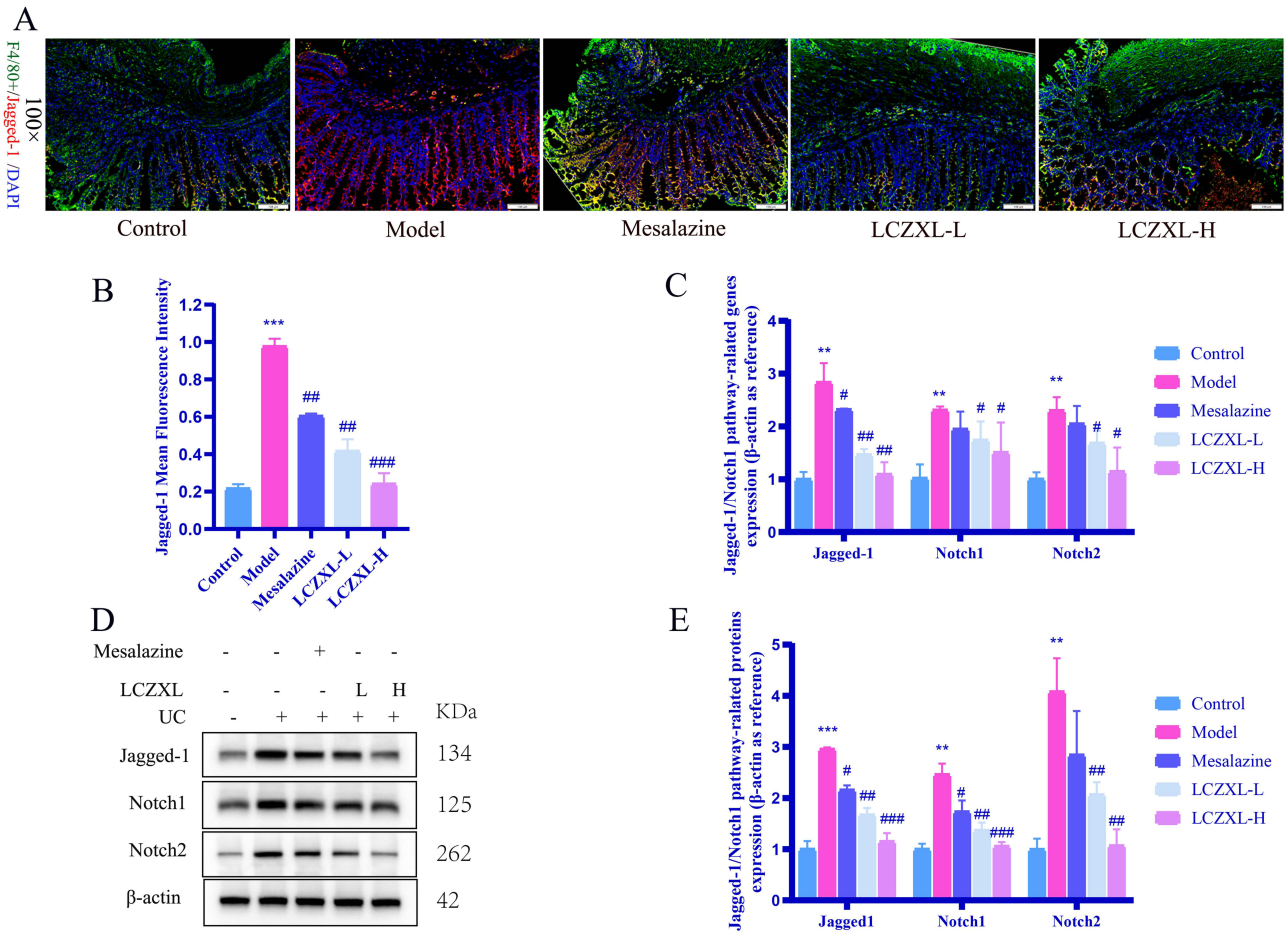

Figure 6 LCZXL enema treatment inhibiting the Jagged-1/Notch1 signaling pathway activation in UC mice. (**A**) IF was used to detect the Jagged-1 expression in the colon (100×), (**B**) Jagged-1 mean fluorescence Intensity. (**C**) The Jagged-1/Notch1 signaling pathway-related gene expression including Jagged-1, Notch1, and Notch2 was quantified by qPCR assay. (**D** and **E**) WB was performed to detect these proteins' expression in the colon too. $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$, compared with model group. mean±SD.

