Icariin Inhibits Intestinal Inflammation of DSS-Induced Colitis Mice Through Modulating Intestinal Flora Abundance and Modulating p-p65/p65 Molecule

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ABSTRACT

Background: *Ulcerative colitis, as a kind of inflammatory bowel disease (IBD) is characterized by abdominal pain. This study aimed to investigate the effect of icariin (ICA) on the intestinal microflora of colitis mice.*

Methods: *Fifteen female C57BL/6 mice were randomly divided into the Control group, dextran sodium sulfate (DSS)-induced colitis (DSS) group, and ICA treatment (DSS+ICA) group. The severity of inflammation in DSS-induced colitis mice was evaluated using disease activity scoring (considering weight-loss percentage, stool-shape change, and stool-bleeding scoring). Pathological changes of mice intestinal tract were evaluated using hematoxylin–eosin (HE) staining. Serum levels of TNF-α and IL-6 were detected with enzyme-linked immunosorbent assay. Expressions of p65 and p-p65 (p-p65/p65 ratio) were analyzed using Western blot assay. 16S rDNA sequencing was used to analyze the abundance and composition of intestinal microflora.*

Results: *Compared with DSS group, ICA significantly improved disease activity ($P < .05$) and reduced inflammatory damage of colon tissues ($P < .05$) in DSS-induced colitis mice. Compared with the DSS group, mice in the ICA group demonstrated significant weight and colon length ($P < .05$). ICA significantly inhibited expressions of IL-6 and TNF-α compared to the DSS group ($P < .05$). p-p65/p65 ratio in the DSS + ICA group was remarkably enhanced compared to the DSS group ($P < .05$). ICA significantly reduced the proportion and activity of Bacteroides, Helicobacteraceae, Turicibacter, and significantly increased that of beneficial microflora (Lactobacillus, Lachnospiraceae, Akkermansia), so as improved damages of colon tissues.*

Conclusion: *ICA can improve intestinal flora abundance and composition of DSS-induced colitis mice, and inhibit tissue damage and inflammatory response through modulating the p-p65/p65 expression.*

Keywords: *Icariin, ulcerative colitis, intestinal microflora, dextran sulfate sodium, inflammation*

INTRODUCTION

Inflammatory bowel disease (IBD), as a kind of colonic mucosal disease, is closely related to the pathogenesis of colorectal cancer.[1,2] IBD mainly includes ulcerative colitis (UC) and Crohn's disease and is characterized by typical symptoms of abdominal pain, diarrhea and/or fever, remarkable features of persistent progress, and recurrent inflammation.[3,4] A relevant study reports that the development of inflammatory enteritis is not caused by a single factor, but by multiple factors, such as environment, genetics, and lifestyle.[5] The pathogenesis of IBD is closely related to the imbalance of intestinal microflora.[6] Changes in intestinal flora structure can cause the activation of the inflammatory signaling pathway and release of inflammatory factors.[6] The comprehensive changes of bacterial species, quantity, distribution, and other factors are closely related to the occurrence of intestinal inflammation.[7]

Many scholars have carried out researches on prevention, treatment, evaluation, and nursing intervention of UC.[8,9] Presently, treatment of UC is mainly conducted through anti-inflammatory drugs, immuno-modulators, and surgical treatments.[8,9] However, the above drugs also demonstrate their limitations, and long-term use would also lead to serious side effects.[10,11] With the rapid development of Traditional Chinese Medicine, natural or traditional herbs have attracted more attention. Traditional

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herbal medicine not only plays an anti-inflammatory effect but also illustrates less side effects. Therefore, traditional herbal medicine is often used to treat some chronic inflammatory diseases, and its role in the prevention and treatment of IBD has been continuously concerned.[12,13]

Icariin (ICA) is a flavonoid extracted from *Epimedium* of *Berberidaceae*. ICA is one of the main bioactive compounds in *Epimedium*, and is the most widely studied monomer in recent years.[14] The previous in vitro and in vivo studies[15-17] have shown that ICA plays critical roles in scavenging ROS, anti-apoptosis, anti-inflammation, anti-cancer, anti-atherosclerosis, neuro-protection, heart protection, and immune protection. However, there is even no study for illustrating the effect of ICA on microbiota till now. The molecular mechanism of ICA may involve in anti-inflammatory/inflammatory cytokines[18] and NF-κβ signal pathway regulation.[17,19]

Chinese herbal medicine can regulate the distribution of intestinal flora.[20] As a kind of Chinese herbal medicine, whether icariin can prevent and treat IBD by regulating intestinal microflora remains to be further studied. It has been reported that ICA has a protective effect on experimental colitis,[21,22] however, the regulatory effect of ICA on IBD or intestinal flora and associated mechanisms have not been fully explored. The purpose of this study was to investigate whether ICA can inhibit the development of mouse colitis induced by dextran sulfate sodium (DSS) through modulating distribution of intestinal flora, and to reveal the potential mechanisms.

MATERIALS AND METHODS

Animals

The male C57BL/6 mice are aggressive and prone to mutual injury, which might lead to failure of the experiment; therefore, the female mice were selected in this study. The specific pathogen-free female C57BL/6 mice (8 weeks and weighting from 18 g to 20 g) (Certificate No. 2015000566355) were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China). The mice were fed and exposed to the circumstances of 12 h/12 h light/night cycle, 22°C and 55% humidity.

