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Original Article

Balsalazine decreases intestinal mucosal permeability of dextran sulfate sodium-induced colitis in mice

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Aim: To investigate the effect of balsalazine treatment on intestinal mucosal permeability in dextran sulfate sodium (DSS)-induced colitis and to determine the mechanism of the balsalazine-induced changes.
Methods: Experimental colitis was induced in C57BL/6J mice by the administration of 5% DSS. Balsalazine was administered intragastrically at doses of 42, 141, and 423 mg/kg. The disease activity index (DAI) score was evaluated and colon tissue was collected for the assessment of histological changes. The amount of malondialdehyde (MDA) in the colon was determined, along with the activity of myeloperoxidase (MPO), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px). Mucosa from the small intestine was collected to determine the levels of tumor necrosis factor (TNF)-α and interferon (IFN)-γ. The mucosa was ultrastructurally examined with transmission electron microscopy and intestinal permeability was assayed using Evans blue.
Results: Balsalazine was found to reduce the DAI score and the histological index (HI) score, decrease the MDA content and the activity of MPO, and increase the activity of SOD and GSH-Px in colitis mice. At the same time, balsalazine ameliorated microvillus and tight junction structure, resulting in a decrease in the amount of Evans blue permeating into the intestinal wall and the levels of TNF-α and IFN-γ in colitis mice.
Conclusion: In colitis mice, the anti-colitis effect of balsalazine results in a decrease in intestinal mucosal permeability. The mechanism of this effect is partly associated with balsalazine's antioxidative and anti-inflammatory effects.

Keywords: balsalazine; dextran sulfate sodium; colitis; permeability; intestinal mucosal barrier

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Introduction

The intestinal mucosal barrier plays a pivotal role in preventing microorganisms and bacterial toxins from entering an organism's bloodstream. However, the barrier becomes impaired during inflammatory bowel disease (IBD). As a result, a large quantity of endotoxin can enter into systemic circulation through the impaired intestinal mucosa[1, 2]. In addition, oxygen free radicals (OFRs) and proinflammatory cytokines are induced, facilitating impairment of intestinal mucosal permeability[3–9]. Oxidants alter cytoskeletal components (such as actin), resulting in disruption of the structural integrity of epithelial cells[10, 11]. In addition, proinflammatory cytokines, including tumor necrosis factor (TNF)-α and interferon (IFN)-γ, disrupt tight junctions[6–9], decrease the transendothelial electrical resistance (TEER), and regulate the expression of intestinal mucosal barrier-associated proteins. Therefore, restoring the impaired intestinal mucosa is beneficial for controlling and reducing the inflammation and immunologic reaction occurring in the intestinal mucosa of patients with IBD[12].

5-aminosalicylate (5-ASA) is used to treat ulcerative colitis (UC) because of its ability to control and relieve inflammation. Sulfasalazine, the first 5-ASA-containing drug, functions by releasing an active component in the colon through the activity of azo reductase expressed by colonic bacterial. Sulfasalazine has been approved for therapeutic usage because of its ability to improve intestinal mucosal permeability[13, 14]. Balsalazine (5-ASA azo bonded to an inert carrier, 4-amino-benzoyl-alanine) can also release 5-ASA through cleavage of the compound by azo reductase expressed by intestinal luminal bacteria. The administration of balsalazine effectively induces remission in patients with mild to moderate UC. However, whether balsalazine improves intestinal mucosal permeability is still unknown[15, 16].

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Previous studies have shown that mucosal barrier dysfunction is a pathophysiological feature of colitis, irrespective of etiology or species[17, 18]. Therefore, dextran sulfate sodium (DSS)-induced colitis is considered an appropriate model of the injured mucosal barrier[19]. The epithelium of the small intestine is at the front line of the intestinal barrier. Thus, increased small-intestine permeability may be an important etiological event in colitis[20]. Therefore, the present study was designed to investigate the effect of balsalazine on small-intestine mucosal permeability in the DSS-induced colitis model and the possible mechanisms of its effects.

