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Curcumin chitosan microsphere improve ulcerative colitis inflammatory response by regulating miR-224-3p/TLR4 axise

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Abstract

Objective: To observe the clinical effect of curcumin chitosan microsphere (CCM) on ulcerative colitis. Methods: Our group selected 75 cases with ulcerative colitis (UC) receiving treatment at The First Affiliated Hospital of Nanchang University as observation group, and 75 cases that were healthy and received examination at the same time as control group. We detected serum miR-224-3p, TLR4, TNF-α and NF-κB levels using the double antibody sandwich ELISA (DAS-ELISA). In animal experiments, our team applied DSS to induce IBD mice models, allocated into control group, model group, sulfasalazine group, curcumin group and CCM high dose group, CCM medium dose group, and CCM low dose group, in total 7 groups (n = 10). After molding, on 3rd day our team began intragastric administration for 10 days. On 14th d, we sacrificed the mice, conducted HE staining, observed changes in the pathological form of bowel tissue in each group, and gave inflammation scores. Taking the colon tissue and serum, our team applied ELISA to detect inflammatory factors such as TNF-α, TLR4, IFN-γ levels in supernate of tissue homogenate, as well as performed western blot to detect SDF-1, CXCR4, miR-224-3p protein expression levels in intestinal tissue. Results: TNF-α, TLR4, NF-κB expressions in observation group were signally elevated while miR-224-3p expression visually decreased. In control group, TNF-α, TLR4, NF-κB expressions in observation group were signally decreased while miR-224-3p expression visually elevated, the difference was significant ($P < 0.05$). After treatment, serum TNF-α, TLR4, NF-κB expressions in sulfasalazine group, curcumin group, CCM low, medium and high dose groups were signally reduced, while IFN-γ expression was elevated significantly, when comparing with those in model control group, the difference was significant ($P < 0.01$). Compared CCM low, medium and high dose groups with sulfasalazine group, there was a significant difference in efficacy ($P < 0.05$). Compared CCM low, medium and high dose groups with curcumin group, there was a significant difference in efficacy ($P < 0.05$). We applied western blot to detect SDF-1, CXCR4, and miR-224-3p protein expression levels, finding that CCM enhanced in SDF-1, CXCR4, and miR-224-3p protein expression levels, with significant differences when comparing with those in model control and curcumin groups. Conclusion: CCM may elevate IFN-γ level and enhance SDF-1, CXCR4, and miR-224-3p protein expression levels through inhibiting TNF-α, NF-κB, and TLR4 expressions, thus reducing inflammatory response as well as damage to colon tissue in mice with UC through anti-inflammatory effects.

Keywords: curcumin chitosan microsphere; ulcerative colitis.

Practical Application: Traditional treatments of IBD patients are mainly through western medicine, but with great side effects, adverse reactions, high recurrence rate after suspension and high price. Modern medicine has found that curcumin, a phenolic material extracted from turmeric in zingiberaceae, has functions of antioxidant, blood lipid reduction, anticoagulant, anti-tumor and removing oxygen radicals. Numerous studies have shown that curcumin has anti-IBD effects. However, curcumin is not easy to prepare tablets, capsules or injections and other dosage forms due to its water insolubility and short metabolic half-life, which greatly limit its clinical application. PLGA and chitosan, a good bio-compatible biodegradable material, can be used as a carrier of sustained drug release, improving the utilization rate of drugs and promoting the absorption of drugs through the small intestine mucosa, and has a good prospect in the sustained drug release for intestinal diseases.