Establishment of Colitis Mouse Model and Trial Grouping

Fifteen female C57BL/6 mice were randomly divided into 3 groups, including the Control group (n = 5), DSS group (n = 5), and DSS + ICA group (n = 5). Control group: The mice were given distilled water to drink freely for 7 days, then were intragastrically administrated with 300 μL 10% ethanol solution every day (here ethanol was used for dissolving the ICA), from 8th day to 15th day. DSS group (model group): The mice were administrated with 2.5% DSS solution (Cat. No. 0216011080, MP Biomedical, Solon, OH, USA) for free drinking for 7 days. From the 8th to the 15th day, in addition to 2.5% DSS administration, mice were also administrated with 300 μL 10% ethanol solution daily, according to the previous study described with a few modifications.[23] DSS+ICA group (model treating group): The solution containing 2.5% DSS was intragastrically given to mice for free drinking in the first 7 days. From the 8th day to the 15th day, 10 mg/kg/day ICA (Cat. No. HY-N0014, MedChemExpress, Momouth Junction, NJ, USA) was intragastrically administrated to mice in addition to 2.5% DSS, as the previous study described with a few modifications[21]. For all above 3 groups, there was no animal died during this study (with a 100% successful rate of DSS model in both DSS group and DSS + ICA group).

General Data Collection

The body weight, feces, and fecal blood were measured and recorded every day. During the experiment, the weight of mice was weighed at a fixed time every day, and the curve of weight change was recorded and drawn. Whether the mice have blood in stool and death were observed every day.

Disease Activity Index (DAI) Evaluation

The weight-loss percentage scoring system of mice included 0 point (0), 1 point (0-5%), 2 points (5-10%), 3 points (10-20%), and 4 points (>20%). The stool-shape change scoring of mice included 0 point (none), 2 points (loose stool), and 4 points (diarrhea). The stool-bleeding scoring of mice included 0 point (none), 1 point (extremely light), 2 points (mild), 3 points (obvious), and 4 points (massive hemorrhage). The average value for the total score of the above 3 results was assigned as DAI value, with a formula of DAI=(body mass index score + stool-shape score + bleeding score)/3. The mice have conducted the DAI scoring at the same time every day. The 0 score was assigned as a minimum value and 4 score was as a maximum value.

Detection of Inflammatory Factors with ELISA

At 15 days post the treatments, mice in each group were collected from the orbital cavity. Then, the blood was coagulated for 2 h at room temperature, centrifuged

at 3000 ×g, 4°C for 10 min to harvest the serum. The serum was stored at -80°C for the following ELISA test. The serum levels of IL-6 and TNF-α were detected using IL-6 ELISA Kit (Cat. No. JYM0060Mo, ColorfulGene BioTech. Co. Ltd., Wuhan, China) and TNF-α ELISA Kit (Cat. No. JYM0218Mo, ColorfulGene BioTech. Co. Ltd., Wuhan, China), respectively, according to protocols of manufacturers. The ELISA was conducted using a microplate reader (Model: 680, Bio-Rad Laboratories, Hercules, CA, USA).

Morphological Observation of Colon

The mice were dissected immediately post the orbital blood collection. The length of isolated colon from anus to caecum was recorded and imaged.

Histological Analysis

The colon from 1.5-2 cm below caecum to 1.5-2 cm above rectum was taken and washed with normal saline. The separated colon was divided into 3 parts, including near caecum part, near anus part, and medium part. The colon tissue was fixed with 4% paraformaldehyde (Beyotime Biotech., Shanghai, China) for 24 h, then dehydrated, paraffin-embedded and cut into sections (with thickness 5 μm) using ultra-thin semiautomatic microtome (HM340E, Thermo Scientific Pierce, Rockford, IL, USA). The above-treated tissue sections were used for hematoxylin–eosin (HE) staining. The sections of colon tissues were stained using the commercial HE Staining Kit (Cat. No. C0105, Beyotime Biotech.) as instructed by the protocol of the manufacturer. The HE staining images were scanned with a Digital Section Scanner (Model: Pannoramic MIDI, 3DHISTECH Ltd., Budapest, Hungary).

Moreover, the pathological scores for the above histological images were conducted as described by the previous study[24] with a few modifications. In order to measure the lung injury score, the sum for each of 5-independent variables, including (A) neutrophils in intestinal villus (0 score: none, 1 score: 1-5, 2 scores >5), (B) neutrophils in interstitial spaces (0 score: none, 1 score: 1-5, 2 scores >5), (C) inner wall and space of intestinal villus (0 score: none, 1 score: 1, 2 scores >1), (D) proteinaceous debris filling airspaces (0 score: none, 1 score: 1, 2 scores >1), (E) intestinal villus wall thickening (0 score: <2×, 1 score: 2×-4×, 2 scores >4×), were weighted based on associated features and were subsequently normalized to amounts of fields evaluated. The final total injury score was assigned as the following formula: Score = (20 × A + 14 × B + 7 × C + 7 × D + 2 × E)/(amounts of fields × 100).

Western Blotting Assay

The intestinal tissues were separated and washed with phosphate-buffered saline (PBS) 2-3 times, cut into pieces, and treated with 150-250 μL nucleoprotein lysate for the ultrasonic lysis (Beyotime Biotech., Shanghai, China). The lysates were centrifuged at 12000 ×g for 5 min to obtain the supernatant. The obtained lysates were quantified with Enhanced BCA Protein Assay Kit (Cat. No. P0010S, Beyotime Biotech.). Then, the lysates were loaded onto SDS-PAGE and electro-transferred onto PVDF membranes (Amersham Biosciences, Piscataway, New Jersey, USA). The PVDF membranes were incubated with rabbit anti-mouse p65 polyclonal antibody (Cat. No. AF5006, 1: 1000, Affinity Biosciences, Cincinnati, OH, USA), rabbit anti-mouse phospho-p65 (p-p65) polyclonal antibody (Cat. No. AF2006, 1:1000, Affinity Biosciences) and rabbit anti-mouse β-actin polyclonal antibody (Cat. No. AF7018, 1:1000, Affinity Biosciences) overnight at 4°C. Then, PVDF membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Cat. No. S0001, 1:1000, Affinity Biosciences) at room temperature for 2 h. Finally, Western blot bands were visualized with BeyoECL Moon Imaging Kit (Cat. No. P0018FS, Beyotime Biotech.) and the staining images were captured with a professional ALPHA Imaging System (ProteinSimple, Santa Clara, CA, USA).