Materials and methods

Animals and reagents

Specific pathogen-free (SPF)-grade C57BL/6J mice, 6–8 weeks old and weighing 20±2 g, were provided by Shanghai Slac Laboratory Animal Co Ltd (Shanghai, China; Certificate No SCXK 2007-0005). The mice were housed in animal facilities with 50% humidity and a 12:12-h light-dark cycle and were fed a standard pellet diet and tap water *ad libitum*. Balsalazine was provided by Shanxi Anter Incorporated. DSS (molecular weight 8000) was purchased from Sigma-Aldrich Co. Kits for detecting Myeloperoxidase (MPO; batch number: 080416), glutathione peroxidase (GSH-Px; batch number: 080320), malondialdehyde (MDA; batch number: 080321), superoxide dismutase (SOD; batch number: 080320) and Evans blue (EB) were all bought from Nanjing Jiancheng Biotechnology Institute (Nanjing, China). The ELISA kits for TNF-α (batch number: 080621) and IFN-γ (batch number: 080510) were obtained from Jingmei Biotech Co Ltd (Shenzhen, China). The main instruments used in this study were an ultraviolet spectrophotometer (752N; Shanghai, China), an enzyme-labeling instrument (ELx800; USA), a transmission electron microscope (TEM; Hitachi, Japan), a light microscope (Olympus; Japan), and a high-speed controllable homogenizer (FSH 2; China).

Experimental protocols

All experiments were performed in accordance with the institutional and national guidelines for the care and use of laboratory animals and were approved by the Ethics Committee of Anhui Medical University. Forty-five C57BL/6J mice were randomly divided into the following five groups: a normal group, a DSS-treated group, and three balsalazine groups treated at doses of 42 mg/kg, 141 mg/kg, and 423 mg/kg according to the study of Kimura *et al*. Balsalazine was resuspended in distilled water and administered intragastrically to the treatment groups once a day from the first day (d1) of the experiment and lasting for 7 days. All the other groups received distilled water as a control[21].

Induction of colitis

Acute colitis was induced in mice by replacing normal drinking water with distilled water containing 5% (*w/v*) DSS and allowing them to drink freely for 7 days[22]. The mice in the DSS group and the three balsalazine groups all drank 5% DSS, and the mice in the control group received regular distilled water.

Evaluation of DAI

Throughout the duration of the experiment, the following parameters were recorded for each mouse daily by two unblinded observers: weight, presence of occult or gross blood in feces, and stool consistency. These parameters were each assigned a score and utilized to calculate an average daily disease activity index (DAI) score for each mouse as previously described[22].

Surgical procedure

The mice were sacrificed by chloral hydrate anesthesia after 7 days of DSS administration. The abdomens were opened along the median line, and the colon was rapidly excised, rinsed gently with ice-cold phosphate-buffered saline, placed on ice, and opened longitudinally. Two continuous pieces were collected after gross morphological changes of colon mucosa were examined. One part of the colon was immediately fixed in 10% neutral buffered formalin for histological analysis. The other colon segment was homogenized for use in the assessment of MDA content, along with the activity of MPO, SOD, and GSH-Px. The homogenate was stored at -20 °C. Small-intestine mucosa was collected for TEM analysis, assessment of permeability by Evans blue and levels of TNF-α and IFN-γ.

Histological assessment

The portion of the colon fixed in 10% neutral buffered formalin was embedded in paraffin for histological analysis. Full-thickness (5 μm) sections were stained with hematoxylin and eosin and examined microscopically by a blinded pathologist who was unaware of the experimental design of the study. Severity of colitis was graded on a scale of 0–4 and expressed as the pathological index according to the standard scoring system[22].

Assessment of TEM

The ileum within 0.5 cm of the ileocecal junction (about 1 cm) was excised with a sharp scalpel. Specimens processed for TEM were fixed in 2.5% glutaraldehyde for four hours at 4 °C, followed by fixation in osmic acid and embedded in Epon. Ultrathin sections were examined by TEM.

Determination of intestinal permeability by Evans blue

The small-intestine sacs were prepared as previously described[23, 24]. Briefly, the small intestine was incised, and the fecal contents were washed out gently with 2−3 mL of PBS. The proximal and distal intestines were ligated, and 0.2 mL of 1.5% (*w/v*) Evans blue (EB) in PBS was infused into the lumen. The sac was then incubated in 20 mL Krebs buffer in 95% O_2 at 37 °C for 30 min. The sac was washed three times in 6 mmol/L acetylcysteine and dried on filter paper at 37 °C for 24 h, followed by incubation with 1 mL of formamide at 50 °C for 24 h. The amount of dye eluted was estimated at a wavelength of 655 nm. The amount of EB permeating the

intestinal wall was calculated based on the standard curve of EB in formamide.