LCZXL Enema Stops the Jagged-1/Fc-Aggravated Colon Injury in UC Mice

To further investigate whether the LCZXL enema alleviated the development of UC by targeting Jagged-1 protein, Jagged-1/Fc was applied to a UC mice model, either alone or in combination with LCZXL. Figure 7A and D demonstrated the colon lengths in each group, indicating that Jagged-1/Fc exacerbated colon shortage in the LCZXL group to a certain extent. HE staining (Figure 7B) and CMDI scores (Figure 7E) revealed that Jagged-1/Fc aggravated colonic injury induced by UC, particularly characterized by complete loss of intestinal epithelium and total depletion of goblet cells, while LCZXL enema ameliorated this condition. And compared to the Jagged-1/Fc + LCZXL group, the LCZXL group exhibited a higher expression of Zonula Occludens-1 (ZO-1) (Figure 7C and F).

LCZXL Enema Inhibited Jagged-1/Fc-Induced M1 Polarization and the Activation of the Jagged-1/Notch1 Signaling Pathway in UC Mice

For mice in UC model, which were treated with Jagged-1/Fc and LCZXL, either individually or in combination, we further examined the expression of M1 and M2 macrophages, as well as Jagged-1 in the colons of each group. Figure 8A–D showed that Jagged-1/Fc exacerbated the UC-induced increase in CD86 and decreased the expression of CD206, while LCZXL treatment inhibited this process. Additionally, Jagged-1/Fc decreased the expression of CD206 in the LCZXL group. Moreover, Jagged-1/Fc enhanced the expression of Jagged-1 in the colons of UC mice. Compared to the LCZXL group, the Jagged-1/Fc+LCZXL group significantly up-regulated Jagged-1 protein expression (Figure 8E and F). These results

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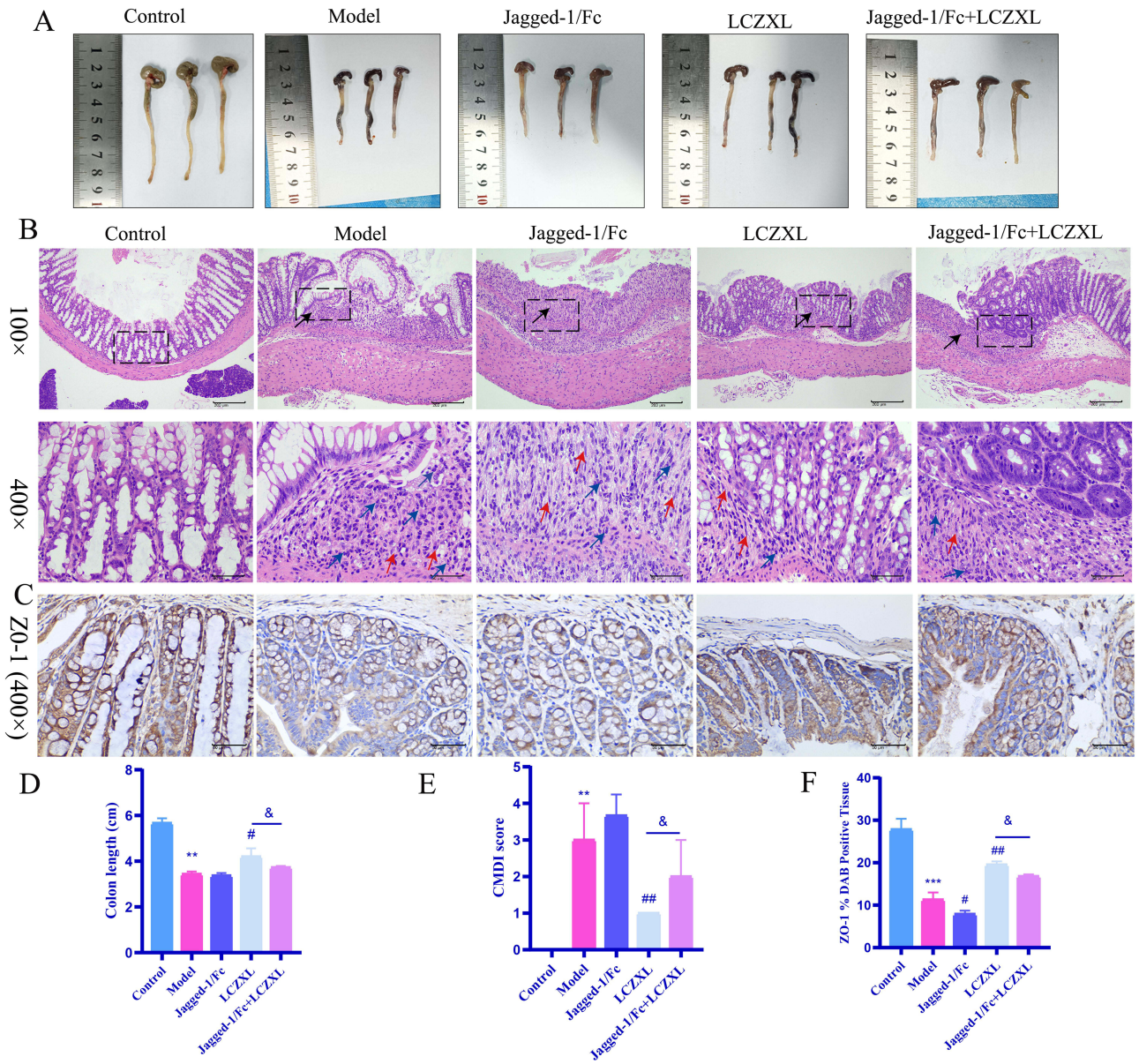