16S rDNA Extraction and Biological Analysis of Intestinal Microflora

The DNA was extracted from mice feces (n = 5) using DNA extraction kit (Cat. No. D0065S, Beyotime Biotech.), according to the manufacturer's protocol. In brief, a total of 200 mg fecal sample was added to a 2 mL centrifugal tube and centrifuged at 13000 r/min and 4°C for 45 min. Then, the obtained supernatants were discarded and precipitates were resuspended with total of 100 mL pre-cooled PBS and treated with phenol. The solutions were then centrifuged at 13 000 r/min and 4°C for 10 min again. Finally, the purified genomic DNA was extracted by adding pre-cooled absolute ethanol. The purity and concentration of total DNA were detected using an ultraviolet spectrophotometer (Mode: Nanodrop 2000 UV, Thermo Electron Corp, Waltham, MA, USA) and sequenced by Shanghai Meiji Company (Shanghai, China). The effective data was clustered on the Meiji cloud platform, and the sequences were

clustered into OTUs (Operational Taxonomic Units). The abundance, diversity, similarity, and composition of bacteria were analyzed eventually.

Statistical Analysis

The continuous variables were illustrated as mean ± standard deviation (SD) and analyzed with professional SPSS software (version: 20.0, SPSS Inc., Chicago, Ull, USA). Tukey's post-hoc test validated analysis of variance was used to compare differences among multiple groups. The $P < .05$ was assigned as the significant difference.

RESULTS

ICA Ameliorated Body Weight Loss Induced by DSS

At 7 days post-treatments with 2.5% DSS, mice demonstrated obvious diarrhea and hematochezia. Compared with the Control group, the body weight of mice in the DSS group significantly decreased from the 12th to the 15th day post-treatments (Figure 1A, $P < .01$). However, compared with DSS group, body weight of DSS + ICA group was significantly increased from the 12th to the 15th day post-treatments (Figure 1A, $P < .05$). These results suggest that ICA could promote the body weight of mice.

ICA Decreased Disease Activity Index of DSS-Colitis Mice

In this study, mice's disease activity index (DAI) was determined according to the formula involving body mass index score, stool-shape score, and bleeding score of mice. At the end of experiment (the 15th day), compared with the Control group, the DAI values of mice in DSS group and DSS+ICA group were remarkably lower (Figure 1B, $P < .05$). Meanwhile, compared with mice in DSS group, the DAI index of mice in DSS + ICA group was significantly decreased at the 15th day post ICA treatment (Figure 1B, $P < .05$).