Detections of colonic and intestinal homogenates

The dissected colon and intestine were excised and all fat and mesenteries were removed. The tissue was subsequently homogenized in physiologic saline and stored at -20 °C temporarily. Detection of the amount of MDA, and the activities of MPO, SOD, and GSH-P_X were determined according to the manufacturer's guidelines. The levels of TNF-α and IFN-γ in small intestine homogenates were determined using ELISA kits.

Statistical analysis

The statistical software used was SPSS11.5. All analyses are expressed as mean±standard deviation (SD). Group comparisons were performed using the one-way analysis of variance (ANOVA) test and correlations were tested by Pearson's rank correlation coefficient. A P value <0.05 was considered statistically significant.

Results

The effect of balsalazine on DSS-induced colitis

Administration of a 5% DSS solution in the drinking water resulted in colitis in these mice. The induction of colitis is reflected by the presence of bloody stool, diarrhea and weight loss. The DAI score increased from day 5 (d 5) and all mice had abnormal stools during the experiment. Balsalazine treatment significantly decreased the DAI score compared with the DSS group (P<0.05, Figure 1).

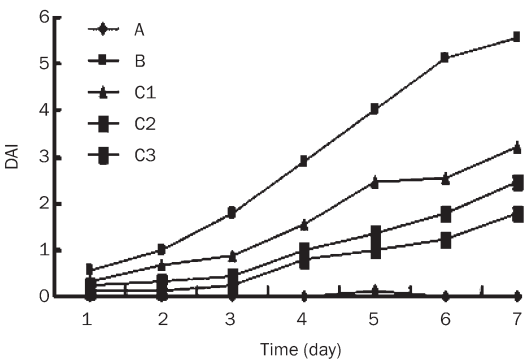

Figure 1. Effects of balsalazine on DAI score with administration of 5% DSS during 7 days (n=9). Animal weights, presence of occult or gross blood in the feces, and stool consistency were recorded daily for each animal to calculate an averaged daily disease activity index (DAI). (A) Normal group; (B) DSS group; (C1) balsalazine 42 mg/kg; (C2) balsalazine 141 mg/kg; (C3) balsalazine 423 mg/kg. DAI was significantly high and increasing daily in the DSS group. DAI in the balsalazine groups were lower than that in the DSS group (P<0.05 from d 5 by ANOVA test).

Colonic mucosal lesions were evaluated at the end of the experiment. Balsalazine attenuated mucosal hyperemia and edema. Additionally, balsalazine reduced the extent of colonic mucosal lesions by reducing the HI score from 7.66±1.00 in the DSS group to 5.66±0.71, 4.11±0.78, and 2.22±0.44 in the 42 mg/kg, 141 mg/kg, and 423 mg/kg balsalazine dosage groups, respectively (Figure 2). As shown in Figure 3B, DSS treatment induced multifocal superficial ulcers in the colon mucosa, inflammatory cells infiltration mainly into the mucosa and the submucosa, along with edema in the submucosa and the folliculus lymphaticus. Furthermore, surface epithelial cells and colonic crypts were damaged and severely lost. However, in the balsalazine treatment groups there was mild inflammation infiltration, integrated surface epithelium, and attenuated crypt cell loss (Figure 3C). These results indicated that balsalazine had significant anti-colitis effects.

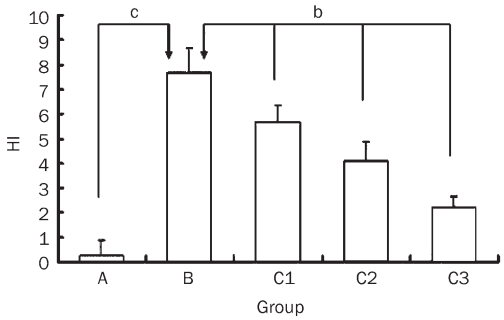

Figure 2. Effects of balsalazine on HI score with administration of 5% DSS after 7 days (n=9). (A) Normal group; (B) DSS group; (C1) balsalazine 42 mg/kg; (C2) balsalzine 141 mg/kg; (C3) balsalazine 423 mg/kg. Severity of colitis was graded on a scale of 0−4 and expressed as the pathological index according to the standard scoring system. HI was significantly high in the DSS group. HI in the balsalazine groups was lower than that in the DSS group. (^bP<0.05, ^cP<0.01 *vs* DSS group by ANOVA test).