1 Introduction

Inflammatory bowel disease (IBD) is a type of recurrent chronic nonspecific intestinal inflammatory disease, like ulcerative colitis (UC) and Crohn's disease (CD) (Sairenji et al., 2017; Flynn & Eisenstein, 2019; Zhang & Li, 2014). In recent years, with residents' lifestyle and eating habits changing, IBD incidence and canceration ratio in China has been increasing year by year, which has become the second killer of the intestine after colorectal cancer, and has been covered by chronic diseases of urban resident basic medical insurance, which seriously affects the health and safety of patients (Malik, 2015; Wright et al., 2018; Taleban et al., 2015). Clinical treatment for IBD is tricky, for there is a lack of specific, targeted drugs (Sheehan & Shanahan, 2017;

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Lin et al., 2019). Therefore, it is very essential for IBD prevention and treatment to actively probe into the action target as well as treatment method of IBD. Combined with pre-work, our team applied CCM to intervene in IBD, systematically observed CCM repair impact on IBD, and explored CCM mechanism in preventing and controlling IBD from its impact on TLR4, TNF-α, IL-1β, IFN-γ, NF-κB expressions in UC rats.

2 Serum miR-224-3p, TLR4 expressions etc. in patients with UC (clinical trials)

2.1 Medical records

Our group selected 75 cases with ulcerative colitis (UC) receiving treatment at The First Affiliated Hospital of Nanchang University as observation group. Inclusion standards: (1) in line with the *Consensus on Diagnosis and Treatment of Inflammatory Bowel Diseases* by the Inflammatory Bowel Disease Group of Chinese Medical Association Digestive Medicine Division; (2) Patients or their families signed the informed consent form. Exclusion criteria: (1) Amid lactation or pregnancy; (2) Combined with diabetes, (3) Accompanied with autoimmune diseases; (4) Combined with kidney, heart, liver and other organ diseases; (5) Combined with serious infections. And we selected 75 cases that were healthy and received examination at the same time as control group.

2.2 Specimen collection & test methods

Our team performed the fasting blood draw of 5 mL from vein in control and observation groups before and after treatment, applied DAS-ELISA to detect serum miR-224-3p, TLR4, TNF-α, NF-κB levels, and Σ960960 enzyme-labeled instrument, to determine the findings, the kits provided by Department of Pathology, the First Affiliated Hospital of Nanchang University.

2.3 Statistical analysis

Our team applied SPSS21.0 software for data analysis, expressed the measurement data as mean ± standard deviation, detected it via t-test, and $P<0.05$ mean the difference was significant.

3 Serum miR-224-3p, TLR4 expressions etc. in rat model with UC (animal experiments)

3.1 Materials & methods

Preparation of curcumin-loaded PLGA microspheres

Our team prepared PLGA microspheres using the multiple emulsion-solvent evaporation technique (W/O/W), dissolved 2 g PLGA in a 10 mL dichloromethane solution, and dispersed 100 mg curcumin into above solution after stirring it at 10,000 rpm for 1 min. Slowly our team put the organism into 400 mL of 1% (w/v) PVA (Av. Mw 30,000-70,000) aqueous solution and stirred them for 3 min at 10,000 rpm. Later we added 500 mL of 0.1% (w/v) PVA aqueous solution to the resulting emulsion, stirred them for decompression for 6 hours at 250 rpm at 25 °C, dried dichloromethane to centrifugally screen microspheres (50 and 100 μm, Fisher Scientific, Pittsburgh, PA), eluted 3 times with sterile double-stilled deionized water, and finally conducted vacuum drying for 24 hours. And we prepared blank microspheres without drugs in the same way.

Preparation of curcumin-loaded PELA microspheres

Our team prepared PELA microspheres using the solvent evaporation method (W/O/W), dissolved 2 g PLGA in a 10 mL dichloromethane solution, and dispersed 100 mg curcumin into above solution after stirring it at 10,000 rpm for 1 min. Slowly our team put the organism into 400 mL of 1% (w/v) PVA (Av. Mw 30,000-70,000) aqueous solution and stirred them for 3min at 10,000 rpm. Later we added 500 mL of 0.1% (w/v) PVA aqueous solution to the resulting emulsion, stirred them for decompression for 6 hours at 250 rpm at 25 °C, dried dichloromethane to centrifugally screen microspheres (50 and 100 μm, Fisher Scientific, Pittsburgh, PA), eluted 3 times with sterile double-stilled deionized water, and finally conducted vacuum drying for 24 hours. And we prepared blank microspheres without drugs in the same way.