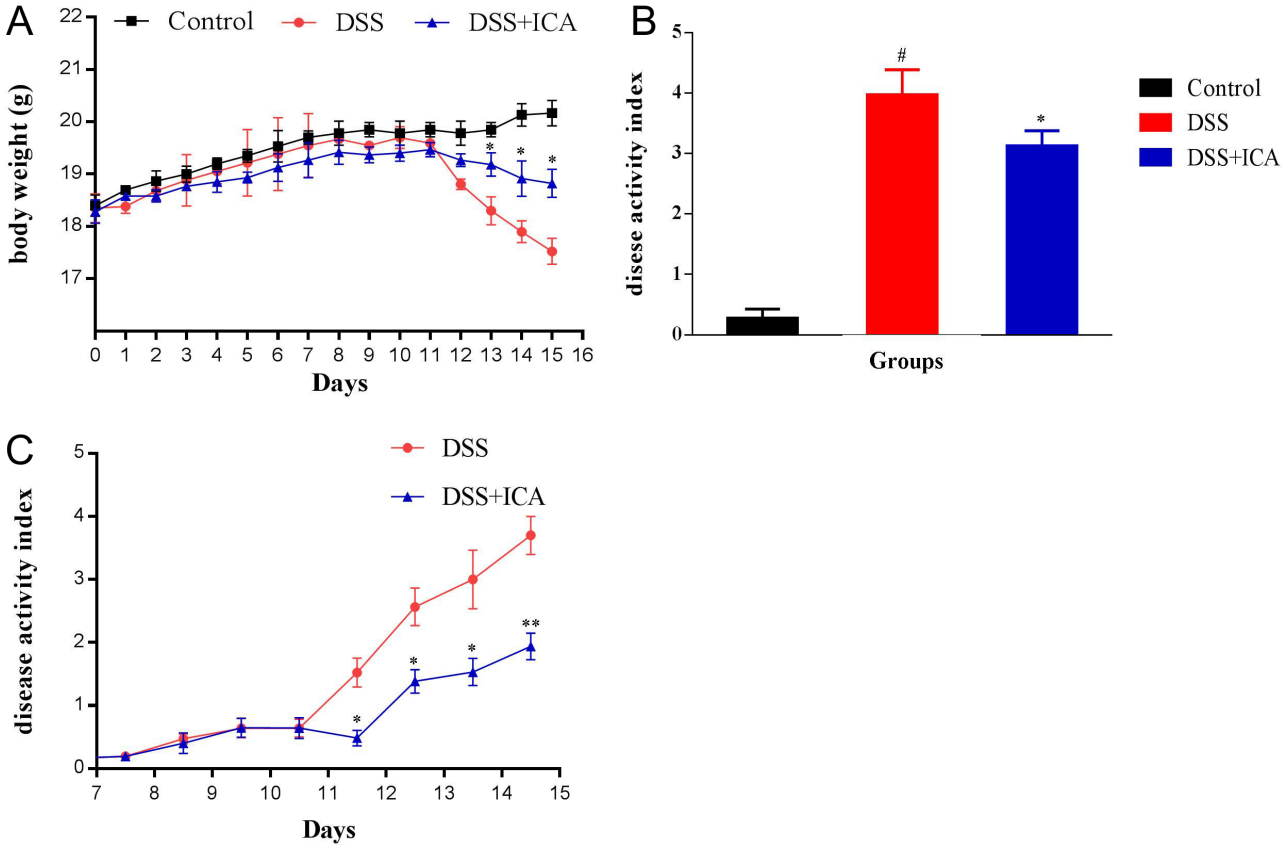

Figure 1. Effects of ICA on body weight and disease activity index of DSS-induced ulcerative colitis mice. A. Statistical analysis for body weight in Control group ($n = 5$), DSS group ($n = 5$) and DSS+ ICA group ($n = 5$) from 0 day to 15th day. B. Statistical analysis for disease activity index in Control, DSS and DSS + ICA group at 15th day. C. Statistical analysis for changes of disease activity index in DSS and DSS + ICA group from 8th day to 15th day. $^{*}P < .05$, $^{**}P < .01$ vs. DSS group at the same time point. $^{\#}P < .05$ vs. Control group.

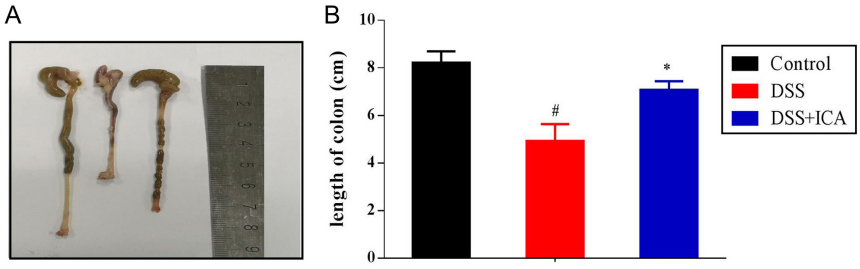

Figure 2. Effects of ICA on length of colon of DSS-induced ulcerative colitis mice. A. Image for the colons in Control group (n = 5), DSS group (n = 5) and DSS + ICA group (n = 5). B. Statistical data and comparison for the length of the colon in different groups. $^{*}P < .05$ vs. DSS group at the same time point. $^{\#}P < .05$ vs. Control group.

Meanwhile, the changes in DAI scores of mice in DSS group and DSS + ICA group were also recorded (Figure 1C). The results showed that DAI score of mice was significantly reduced in DSS + ICA group compared to that in DSS group (Figure 1C, $P < .05$) from 12th to 15th day post-treatments. Moreover, the symptoms of diarrhea and fecal blood in DSS + ICA group were also improved. These results suggest that ICA could improve disease activity.

ICA Prolonged Colon Length of DSS-Colitis Mice

In this study, colon length was used as an objective index to reflect the severity of inflammation; the more serious of colitis, the shorter length of the colon.[25] Therefore, the colon length of mice was recorded in this study (Figure 2A). The statistical analysis results indicated that the colon length of mice in the DSS group was remarkably shorter than that in the Control group (Figure 2B, $P < .05$). The colon length of mice in the DSS + ICA group was significantly prolonged compared to that of mice in the DSS group (Figure 2B, $P < .05$). Therefore, DSS treatments aggravated the severity of colonic inflammation, while the addition of ICA could alleviate colonic inflammation of DSS-colitis mice.

ICA Improved Pathological Structure of Colon Tissues in DSS-Colitis Mice

The histological evaluation of the colon in Control group showed that the structure of colon tissues was normal without obvious histological changes (Figure 3A). However, DSS-colitis mice demonstrated severe colonic ulcer and tissue damage. Meanwhile, colonic tissues of DSS mice had obvious hyperemia and edema, and demonstrated loss of histological structure and disintegration of epithelial cells (Figure 3A). The epithelial barrier of colon tissue was broken and a large number of crypts were decreased. Additionally, the granulosa cells and monocytes in colon tissues infiltrated into the mucosa and sub-mucosa (Figure 3A). Compared with DSS group, the severity of colonic ulcer and colon injury in the ICA + DSS group was obviously lighter, and the intestinal wall depression was not obvious (Figure 3A). Meanwhile, colon tissue structure of DSS + ICA group was relatively complete (Figure 3A). According to the pathological analysis for histological images, the DSS group's pathological score was significantly higher than that in Control group (Figure 3B, $P < .05$). However, DSS + ICA group demonstrated a significantly lower pathological score compared to that of DSS group (Figure 3B, $P < .05$). These findings suggest that ICA reduces the severity of colon injury in DSS-colitis mice.

ICA Inhibited NF-κB Signaling Pathway

In this study, the expression of key proteins (p65 and p-p65) of the NF-κB signaling pathway[26] was detected with a Western blot assay (Figure 4A). The results illustrated that expression of p65 (Figure 4B, $P < .05$) and p-p65 (Figure 4C, $P < .05$) in colitis tissues in DSS group was significantly higher compared to that in the Control group. However, expression of p65 (Figure 4B, $P < .05$) and p-p65 (Figure 4C, $P < .05$) in colitis tissues in DSS + ICA group was significantly lower compared to that in DSS group. Moreover, the ratio of p-p65/p65 in DSS group was significantly reduced compared to that in Control group (Figure 4D, $P < .05$). The ratio of p-p65/p65 in DSS + ICA group was also remarkably enhanced compared to that in DSS group (Figure 4D, $P < .05$). The above results demonstrated that ICA could reduce the inflammatory response by inhibiting the NF-κB signal pathway in DSS-colitis mouse models.