The effect of balsalazine on intestinal mucosal barrier function in DSS-induced colitis

The integrity of the intestinal mucosal barrier is the result of the structure of the intestinal epithelial cells and the intercellular tight junctions. Compared with the control group (Figure 4A), the DSS group (Figure 4B) displayed the following changes: a focal reduction of intestinal microvillus, disarrangement of the epithelial surface, irregular widening of the intercellular space, decurtated and broader junctional complexes, and partial surface epithelium injury, even detachment of the epithelium. In contrast, the balsalazine-treated groups (Figure 4C, 4D, and 4E) displayed an attenuation of the surface epithelium injury, regular and intensive microvilli, and ameliorated tight junctions.

Simultaneously, the amount of EB permeating into the intestinal wall in the DSS-induced group was 281.43±7.06 μg/g, which was markedly higher than that in the control group (90.07±5.20 μg/g, P<0.01). These data indicate that intestinal mucosal permeability was increased in DSS-induced colitis mice. The treatment of DSS-induced colitis mice with 42 mg/kg, 141 mg/kg, and 423 mg/kg of balsalazine lowered the amount of EB permeating into the intestinal wall to

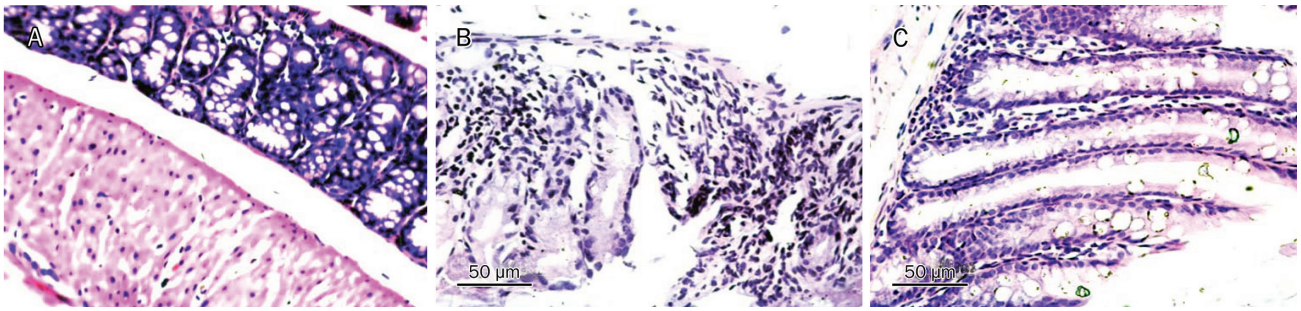

Figure 3. Histopathologic features of the colon in association with colitis (HE×400). A: normal group, B: DSS group, C: balsalazine group. No histological damage was seen in the normal group. The DSS group showed severe inflammatory cells infiltration in mucosa and submucosa, and the surface epithelial cell and crypts were damaged and lost severely. The balsalazine group showed mild inflammation, integrated surface epithelium, and attenuated crypt cell loss.