Figure 7 LCZXL enema stopping the Jagged-1/Fc-aggravated colon injury in UC mice. (**A**) The measurement of colon length. (**B**) HE staining of the colon (100× and 400×). The black arrows indicate colonic fibrosis, the blue arrows indicate neutrophil infiltration and the red arrows indicate fiber cells. (**C**) ZO-1 IHC of staining of the colon (400×). (**D**) The colon length count. (**E**) CMDI score. (**F**) ZO-1% DAB positive tissue count. $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with model group. $^{\&}P < 0.05$, compared with the Jagged-1/Fc + LCZXL group. mean±SD.

suggested that LCZXL may inhibit M1 macrophage polarization by suppressing the Jagged-1/Notch1 signaling pathway, thereby reducing the release of pro-inflammatory cytokines and achieving a therapeutic effect in UC.

Discussion

In this study, LCZXL-H enema expressed a strong therapeutic effect, via inhibiting the loss of epithelium and remodeling the structure of goblet cells. UC can cause severe colonic damage and as tissue damage occurred, various signaling molecules were released, attracting immune cells, which included neutrophils, monocytes and macrophages to migrate to the site of injury, leading to an inflammatory cell infiltrate. Macrophages developed from monocytes played a crucial role in regulating inflammatory progression as a key component of the inflammatory infiltrate. Therefore, the aim of this study