ICA Reduced Serum Levels of Pro-Inflammatory Cytokines in DSS-Colitis Mice

Serum levels of pro-inflammatory cytokines (TNF-α and IL-6) of DSS-colitis mice were examined using ELISA. The results showed that serum levels of IL-6 (Figure 5A, $P < .05$) and TNF-α (Figure 5B, $P < .05$) in DSS group were significantly higher compared to those in the Control

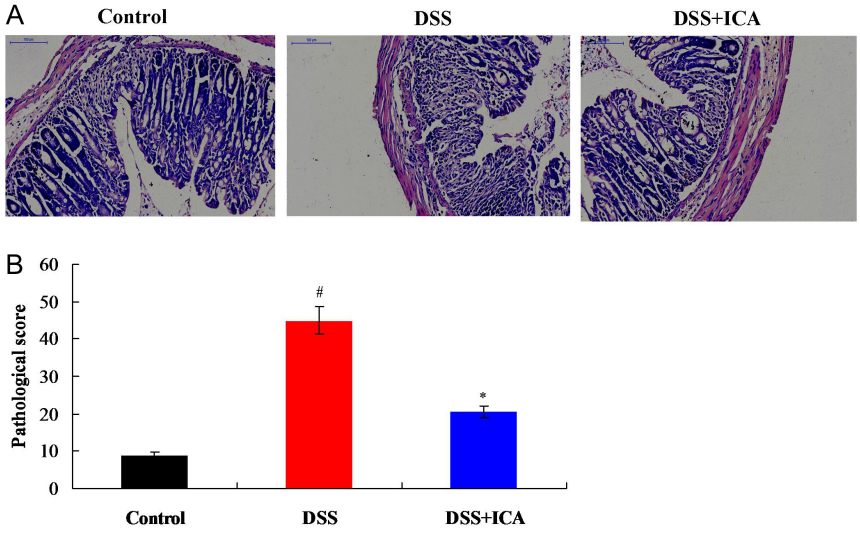

Figure 3. Evaluation for histopathological characteristics of ICA treated DSS-induced colitis mice using HE staining. A. Histopathological images of colitis mice in the Control group (n = 5), DSS group (n = 5) and DSS + ICA group (n = 5). B. Pathological scores of colitis mice in different groups. $^{*}P < .05$ vs. DSS group at the same time point. $^{\#}P < .05$ vs. Control group.

group. Furthermore, compared with DSS group, the serum levels of IL-6 (Figure 5A, $P < .05$) and TNF-α (Figure 5B, $P < .05$) in DSS + ICA group were significantly lower.

Effect of ICA on Composition of Intestinal Microflora in Phylum Level in DSS-Colitis Mice

In this experiment, DNA contents in intestinal microflora were more than 100 ng, and the OD_{260}/OD_{280} ratio was between 1.8 and 2.0. The results suggest that the purity and quality of DNA meet the experimental requirements. The results illustrated that when the sequence number exceeded 30 000, only a small amount of OTUs would be generated by increasing the sequence (Figure 5). Therefore, these results suggest that the sequencing amount is reasonable.

To analyze the relationship between the pathogenesis of colitis and intestinal microflora changes, we compared

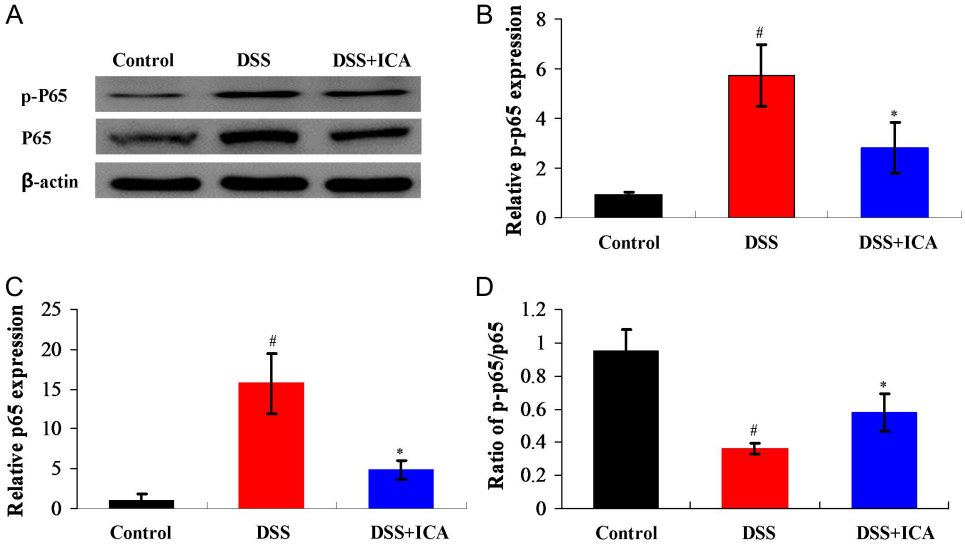

Figure 4. Determination for p65 and p-p65 expressions in colon tissues of DSS-induced colitis mice. A. Western blot images for p65 and p-p65 expressions in the Control group (n = 5), DSS group (n = 5) and DSS + ICA group (n = 5). B. Statistical data and comparison for p-p65 expression in 3 groups. C. Statistical data and comparison for p65 expression in 3 groups. D. Statistical analysis for ratios of p-p65/p65 in all 3 groups. $^{*}P < .05$ vs. DSS group at the same time point. $^{\#}P < .05$ vs. Control group.