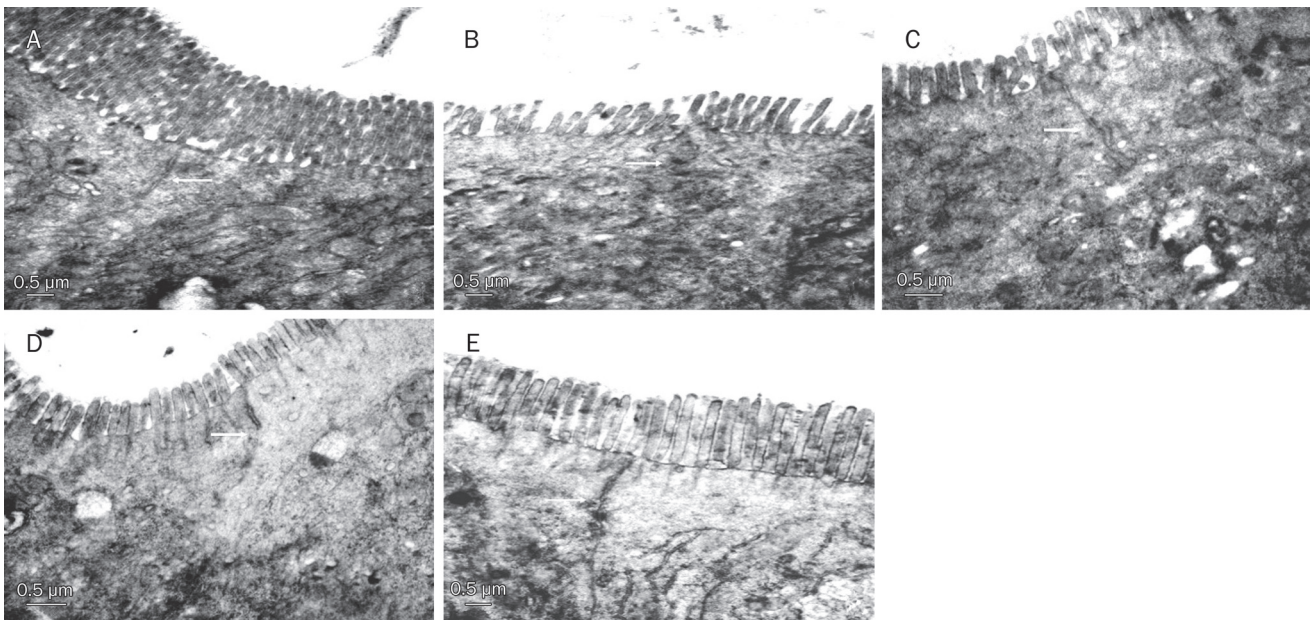

Figure 4. Effects of balsalazine on ileal mucosal epithelial structure under TEM (×20 000). A: normal group, B: DSS group, C, D, E: balsalazine 42, 141, and 423 mg/kg, respectively. No obvious microstructural damage was seen in the normal group. In the DSS group, reduced and shorter microvillus, irregular widening of the intercellular space, decurtated and broaden junctional complexes were seen. The balsalazine groups showed a much more regular and intensive microvillus, and ameliorated tight junctions dose-dependently.

210.99±6.55 μg/g, 166.30±7.14 μg/g, and 110.47±6.78 μg/g, respectively (P<0.05). These results indicate that balsalazine treatment protects the intestinal mucosal barrier in this colitis model.

The effect of balsalazine on intestinal mucosal oxidative damage in the DSS-induced colitis model

Compared with the control group, administration of DSS increased the amount of MDA and the activity of MPO in the colon, while the activity of both SOD and GSH-Px was decreased (P<0.01). All three dosages of balsalazine reversed the DSS-induced changes described above (P<0.05, Table 1). The correlation coefficients between DAI and either MDA, SOD or GSH-Px were 0.718, -0.837, and -0.839, respectively. The correlation coefficients between HI and either MDA, SOD, or GSH-Px were 0.818, -0.946, and -0.957, respectively. The correlation coefficients between the amount of EB and either MDA, SOD, or GSH-Px were 0.842, -0.960, and -0.900, respectively. These results indicated that balsalazine could scavenge free radicals and attenuate the oxidative damage. In addition, there was a strong correlation between oxidative indexes and the amount of EB.

The effect of Balsalazine on intestinal TNF-α and IFN-γ levels in DSS-induced colitis

Compared with the control group, DSS administration significantly increased the levels of intestinal TNF-α and IFN-γ (P<0.01). These data indicate significant intestinal inflammation in the DSS group. However, the levels of intestinal TNF-α and IFN-γ were much lower in the balsalazine treated groups

Table 1. Effects of balsalazine on the content of MDA and the activities of MPO, SOD, and GSH-Px in colon mucosa. n=9. Mean±SEM. $^bP<0.05$, $^cP<0.01$ *vs* DSS group by ANOVA test.

Group	Dose (mg/kg)	MPO (U/g)	MDA (nmol/mg)	SOD (U/mg)	GSH-Px (U/mg)
Normal	–	0.99±0.18^c	1.93±1.27^c	202.84±6.85^c	42.91±0.27^c
DSS	–	2.18±0.80	8.16±1.16	55.81±4.14	7.09±0.16
Balsalazine	42	1.83±0.34^b	4.78±0.93^b	100.28±10.32^b	18.75±0.24^b
	141	1.66±0.20^b	3.25±1.62^b	111.96±7.15^b	21.82±0.19^b
	423	1.17±0.18^b	2.38±1.12^b	167.84±8.93^b	34.73±0.44^b

The mice were sacrificed after 7 days of 5% (*w/v*) DSS administration. The dissected colon were excised, removed all fat and mesenteries, and subsequently homogenized in physiologic saline, stored at -20 ºC temporarily for the detections of the content of MDA and the activities of MPO, SOD, and GSH-Px as described by kits.

Table 2. Effects of balsalazine on the levels of TNF-α and IFN-γ in small intestine. n=9. Mean±SEM. $^bP<0.05$, $^cP<0.01$ *vs* DSS group by ANOVA test.