Preparation of curcumin conjugated chitosan microspheres

a. Taking a certain amount of chitosan (0.5 mg/mL-4 mg/mL) to dissolve in 1% acetic acid solution and to adjust the pH to 5 with NaOH solution; b. Filtering in turn with filter paper with a thickness of 0.45 m and 0.22 m; c. Taking a certain amount of sodium tripolyphosphate (TPP) to dissolve with double-stilled water, and to configure as 0.54 mg/mL TPP solution; d. Filtering with filter paper with thicknesses of 0.45 m and 0.22 m in turn; e. TPP solution and CS solution in a 5:1 ratio, making TPP solution slowly drip (30 drops/min) into the chitosan solution, observing the experimental phenomenon. When there were microspheres, the solution changed from clarification to be with blue light, and a little bubble. Later we stirred it with magnetic force for 30 min, centrifuged at 15000 r/min for 15 min. Our team washed the resulting sediment via pure water three times, and collected the sediment to perform subatmospheric freeze-drying.

Determination of drug-loading and encapsulation efficiency

Our team precisely weighed homemade 1mg curcumin microspheres, added 1 mL of glacial acetic acid to make it fully dissolved under ultrasound, centrifuged it, diluted the supernate with HPLC mobile phase 50 times, and measured it. We put the resulting peak area into the standard curve to calculate curcumin concentration in solution so that we could the curcumin content in microspheres. The drug-loading and encapsulation efficiency microspheres were calculated by following formulas: Drug-loading efficiency = (drug content in microspheres/microsphere content) ×100%, Encapsulation efficiency = (drug content in microspheres /total drug content) ×100%

IBD model establishment

Our team selected 70 SPF BALB/c mice, and induced the IBD mouse model via DSS, which were by random allocated into normal control group, model group, sulfasalazine group,

curcumin group and CCM high dose group, CCM medium dose group, and CCM low dose group, in total 7 groups (n = 10). Each group received cage feeding, free to take water, the lighting circle and dark period were 12h, and the temperature was controlled at (23 ± 2) °C. Model group, positive control group, curcumin group and CCM groups were given free drinking of 5% DSS for 7d to induce IBD, daily observation of fecal characteristics, occult blood and general conditions. The control group received free drinking of pure water.

Administration in experimental groups

Control group: normal drinking and diet; Model group: free drinking water with 5% DSS, normal diet; Sulfasalazine group: free drinking water with 5% DSS + feeding containing CMM (5g/L); Curcumin group: free drinking water with 5% DSS + feeding containing curcumin (20g/L); CCM Low dose group: free drinking water with 5% DSS + feeding containing CCM (10g/L); CCM medium dose group: free drinking water with 5% DSS + feeding containing CCM (20g/L); CCM high dose group: free drinking water with 5% DSS + feeding containing CCM (40g/L).

3.2 Observation indicators

Intestinal morphology & inflammation scores

After the last weighing, our team performed abdominal anesthesia in each group through 0.7% barbiturate (10 μL/g), and applied the posterior dislocation of the cervical spine to kill the mice, placed intestinal tissue in 10% neutral Formalin solution to fix for 24 hours, and conducted conventional paraffin slices. In accordance with HE staining, our team dyed the slices, and observed the pathological morphological changes in the intestinal tissues of each group under optical microscope, and gave relative inflammation scores.

Histological injury score

Based on the criteria developed by Dieleman et al., we calculated the length of the damage point of the colon mucous membrane as the colon injury score. 1 score: ≤ 0.5mm; 2 scores: ≤ 1mm; 3 scores: ≤ 2mm, by analogy, when the damage point width was more than 2 mm, the score doubled.