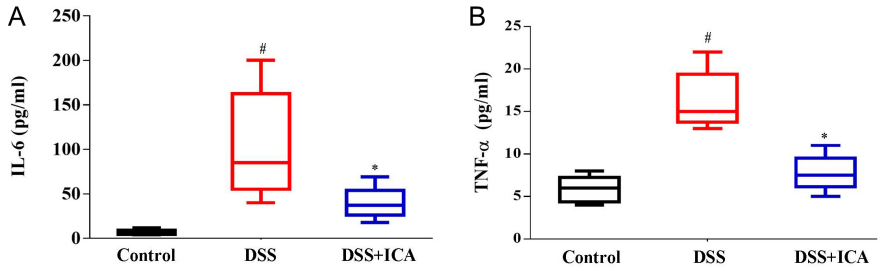

Figure 5. Changes of inflammatory cytokines (IL-6 and TNF-α) in serum of DSS-induced colitis mice. A. Statistical data and comparison of serum IL-6 levels in Control group (n = 5), DSS group (n = 5) and DSS + ICA group (n = 5). B. Statistical data and comparison of serum TNF-α levels in Control, DSS, and DSS + ICA group. *P < .05 vs. DSS group at the same time point. #P < 0.05 vs. Control group.

the classification information of intestinal microflora in each group at the classification level of *phylum, family*, and *genus*. For the level of phylum, *Bacteroidetes* and *Firmicutes* were the main *phylum* of intestinal microflora. Compared with the Control group, the abundance of *Bacteroidetes* was decreased (73.87% vs. 26.42%), and that of *Firmicutes* was increased (19.4% vs. 33.51%) (Table 1). After intervention with high concentration ICA, the proportion of each *phylum* in intestinal microflora was similar to that of the Control group (Table 1). In this study, the top 6 species at *phylum* level in each group included *Bacteroidetes phylum*, *Firmicutes phylum*, *Proteobacteria phylum*, *Verrucomicrobia phylum*, *Tenericute phylum* and *Deferribacteres phylum* (Table 1). Therefore,, the dominant *phylum* of each group was the same.

However, compared with Control group, sequencing and composition ratio of dominant bacteria at *phylum* level in DSS group demonstrated an obvious change. Intestinal microflora changes mainly included significantly decreased *Bacteroidetes*, significantly increased *Firmicutes*, *Proteobacteria*, *Verrucomicrobia*, *Tenericute*, and *Deferribacteres* (Table 1). Compared with the Control group and DSS group, the *phylum* level sequencing and the composition ratio of DSS + ICA group demonstrated significant differences. The differences of intestinal microflora mainly manifested in *Tenericute* (DSS + ICA group: 0.42%, DSS group: 1.91%, Control group 0.55%), *Bacteroidetes* (DSS + ICA group: 25.44%, DSS group:

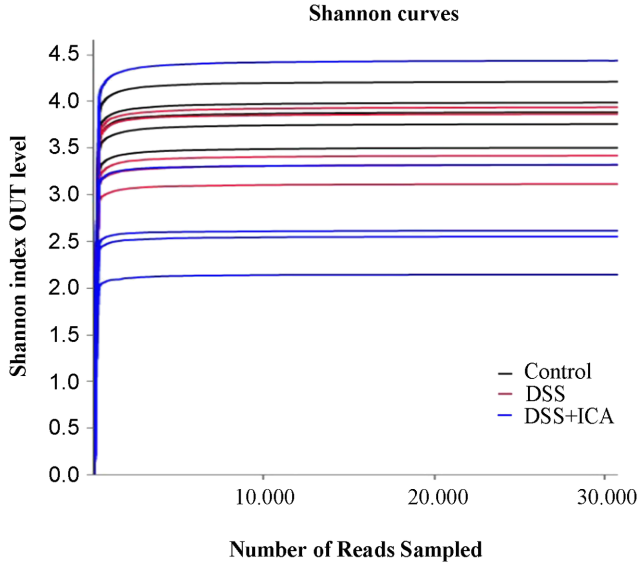

Figure 6. Sample dilution curve for the sequencing amount triggered OTUs.

Table 1. Changes of Intestinal Flora in Mice-Top 6 Species at the Phylum Level

Control group		DSS group		DSS + ICA group	
Phylum	Constituent ratio	Phylum	Constituent ratio	Phylum	Constituent ratio
Bacteroidetes	73.87	Firmicutes	33.51	Proteobacteria	33.90
Firmicutes	19.40	Proteobacteria	30.20	Firmicutes	35.26
Proteobacteria	4.41	Bacteroidetes	26.42	Bacteroidetes	25.44
Verrucomicrobia	1.49	Deferribacteres	6.66	Verrucomicrobia	4.47
Tenericute	0.55	Tenericute	1.91	Tenericute	0.42
Deferribacteres	0.08	Verrucomicrobia	1.74	Deferribacteres	0.14