Group	Dose (mg/kg)	TNF-α (pg/g)	IFN-γ (pg/g)
Normal	–	90.50±7.62^c	88.38±5.93^c
DSS	–	177.25±17.82	199±15.36
Balsalazine	423	125.5±13.59^b	119.38±13.93^b

The mice were sacrificed after 7 days of 5% (*w/v*) DSS administration. The dissected small intestine were excised, removed all fat and mesenteries, and subsequently homogenized in physiologic saline, stored at -20 ºC temporarily. The contents of TNF-α and IFN-γ were determined as indicated in the ELISA kits.

than that in the DSS group ($P<0.05$, Table 2). The correlation coefficients between the amount of EB and the levels of intestinal TNF-α or IFN-γ were 0.887 and 0.948, respectively. These results indicate that balsalazine reduces the level of proinflammatory cytokines and attenuates the intestinal inflammation.

Discussion

In the present study, the treatment of colitis mice with balsalazine resulted in an obvious anti-colitis effect. Physiologically, balsalazine attenuated or prevented bloody stool, diarrhea, and weight loss. In addition, the administration of balsalazine decreased both the DAI and the HI score and lowered MPO activity in the DSS-induced model of colitis. The effect of balsalazine on intestinal mucosal permeability is usually reflected by changes in intestinal function and structure. An increase in the amount of EB permeating into the intestinal wall, a disruption in the epithelial cell borders, and damage to the intestinal epithelial cells and intercellular tight junctions all indicated that intestinal mucosal permeability was increased in the DSS group[19, 23]. Administration of balsalazine significantly decreased the DSS-induced increase in intestinal mucosal permeability. Therefore, these results indicate that the beneficial effects of balsalazine on the intestinal mucosal barrier may lead to depression of the intestinal inflammatory pathology and subsequent attenuation of colonic inflammation. However, the underlying mechanisms of the intestinal mucosal permeability improved by balsalazine treatment have not been studied. Understanding these mechanisms will contribute to elucidating the roles of balsalazine in the pathogenesis of UC.

Oxygen free radicals (OFR) play an important role in the pathogenesis of UC[25] and have been shown to damage the intestinal mucosal barrier[26]. The loss of antioxidant defenses may severely compromise the inflamed mucosa and render it more susceptible to the oxygen-induced injury and thereby hinder the recovery of the mucosa and the return of the integrity of the epithelial cell layer. Thus, it increased the intestinal mucosal permeability[27, 28], while antioxidants treatment is beneficial to ameliorate intestinal mucosa permeability[29–34]. In the present study, balsalazine attenuated intestinal oxidative damage markedly by decreasing MDA content, along with increasing SOD and GSH-Px activity. Thus, considering the strong correlation between oxidative indexes and EB, we presume that the anti-oxidation effect of balsalazine might contribute to the improvement of the intestinal mucosal permeability.

IFN-γ and TNF-α are considered the most important cytokines in the pathogenesis of UC. The interaction between IFN-γ, TNF-α, and the mucosal immune system may lead to the disruption of the epithelial barrier. TNF-α can also stimulate the synthesis of OFR and other inflammatory mediators and thus amplify the disruption of epithelial barrier[35]. Furthermore, apoptosis of ileal enterocytes can be prevented by both anti-TNF strategies and in TNFR-1$^{-/-}$ animals[36, 37]. In our study, balsalazine decreased the levels of intestinal IFN-γ and TNF-α. These results are similar to the results described by Fiorucci *et al*[38]. Furthermore, Kim *et al* showed that 5-ASA attenuated TNF-dependent NF-κB activation, which may be critical during intestinal inflammation[39]. Considering these results, and the strong correlation between the intestinal levels of IFN-γ and TNF-α and the amount of EB, we hypothesize that balsalazine protects the intestinal mucosal barrier partly by inhibiting the proinflammatory cytokines.

In conclusion, the beneficial anti-colitis effect of balsalazine treatment was due to its ability to restore both the structure and function of the intestinal mucosal barrier. The mechanisms by which balsalazine restored the integrity of the

intestinal mucosal barrier are partially associated with the antioxidative and anti-inflammatory effect of balsalazine. However, these mechanisms require further investigation. More advanced methods, including molecular biological techniques for detecting changes in the intestinal mucosal barrier-associated proteins, will be applied in future studies.

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Author contributions

Xiao-chang LIU designed and carried out the study. Qiao MEI and Jian-ming XU designed the study. Jing HU carried out part of study together with Xiao-chang LIU.

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