

Ameliorative effects of sodium ferulate on experimental colitis and their mechanisms in rats

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Abstract

AIM: To investigate the ameliorative effects of sodium ferulate (SF) on acetic acid-induced colitis and their mechanisms in rats.

METHODS: The colitis model of Sprague-Dawley rats was induced by intracolonic enema with 8 % (V/V) of acetic acid. The experimental animals were randomly divided into model control, 5-aminosalicylic acid therapy group and three dose of SF therapy groups. The 5 groups were treated intracolonic with normal saline, 5-aminosalicylic acid (100 mg·kg⁻¹), and SF at the doses of 200, 400 and 800 mg·kg⁻¹ respectively and daily (8: 00 am) for 7 days 24 h following the induction of colitis. A normal control group of rats clystered with normal saline instead of acetic acid was also included in the study. Pathological changes of the colonic mucosa were evaluated by the colon mucosa damage index (CMDI) and the histopathological score (HS). The insulted colonic mucosa was sampled for a variety of determinations at the end of experiment when the animals were sacrificed by decapitation. Colonic activities of myeloperoxidase (MPO) and superoxide dismutase (SOD), and levels of malondialdehyde (MDA) and nitric oxide (NO) were assayed with ultraviolet spectrophotometry. Colonic contents of prostaglandin E₂ (PGE₂) and thromboxane B₂ (TXB₂) were determined by radioimmunoassay. The expressions of inducible nitric oxide synthase (iNOS), cyclo-oxygenase-2 (COX-2) and nuclear factor kappa B (NF-κB) p65 proteins in the colonic tissue were detected with immunohistochemistry.

RESULTS: Enhanced colonic mucosal injury, inflammatory response and oxidative stress were observed in the animals clystered with acetic acid, which manifested as the significant increase of CMDI, HS, MPO activities, MDA and NO levels, PGE₂ and TXB₂ contents, as well as the expressions of iNOS, COX-2 and NF-κB p65 proteins in the colonic mucosa, although the colonic SOD activity was significantly decreased compared with the normal control (CMDI: 2.9±0.6 vs 0.0±0.0; HS: 4.3±0.9 vs 0.7±1.1; MPO: 98.1±26.9 vs 24.8±11.5; MDA: 57.53±12.36 vs 9.21±3.85; NO: 0.331±0.092 vs 0.176±0.045; PGE₂: 186.2±96.2 vs 42.8±32.8; TXB₂: 34.26±13.51 vs 8.83±3.75; iNOS: 0.365±0.026 vs 0.053±0.015; COX-2: 0.296±0.028 vs 0.034±0.013; NF-κB p65: 0.314±0.026 vs 0.039±0.012; SOD: 28.33±1.17 vs 36.14±1.91; *P*<0.01).

However, these parameters were found to be significantly ameliorated in rats treated locally with SF at the given dose protocols, especially at 400 mg·kg⁻¹ and 800 mg·kg⁻¹ doses (CMDI: 1.8±0.8, 1.6±0.9; HS: 3.3±0.9, 3.1±1.0; MPO: 63.8±30.5, 36.2±14.2; MDA: 41.84±10.62, 37.34±8.58; NO: 0.247±0.042; 0.216±0.033; PGE₂: 77.2±26.9, 58.4±23.9; TXB₂: 18.07±14.83; 15.52±8.62; iNOS: 0.175±0.018, 0.106±0.019; COX-2: 0.064±0.018, 0.056±0.014; NF-κB p65: 0.215±0.019, 0.189±0.016; SOD: 32.15±4.26, 33.24±3.69; *P*<0.05-0.01). Moreover, a therapeutic dose protocol of 800 mg·kg⁻¹ SF was observed as effective as 100 mg·kg⁻¹ of 5-ASA in the amelioration of colonic mucosal injury as evaluated by CMDI and HS.

CONCLUSION: Administration of SF intracolonic may have significant therapeutic effects on the rat model of colitis induced by acetic acid enema, which was probably due to the mechanism of antioxidation, inhibition of arachidonic acid metabolism and NF-κB expression.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a collection of chronic idiopathic inflammatory disorders of the intestine and/or colon. Although the pathophysiology of these disorders is not known with certainty, a growing body of experimental and clinical data suggested that this kind of chronic gut inflammation might be resulted from a maladjusted immune response to certain bacterial antigens^[1-3]. This uncontrolled immune system activation results in a sustained overproduction of cytokines, eicosanoids, oxygen and nitrogen reactive species, which has been thought to be the major causative factors leading to intestinal and/or colonic injury and dysfunction in inflammatory bowel disease.

Based on this hypothesis, current therapeutic strategy for IBD has therefore focused on the use of anti-inflammatory agents, aminosalicylates and corticosteroids^[1,4-9]. However, some notable limitations are found existing in the management of IBD patients and meanwhile some patients are refractory to these therapies. Long term use of glucocorticoids, for example, although effective in suppressing active inflammation, was associated with high rates of relapse and unacceptable toxicity^[10-12]. On the other hand, 6-mercaptopurine and its prodrug azathioprine are effective in inducing and maintaining remission, however, a significant number of patients were resistant or intolerant to thiopurines^[10]. All these suggest that seeking for more preferable drugs harboring better therapeutic results and less side-effects for the treatment of patients with IBD is urgently necessary.

Sodium ferulate (SF) is one of the effective components of *Radix Angelica sinensis*. Many researches have revealed that

SF possesses plenty of beneficial pharmacological effects such as inhibiting the production of inflammatory mediators, improving endothelial function, relieving oxidative stress and safe in use^[13-18]. We presumed that SF might be a possible candidate to be used for the treatment of IBD, which to our knowledge has not been investigated in previous studies. The present study was therefore conducted to confirm our hypothesis with an effort to demonstrate the therapeutic effects of sodium ferulate on experimental colitis.