Western Blotting for testing SDF-1, CXCR4, miR-224-3p levels in mice

Our team collected colon tissue from each group and applied Western blot to detect SDF-1, CXCR4, and miR-224-3p protein expression levels in intestinal tissues.

Inflammatory factor expression level

Our team collected the colon tissue of each group, prepared tissue homogenate, applied ELISA to detect inflammatory factor level, such as TNF-α, IL-1β, IFN-γ and so on in supernate of tissue homogenate, which was carried out based on the kit instructions.

Statistical methods

Our team applied SPSS 17.0 to perform statistical analysis to represent the count data in frequency, with $\bar{x} \pm S$ representing measurement data, applied *t* test for comparison between the two groups, and compared the cure rate via x2 test.

4 Results

4.1 Changes in serum miR-224-3p, TLR4, TNF-α, NF-κB in control and observation groups

TNF-α, TLR4, NF-κB expressions were elevated significantly in observation group, while miR-224-3p was signally. TNF-α, TLR4 and NF-κB expressions were decreased signally in the control group, while miR-224-3pB expression was significantly higher, with significant difference ($P < 0.05$, Table 1).

4.2. Pathological findings

Colon mucous membrane was seriously damaged in model group, some with defects, intramucosal glands arranged in disorders or with deformation; CCM low, medium and high dose group and sulfasalazine group with mild lesions, and more complete mucous membrane; Normal control group with regular colon tissue structure, and glands arranged neatly, as detailed in Figure 1 below.

4.3 Comparing colon injury scores in each group

The model group, sulfasalazine group, CCM low, medium and high dose groups all showed ulcer formation, and model control group had the highest scores. After 2-to-4-week-treatment, comparing sulfasalazine group, curcumin group and CCM low, medium and high dose groups, the colonic mucosal morphology injury scores were signally reduced with a significant difference ($P < 0.01$) compared with the model control group. Comparing sulfasalazine group, and CCM low, medium and high dose groups, there was significant difference in efficacy ($P < 0.05$). Comparing CCM low, medium and high dose groups with curcumin group, there was significant difference in efficacy ($P < 0.05$) (Table 2).

4.4 TNF-α, TLR4, IFN- γ, and NF-κB levels in each group

After treatment, TNF-α, TLR4, and NF-κB levels in sulfasalazine group, curcumin group and CCM low, medium and high dose groups were visually decreased, while IFN- γ level was visually elevated. There was significant difference in comparing those in sulfasalazine group, curcumin group and CCM low, medium and high dose groups with those in model control group($P < 0.01$). Comparing sulfasalazine group with CCM low, medium and high dose groups, there was significant difference in efficacy ($P < 0.05$). Comparing CCM low, medium and high dose groups with curcumin group, there was significant difference in efficacy ($P < 0.05$) (Table 3).

4.5 Western Blotting for detecting SDF-1 and CXCR4 levels in each group

Our work applied Western blot to detect SDF-1α/CXCR4 signaling pathway activation, finding that SDF-1/CXCR4 signaling pathway

Table 1. Changes in serum miR-224-3p, TLR4, TNF-α, NF-κB of two groups of ($\bar{x}$ ± S).

group N	miR-224-3p	TLR4	TNF-α	NF-κB
Control group	75 26.3 ± 18.2	7.4 ± 1.2	6.5 ± 1.3	7.7 ± 1.4
Observation group	75 6.6 ± 2.4*	111.2 ± 22.5*	107.4 ± 7.5*	121.0 ± 11.3*

Note: compared with control group, *$P < 0.05$.