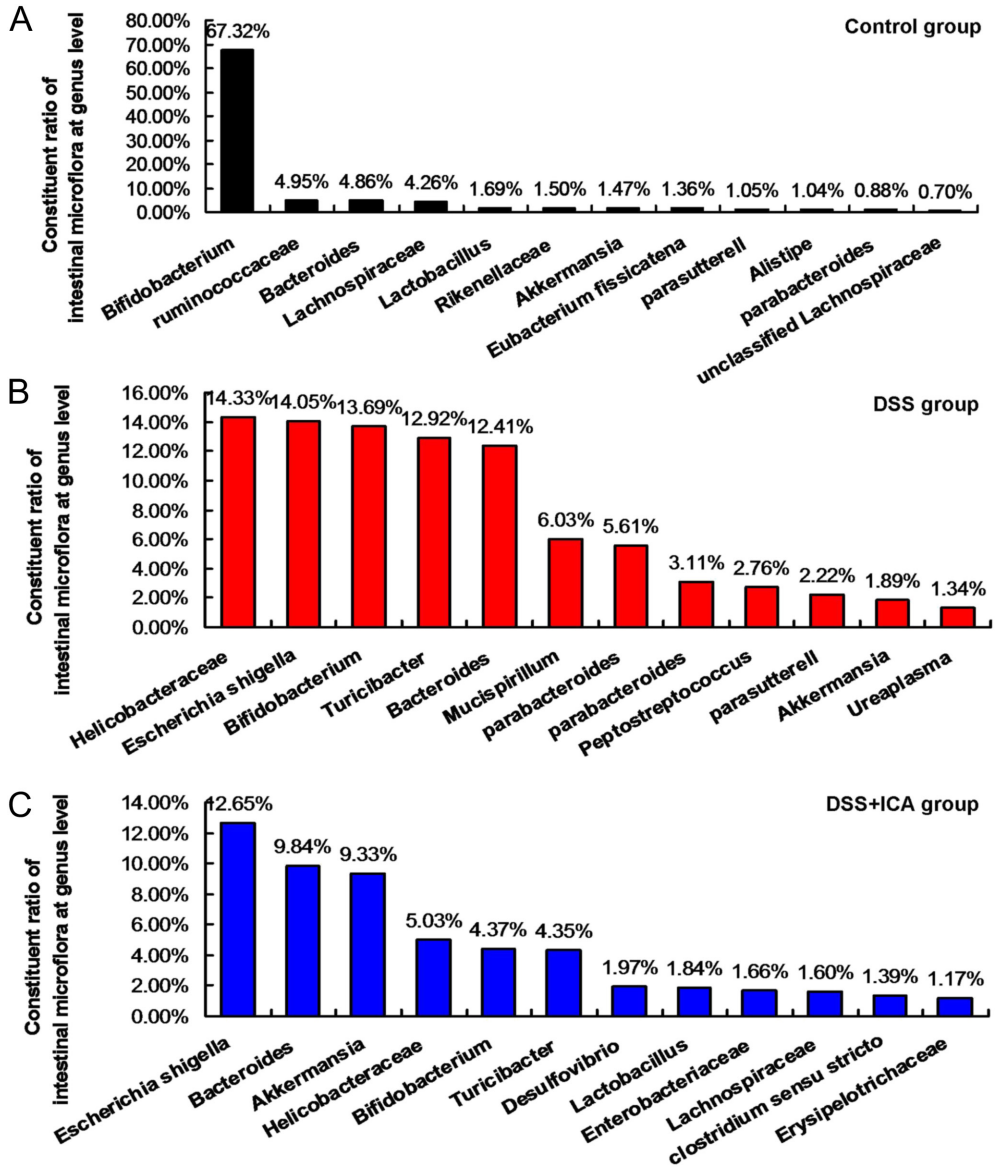

Figure 7. Changes of intestinal microflora in mice-Top 12 species at the genus level. A. Constituent ratio of intestinal microflora in Control group (n = 5). B. Constituent ratio of intestinal microflora in DSS group (n = 5). C. Constituent ratio of intestinal microflora in DSS+ICA group (n = 5).

26.42%, Control group: 73.87%) and *Deferribacteres* (DSS + ICA group: 0.14%, DSS group: 6.66%, Control group: 0.08%) (Table 1). Also, the order of sequencing and composition ratio of intestinal microflora in DSS + ICA group was close to that in the Control group (Table 1).

ICA Increased Activity of Beneficial Microflora and Decreased Pathogenic Intestinal Microflora of Dss-Colitis Mice

At the level of *genus*, the first 12 *genus* were shown in Figure 7. Our results showed that the percentage of *Helicobacteriaceae* in DSS group was the highest (14.33%), followed by *Escherichia coli-Shigella* (14.05%), *Bifidobacterium* (13.69%), *Turicibacter* (12.92%), *Bacteroides* (12.41%), and *Mucispirillum* (6.03%). Post the treatment of ICA, the predominant bacteria in the intestine of mice were probiotics, including *Escherichia coli-Shigella* (12.65%), *Bacteroides* (9.84%), *Akkermansia* (9.33%), *Helicobacteriaceae*

(5.03%), *Bifidobacterium* (4.37%), *Turicibacter* (4.35%), and *Desulfovibrio* (1.97%). In the Control group, *Bifidobacterium* (67.32%), *Ruminococcaceae* (4.95%), and *Bacteroides* (4.86%) were the most common. At the classification level of *family* and *genus*, under the intervention of ICA, the intestinal microflora of colitis mice, including *Helicobacteriaceae* (5.03% in ICA group and 14.33% in DSS group), *Bacteroides* (9.84% in ICA group and 12.41% in DSS group), and *Turicibacter* (4.35% in ICA group, 12.92% in DSS group) were significantly decreased in mice intestine (Figure 7). However, the beneficial microflora, including *Lachnospiraceae* (1.6% in ICA group and not detected in DSS group), *Akkermansia* (9.33% in ICA group and 1.89% in DSS group), and *Lactobacillus* (1.84% in ICA group and not detected in DSS group), were increased significantly (Figure 7). Therefore, the 16S rRNA gene sequencing demonstrated that ICA increased activity of beneficial microflora and decreased a few intestinal microflora of colitis mice.