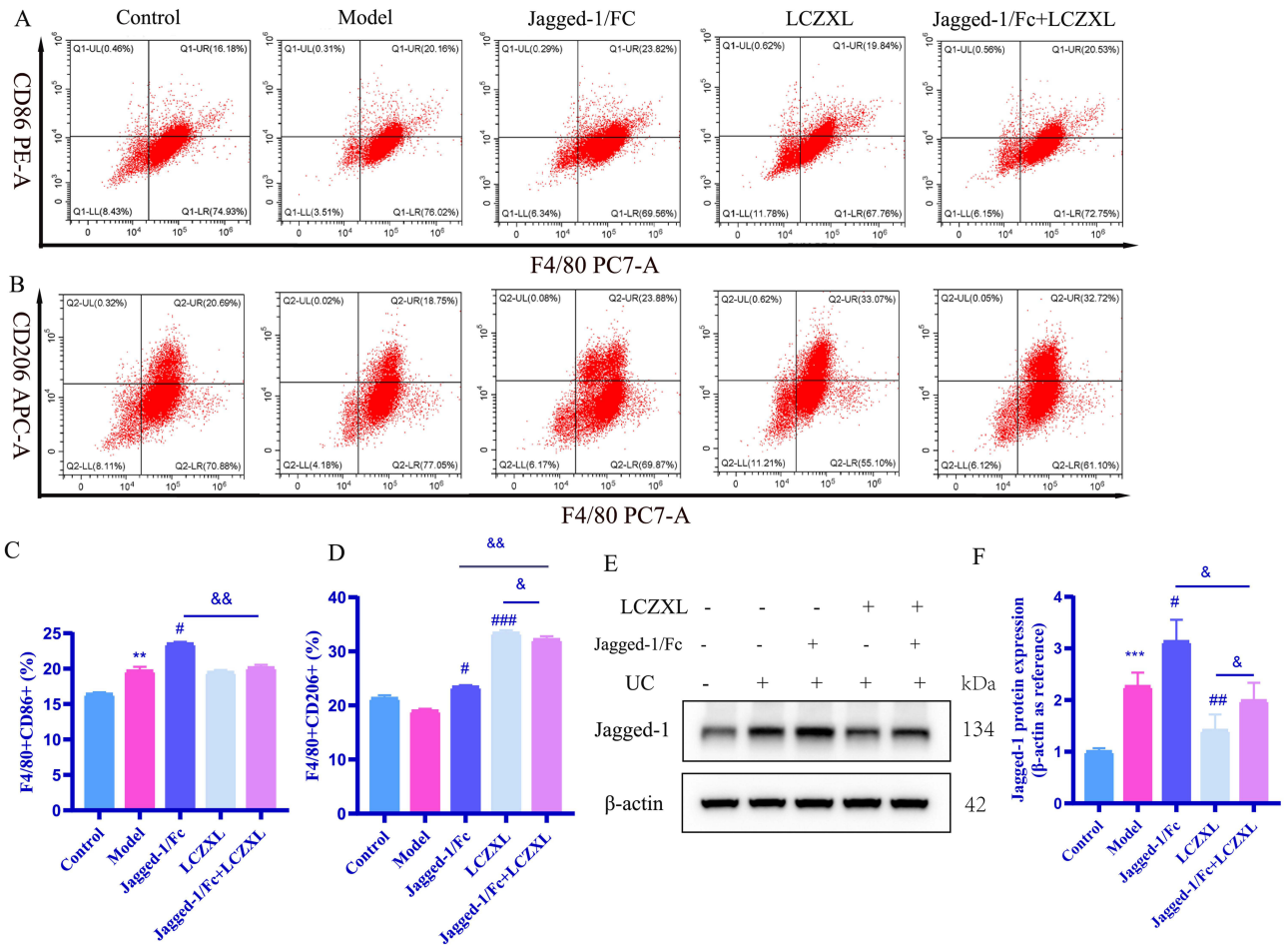

Figure 8 LCZXL enema inhibited Jagged-1/Fc induced M1 polarization and the activation of the Jagged-1/Notch1 signaling pathway in UC mice. Flow cytometry was used to detect the serum level of (**A**) CD86 and (**B**) CD206. Comparison of each group Flow cytometry to detect changes in (**C**) CD86 and (**D**) CD206. (**E** and **F**) WB was performed to detect Jagged-1 protein expression in the colon. $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$, compared with model group. $^{\&}P < 0.05$ and $^{\&\&}P < 0.01$, compared with the Jagged-1/Fc + LCZXL group. mean±SD.

was to explore the potential molecular mechanisms of LCZXL enema for the treatment of UC, focusing on macrophage polarization.

The Notch signaling pathway was initially discovered in fruit flies and was later found to be widely present in vertebrates and invertebrates. In mammals, there are four types of Notch receptors (Notch1–4); Notch1 is primarily distributed in the intestine. There are five types of Notch ligands (Delta-like 1, 3, and 4, and Jagged 1 and 2). The Notch signaling pathway plays an important role in the mucosal barrier dysfunction, especially the chemical and physical barriers.[23] In patients with UC, increased M1 and decreased M2 polarization occurred with high transmission of the Notch1 signaling pathway. Inhibiting Notch1 significantly reduces M1 polarization, increases M2 polarization and anti-inflammatory markers, and restores the M1/M2 balance.[10] And Notch signaling pathway promotes intestinal stem cell differentiation into the absorptive lineage, resulting in a reduction in goblet cells.[24] This mechanism induces colonic epithelial proliferation in a Notch-dependent manner, which is associated with the pathogenesis of colitis-associated colorectal cancer.[25] Blocking the Notch signaling pathway, can improve the barrier function of the mucosal mechanical barrier by upregulating the expression of Zonula occludens-1 and occludin and downregulating claudin2 expression.[9]

TNF-α, IL-6 and IL-1β have been reported to be closely associated with M1 macrophages. They were secreted by M1 macrophages during inflammation and contributed to the development of the inflammatory response.[26] By contrast, anti-inflammatory cytokines such as IL-4 and IL-10 stimulated macrophage differentiation to the M2 phenotype, participated in tissue repair, and prevented excessive inflammatory reactions.[27] Our findings demonstrated that LCZXL enema

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treatment increased the expression levels of IL-4 and IL-10 and decreased the levels of TNF-α, IL-6 and IL-1β. In addition, it was shown that inducible nitric oxide synthase (iNOS) was expressed under inflammatory conditions, leading to the production of large amounts of nitric oxide (NO), which modulated immunity.[28] iNOS and CD86 were usually expressed at high levels in M1 macrophages and were often used as molecular markers for M1 in research. In contrast, Arg1 and CD206 were unique markers for M2 macrophages. Our results showed that LCZXL enema treatment effectively reduced the expression of iNOS and CD86 in the colon, while upregulating Arg1 and CD206. These results suggested that LCZXL enema promoted macrophage differentiation towards the M2 type, thus helped to repair the intestinal barrier. However, the underlying macroscopic mechanisms needed to be further investigated.