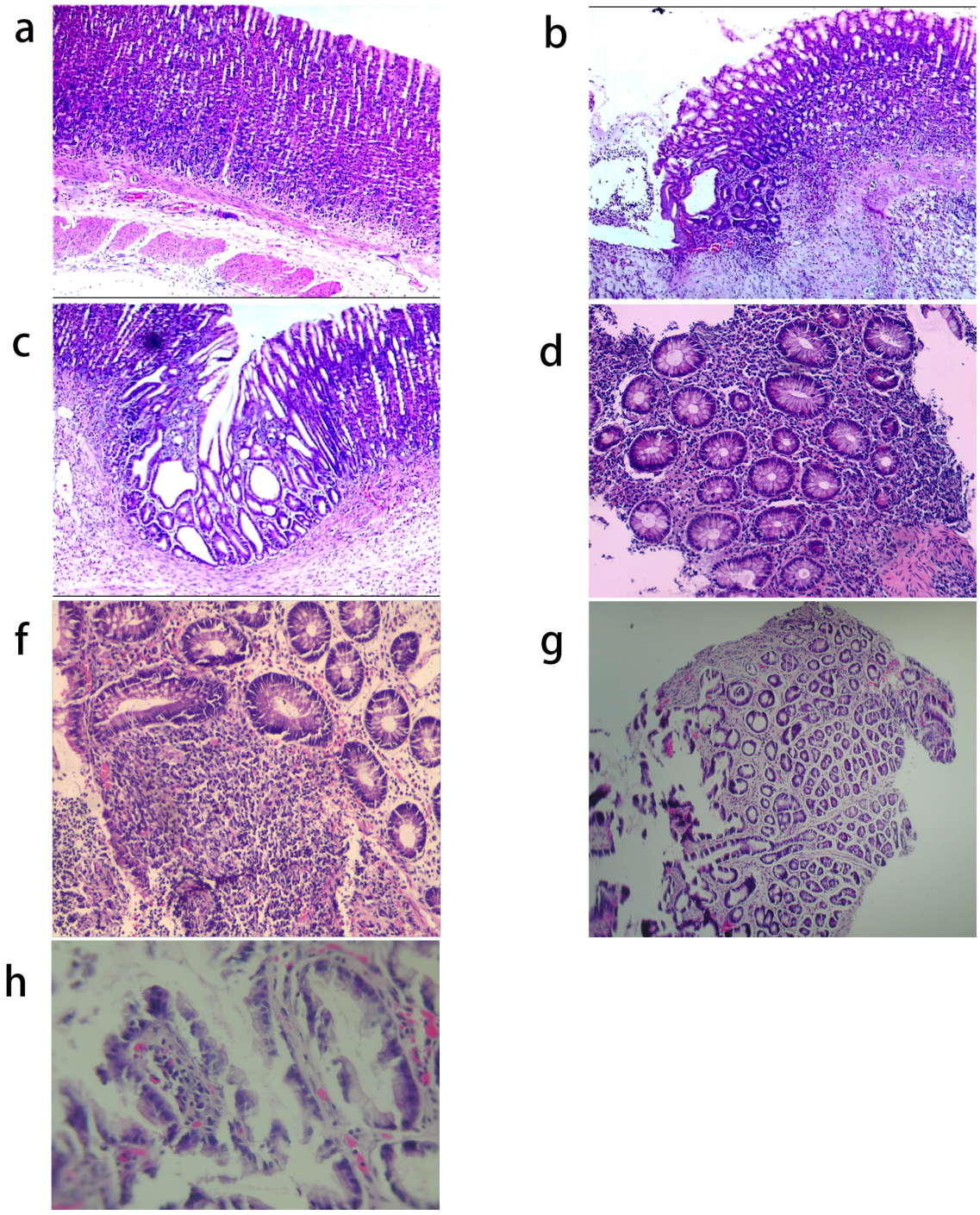

Figure 1. Pathological findings of colon mucous membranes in mice in each group. a Normal control group HE staining(×100), b CCM low dose group HE staining(×100), c CCM medium dose group HE staining (×100), d CCM high dose group HE staining (×100), e Model control group HE staining (×100), f Curcumin group HE staining (×100), g Sulfasalazine group HE staining (×100).

was enhanced in CCM low, medium and high dose groups,when comparing those in model control group, there was significant difference (Figure 2).