DISCUSSION

IBD is usually considered as a major high-risk factor in the development of colon cancer; therefore, many strategies have been taken to prevent IBD in the treatment of colon cancer.[27,28] Non-steroidal anti-inflammatory drugs are usually used to treat IBD, but long-term use can cause many side effects, including nausea, abdominal pain and gastritis.[29] However, in comparison, the Traditional Chinese Medicine demonstrates several advantages and without or less of the above side effects.[30] ICA has been proven to be characterized by a good ability to regulate intestinal microflora and anti-inflammatory effect.[31] The previous study[21] also provided evidence that ICA could improve DSS-induced colitis; however, the underlying mechanism remains to be further revealed.

This experiment mainly investigated the effects of ICA on DSS-induced colitis, inflammatory response, and regulation of microflora. Our results showed that ICA could alleviate inflammation by regulating the composition of intestinal microflora and then inhibiting the expression of TNF-α and IL-6 mediated by NF -κB signaling pathway, consistent with the previous results.[32]

The imbalance of intestinal microorganisms is closely related to the occurrence and development of chronic IBD.[33] ICA can regulate intestinal microorganisms and reduce intestinal inflammation induced by DSS in mice.[21,22] A previous study[34] reported that *Helicobacteraceae* was significantly increased in intestines of IBD patients, while *Rikenellaceae* was significantly decreased in spontaneously formed inflammatory associated colorectal cancer in mice. Cui et al.[35] found that *Lachnospiraceae* significantly decreased in colon cancer patients' intestines, while some OTUs belonging to Lachnospiraceae demonstrated maintaining intestinal health. Therefore, the increase of *Lachnospiraceae* and *Rikenellaceae* in the intestine of mice may promote the anti-inflammatory and even the anti-cancer effects of ICA. Compared with the DSS group, numbers of main intestinal microflora of colitis mice, including *Helicobacteraceae*, *Bacteroides*, and Tricibacter of ICA intervention group, significantly decreased. However, the beneficial microflora, including *Lachnospiraceae* (ICA+DSS group vs. DSS group: 1.6% vs. 0), *Akkermansia* (ICA+DSS group vs. DSS group: 9.33% vs. 1.89%) and *Lactobacillus* (ICA+DSS group vs. DSS group: 1.84% vs. 0) were remarkably increased compared to that in DSS group. The 16S rRNA gene sequencing demonstrated that ICA increased activity of beneficial microflora and decreased a few intestinal microflora of colitis mice. Therefore, ICA may play critical roles in protecting against inflammation of intestinal issues of DSS-induced colitis mice.

Moreover, *Helicobacteraceae* was less in normal mice (less than 0.7%); however, the proportion of which in DSS-induced colitis mice was increased to 14.33% and decreased to 5.03% post the ICA treatment. *Tricibacter* is the core microflora in the intestine of DSS-induced colitis mice, even achieving 12.92%, which can be reduced to 4.35% post the ICA treatment. *Bacteroides* was less in normal mice (4.86%); however, the proportion of DSS-induced colitis mice was increased to 12.41% and decreased to 9.84% post the ICA treatment.

NF-κB, as a transcription factor, the abnormal activation of which is one of the pathophysiological mechanisms for the UC.[36] p65 is an important member of NF-κB family and the activation that leads to the release of many inflammatory factors.[35] Our results showed that treatment of ICA significantly affected the expression of p65 and p-p65 protein, significantly increased body weight and intestinal length of mice, and remarkably improved bloody stool and crypt injury. Inflammation is one of the main inducing factors in IBD pathogenesis, which can cause intestinal tissue damage. According to a previous study,[37] TNF-α, IL-1β, IL-6, IL-17 play important roles in the inflammatory response. In the present

research, the levels of cytokines in the serum of mice were detected using ELISA, and the results showed that levels of TNF-α and IL-6 were significantly lower in DSS + ICA group compared to that in DSS group. These results suggest that ICA could alleviate the inflammatory response of DSS-induced colitis mice.

There are also a few limitations in this study. Firstly, serum lipopolysaccharides levels in different groups have not been clarified. The alteration of intestinal microflora might induce serum LPS and might cause inflammation, which is helpful to clarify mechanism of ICA. Secondarily, according to our results, there might be some associations between alteration of intestinal microflora and inflammation, however, which are have not been analyzed. Thirdly, the specific mechanism for the effects of ICA on microflora has not been fully investigated in this study. Fourthly, as well known, colitis patients and animal models usually demonstrate a reduction in the overall density of gut microorganisms. However, the effects of ICA treatment on total bacterial richness and bio-density of intestinal microflora have not been investigated. Fifthly, the effect of ICA on the balance of intestinal microorganisms (microflora) has not been clarified in DSS-induced colitis mice, which would be investigated using principal component analysis cluster analysis in the following study. Sixth, the effects of ICA treatment on microflora in normal mice have not been investigated. Finally, the dose-dependent effects of ICA on the DSS-induced colitis and gut flora changes have not been clarified. Therefore, we cannot identify whether there is a dose-dependent effect and correlation between ICA treatment and gut flora, which need to be clarified in future investigations.

In conclusion, this study proved that ICA could improve the intestinal flora abundance and composition of DSS-induced colitis mice, and inhibit tissue damage and inflammatory response. Therefore, ICA might be a promising strategy for treating UC clinically.

Ethics Committee Approval: All experiments with mice were conducted in compliance with the guidance that is approved by Institutional Care and Use Committee (IACUC) of Nanjing University of Chinese Medicine.

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