Studies have showed that CD86 on M1 macrophages interacted with CD28 on T cells to provide the necessary stimulatory signals to promote cell activation and proliferation.[29] However, recent studies in allergic airway disease suggested that Jagged-1 may affect the immune function of peripheral T cells by targeting Notch receptors,[30] indicating a potential link between the Jagged-1/Notch signaling pathway and macrophage polarization. However, this interaction has not been demonstrated in UC. In this study, we used Jagged-1/Fc with or without LCZXL by enema for the treatment of UC. Pathological damage to the colon of UC mice was even more severe with Jagged-1/Fc treatment, but LCZXL enemas effectively rescued this damage. Furthermore, LCZXL enema reversed Jagged-1/Fc-induced M1 macrophage polarization and improved the intestinal barrier, specifically increasing Zo-1 protein expression in the colon. Evidence suggested that Zo-1 played an important role in maintaining the function of the intestinal barrier as a tight junction protein.[31] Moreover, our findings suggested that LCZXL enema inhibited Jagged-1/Fc-stimulated activation of the Jagged-1/Notch signaling pathway. These findings suggested that LCZXL enema may improve UC by inhibiting activation of the Jagged-1/Notch signaling pathway and promote M2 polarization.

Puhuang, Baiji Fen, Chishizhi, and Qingdai were the ingredients of the LCZXL enema. In 2021, Study has demonstrated that Pollen Typhae (an extract of Puhuang) could inhibit the expression of iNOS and cyclooxygenase-2 (COX2) in macrophages.[32] A large of evidence has proven that Qingdai was an effective therapy for UC.[33,34] A network pharmacological study on Qingdai showed that the active ingredients of Qingdai were enriched in many immune-related signaling pathways, such as IL-17 signaling pathway, T-cell receptor signaling pathway, TNF signaling pathway, and Th1 and Th2 cell differentiation.[17] From the theory of ancient Chinese medicine, both Baiji Fen and Chishizhi had the therapeutic effect of reducing swelling and stopping bleeding. The combination of Puhuang, Baiji Fen, Chishizhi, and Qingdai was more beneficial in the treatment of UC than the single administration. Thus, previous studies were consistent with our results that the effect of LCZXL enema on UC was due to the anti-inflammatory effect of macrophage polarization. Our study also has some shortcomings, without validation in cells regarding its molecular mechanism and the inflammatory mechanisms of UC alterated the degree of neutrophil infiltration about myeloperoxidase (MPO) activity[35,36] and the Nrf2 pathway, a regulator of the antioxidant defense system,[37] which help to better explain the mechanism of action of the LCZXL enema in the treatment of UC.

Conclusion

Our study proved that LCZXL Enema could inhibit the M1 Macrophage Polarization and promote M2 macrophage polarization of ulcerative colitis via regulating the Jagged-1/Notch1 signaling pathway. LCZXL enema has very superior potential for the treatment of UC.

Abbreviations

UC, ulcerative colitis; LCZXL, LianChuang ZhiXue Liquid; LCZXL-L, Low dose of LCZXL; LCZXL-H, High dose of LCZXL; CMDI, colon mucosal damage index; DAI, disease activity index; HE, hematoxylin and eosin; IHC, immunohistochemistry; iNOS, inducible nitric oxide synthase; Zo-1, Zonula occludens-1; Jagged-1/Fc, Recombinant human Jagged-1 protein/ Fc Chimera Active; ROS, Reactive oxygen species; NO, nitric oxide; COX-2, cyclooxygenase-2.

Data Sharing Statement

The data that supported the findings of this study are available from the corresponding author upon reasonable request.

Ethics Approval and Consent to Participate

All methods were carried out in accordance with relevant guidelines and regulations. The animal experiment was executed with the agreement from the Experimental Animal Ethics Committee, Shaanxi University of Chinese Medicine (Approval SUCMDL20240307048). All methods were reported in accordance with ARRIVE guidelines.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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