4.6 Western Blotting for detecting miR-224-3p level in each group

Our work applied Western blot to detect miR-224-3p signaling pathway activation, finding that miR-224-3p signaling pathway was enhanced in CCM low, medium and high dose groups,when comparing that in model control and curcumin groups, there were significant differences (Figure 3).

Table 2. Comparison of colon injury scores in mice in each group ($\bar{x}$ ± s, n = 20).

	Histological injury scores	Gross morphology injury scores
Normal control group (n = 10)	--	--
Model control group (n = 10)	12.21 ± 1.21	811 ± 1.04
sulfasalazine group (n = 10)	8.52 ± 1.23	6.51 ± 1.02
CCM low dose group (n = 10)	6.11 ± 1.12*ba	5.32 ± 1.05*ba
CCM medium dose group (n = 10)	5.31 ± 1.02*ba	4.02 ± 1.02*ba
CCM high dose group (n = 10)	3.03 ± 1.12*ba	2.74 ± 1.02*ba
Curcumin group (n = 10)	7.83 ± 1.21*	6.73 ± 1.01*

Note: compared with model control group, *$P < 0.01$; compared with sulfasalazine group, $^{b}P < 0.05$; compared with curcumin group, $^{a}P < 0.05$.

5 Discussion

With the further research on IBD, the role of enterocyte tight junction (TJ) in IBD occurrence and development has been recognized. TLR4 signaling pathway is transduced by a signal mediated by TLR4, and it is available to induce the activity of many rapid reaction genes, thus producing many effector molecules, which will participate in the body's defense response amid IBD pathogenesis (Dejban et al., 2021; Chen et al., 2019; Wang et al., 2020; Liu et al., 2020a). Inflammatory response mediated by TLR4 signaling pathway can affect the enterocyte TJ, destroy the intestinal mucosa mechanical barrier, and elevate intestinal epithelial permeability (Kim et al., 2012; Tang et al., 2021; Li et al., 2020; Liu et al., 2020b). Studies have shown that TLR4 is expressed only in small amounts in normal intestinal epithelial cells, while in intestinal mucosa in patients with UC and CD, TLR4 is over-expressed in intestinal epithelial cells. Therefore, protecting the intestinal epithelial cell TJ and improving the function of the intestinal epithelial barrier are a new strategy for the current clinical treatment of IBD. The abnormal activation of TLR4 and the destruction of epithelial TJ mediated by the activation of its downstream signaling

Table 3. Changes in NF-κB, TLR4, IFN- γ, and TNF-αin each group ($\bar{x}$ ± s).

	NF-KB (pg/mL)	TLR4 (pg/mL)	IFN- γ (pg/mL)	TNF-α (pg/mL)
Normal control group (n = 10)	8.72 ± 2.41	8.41 ± 2.12	7.21 ± 2.21	9.13 ± 2.32
Model control group (n = 10)	94.94 ± 6.86	73.21 ± 2.32	150.11 ± 8.72	78.42 ± 6.21
sulfasalazine group (n = 10)	85.21 ± 10.71*	60.21 ± 1.216*	97.21 ± 5.926*	65.21 ± 7.246*
CCM low dose group (n = 10)	75.41 ± 5.21*ab	46.21 ± 1.11*ab	70.21 ± 6.01*ab	51.37 ± 5.31*ab
CCM medium dose group (n = 10)	67.66 ± 13.46* ab	40.12 ± 2.11*ab	60.75 ± 6.21*ab	42.74 ± 6.21*ab
CCM high dose group (n = 10)	36.41 ± 12.31* ab	30.12 ± 2.21* ab	49.21 ± 6.14*ab	37.21 ± 6.25*ab
Curcumin group (n=10)	80.31 ± 4.216*b	53.21 ± 1.11*b	89.21 ± 6.01*b	59.34 ± 7.016*b

Note: compared with model control group, *$P < 0.01$; compared with sulfasalazine group, $^{b}P < 0.05$; compared with curcumin group, $^{a}P < 0.05$.

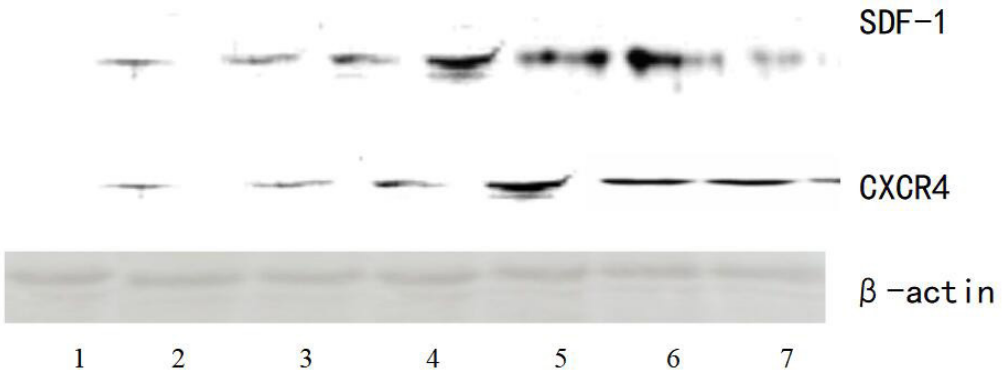

Figure 2. SDF-1/CXCR4 expression in each group. 1 normal control group; 2 model control group; 3 curcumin group; 4 CCM low dose group; 5 CCM medium dose group; 6 CCM high dose group; 7 sulfasalazine group.

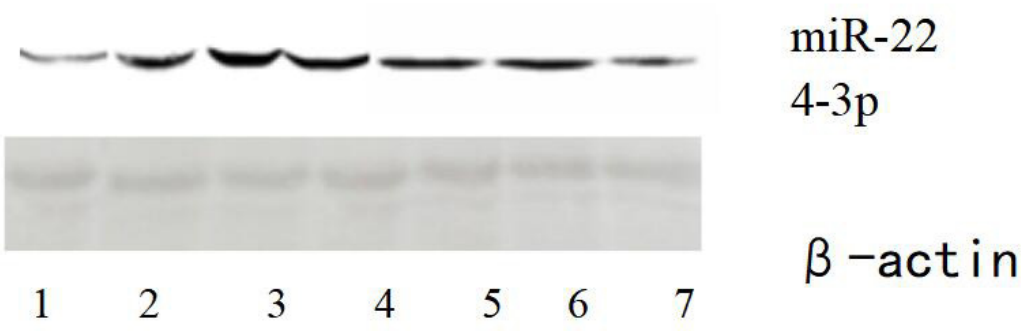

Figure 3. miR-224-3p expression in each group. 1 normal control group; 2 curcumin group; 3 CCM low dose group; 4 CCM medium dose group; 5 CCM high dose group; 6 sulfasalazine group; 7 model control group.

pathway are the key links in IBD onset. Regulating epithelial TJ mediated by the TLR4 signaling pathway may be a new way to prevent and control IBD. The SDF-1/CXCR4 axis acts pivotally in the pathophysiological process of post-ischemic injury repair, angiogenesis, tumor angiogenesis and post-endometrial injury repair. Stromal cell-derived factor (SDF-1 also known as CXCL12) is a key factor in chemotactic EPCs, which can be combined with the CXCR4 expressed on EPC to be chemotactic EPCs (Janssens et al., 2018; Mousavi, 2020). SDF belongs to CXC-type chemotactic factors and is allocated into two proteins (SDF-1α & SDF-1β). SDF-1αis a member of the chemotactic factor CXC family and acts pivotally in the process of EPCs chemotaxis and homing. SDF-1α can be expressed in various tissues and cells, mainly expressed in bone marrow stromal cells, and also bone marrow endothelial cells and hematopoietic stem cells (CD34+ & CD38+), in addition to chondrocyte, endothelial cells and various inflammatory cells. MiR-224-3p is a member of the miR-224 family, and there are relatively few studies on miR-224, instead focusing on its role in tumor-related diseases. miR-224 is located on the human X chromosome, and numerous studies have confirmed that miR-224 is elevated in liver, breast, and pancreatic cancers (Yang et al., 2020; Zhu et al., 2018; Cheng et al., 2018).

At present, the traditional treatment of IBD patients is mainly through western medicine, such as aminosalicylic acid preparations, glucocorticoids, immunosuppressants and other drugs, but with great side effects, adverse reactions, high recurrence rate after suspension and high price (Mazieiro et al., 2018; Pithadia & Jain, 2011). Therefore, the search for safer and more efficient IBD drugs is an urgent problem to be solved clinically. Curcumin is a phenolic material extracted from turmeric in zingiberaceae, which is anti-inflammatory, and pain-relief, promoting blood circulation for removing blood stasis in traditional Chinese medicine, and modern medicine has also found that curcumin has functions of antioxidant, blood lipid reduction, anticoagulant, anti-tumor and removing oxygen radicals. Numerous studies have shown that curcumin has anti-IBD effects. However, due poor stability, curcumin is not easy to dissolve in water with short metabolic half-life in vivo (Mazieiro et al., 2018). So, it is not easy to prepare tablets, capsules or injections and other dosage forms, greatly limiting its clinical application. PLGA and chitosan as a good bio-compatible biodegradable material, can be used as a carrier of sustained drug release, improving the utilization rate of drugs and promoting the absorption of drugs through the small intestine mucosa, and has a good prospect in the sustained drug release for intestinal diseases (Ghattamaneni et al., 2018).

In the course of treating IBD with CCM, our research team found that TNF-α, TLR4, NF-κB expressions were significantly elevated while miR-224-3p expression was decreased signally in observation group. TNF-α, TLR4, NF-κB expressions were decreased significantly while miR-224-3p expression was elevated signally in control group, and the difference was statistically significant ($P < 0.05$). After treatment, serum TNF-α, TLR4 and NF-κB levels were decreased visually while IFN-γ level was elevated signally in sulfasalazine group, curcumin group, CCM low, medium and high dose groups, and the differences were statistically significant ($P < 0.01$). Comparing CCM low, medium and high dose groups with sulfasalazine group, there was a significant difference in efficacy ($P<0.05$). Comparing CCM low, medium and high dose groups with curcumin group, there was a significant difference in efficacy ($P < 0.05$). We applied Western blot to detect SDF-1, CXCR4, and miR-224-3p protein expression levels, finding that CCM enhanced SDF-1, CXCR4, and miR-224-3p protein expression levels, with significant differences from model control and curcumin groups.

The above findings implied that TLR4 signaling pathway activation mediates the epithelial TJ, but regulating intestinal epithelial TJ mediated by TLR4 signaling pathway activation is likely to be a new idea and strategy for IBD prevention and treatment. CCM preparations may reduce damage caused by IBD to intestinal epithelial TJ through inhibiting TLR4 signaling pathway activation and TNF-α, IFN-γ, and NF-κB expression, as well as blocking the activation of its downstream multiple signaling pathway. Additionally, CCM can enhance SDF-1, CXCR4, and miR-224-3p protein expression levels, and plays an anti-inflammatory role in the intestinal mucosa, through anti-inflammatory action to reduce the damage to colon tissue in UC rats, thereby inhibiting or reversing IBD progression,

which also suggests that the latter can be used as a potential therapeutic target for inflammatory enteritis.

Conflict of interest

The authors declare that they have no competing interests.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

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Not applicable.

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