MATERIALS AND METHODS

Animals

Healthy Sprague-Dawley rats of both sexes, weighing 250±30 g, from the Animal Center, Academy of Hubei preventive medical sciences were employed in the present study. The animals were fed on standard rat chow, allowed access to tap water *ad libitum* and acclimatized to the surroundings for one week prior to the experiments. The study protocol was in accordance with the guideline for animal research and was approved by the Ethical and Research Committee of the hospital.

Reagents

Acetic acid was obtained from Wuhan Chemical Corp. SF injection was provided by Pharmaceutical Factory of Zhongnan Hospital, Wuhan University (Lot. 20011219). 5-aminosalicylic acid (5-ASA) was purchased from Guoyi Pharmaceutical Ltd (Lot. 20029477). Polyclonal rabbit anti-rat-iNOS, COX-2 and NF-κB p65 were from Santa Cruz Company. S-P kits were obtained from Beijing Zhongshan Biological Technology CO Ltd. PGE₂ and TXB₂ radioimmunoassay kits were provided by Radioimmunity Institute of PLA General Hospital. MPO, SOD, MDA, NO detection kits were purchased from Nanjing Jiancheng Bioengineering Institute. Other reagents used in the present study were all of analytical grade.

Experimental protocol

The preparation of rat model of colitis was described in the literature^[19,20]. Briefly, after rats were anesthetized with ether, a flexible plastic catheter with an outer diameter of 2 mm were inserted rectally into the colon with the aim to place the catheter tip at 8 cm proximal to the anus. Twenty seconds following enema with 2 ml of 8% (V/V) acetic acid through the catheter, colon lumen was rinsed twice with 5 ml of normal saline. The experimental animals were randomly divided into normal control, model control, 5-aminosalicylic acid therapy and three dose SF therapy groups, and treated intracolonicly with normal saline, normal saline, 5-aminosalicylic acid (100 mg·kg⁻¹), and SF 200, 400 and 800 mg·kg⁻¹ respectively and daily (8:00 am) for 7 days 24 h following the induction of colitis. Then the colon mucosa damage index (CMDI) and the histopathological score (HS) were evaluated and the colon mucosa was sampled for a variety of determinations and observations after the animals were sacrificed by decapitation.

Assessment of CMDI and HS

The colon segment taken from 10 cm proximal to anus of the sacrificed rats was excised longitudinally, rinsed by saline buffer and fixed on a wax block. CMDI and HS were assessed respectively by two independent researchers. The scoring formula for assessment of CMDI was reported in previous literature^[20]. That is, 0: normal mucosa, no damage on the mucosal surface; 1: mild hyperemia and edema, no erosion or ulcer on the mucosal surface; 2: moderate hyperemia and edema, erosion appearing on the mucosal surface; 3: severe hyperemia and edema, necrosis and ulcer appearing on the mucosal surface with the major ulcerative area extending less

than 1 cm; 4: severe hyperemia and edema, necrosis and ulcer appearing on the mucosal surface with the major ulcerative area extending more than 1 cm. For histopathological examination, the colonic samples were fixed in 4% neutral buffered paraformaldehyde overnight and 4-μm thick sections were routinely prepared and stained with haematoxylin and eosin. The colonic pathological changes were observed and evaluated by two independent researchers with a modified histopathological score formula^[19] as: (1) Infiltration of acute inflammatory cells: 0, without; 1, mild; 2, severe. (2) Infiltration of chronic inflammatory cells: 0, without; 1, mild; 2, severe. (3) Fibrin deposition: 0, negative; 1, positive. (4) Submucosal edema: 0, without; 1, focal; 2, diffuse. (5) Necrosis of epithelial cells: 0, without; 1, focal; 2, diffuse. (6) Mucosal ulcer: 0, negative; 1, positive.

Determination of colonic MPO, SOD activities and MDA, NO contents

For determination of colonic MPO activity, the sampled tissues were homogenized (50 g·L⁻¹) in 50 mmol·L⁻¹ ice-cold potassium phosphate buffer (pH 6.0) containing 0.5% of hexadecyltrimethylammonium bromide. The homogenate was frozen and thawed thrice, then centrifuged at 4 000 rpm for 20 min at 4 °C. The MPO activity in the supernatant was measured by the assay kit according to its provider's instructions. The colonic samples for the determination of SOD activity, MDA and NO contents were homogenized in ice-cold PBS (pH 7.4) and centrifuged at 3 000 rpm for 10 min at 4 °C. The resulting supernatant was stored at 20 °C until analysis with corresponding assay kits according to the manufacturer's guide.

Measurement of colonic PGE₂ and TXB₂ level

Colonic specimens used for the assay of arachidonic acid metabolites were prepared according to the protocol by Raab *et al*^[21] and Taniguchi *et al*^[22], and their levels of PGE₂ and TXB₂ were measured by using the corresponding radioimmunoassay kits following the manufacturer's instructions.

Detection of colonic iNOS, COX-2 and NF-κB p65 expression

The immunohistochemical methods for formalin-fixed and paraffin-embedded sections were described previously^[23]. The determinations of colonic iNOS, COX-2 and NF-κB p65 expression were all performed with S-P technique following the recommendations of assay kit providers. Briefly, polyclonal rabbit anti-rat-iNOS, COX-2 and NF-κB p65 were diluted with PBS to 1:150, 1:120, 1:200 respectively and used as the primary antibody in the corresponding detections. For each of these determinations, dewaxed sections were incubated first with the primary antibody overnight at 4 °C after antigen retrieval. The binding of antibodies to their antigenic sites in the tissue sections was further amplified with biotinylated goat anti-rabbit antibody and followed by reaction with 3,3'-diaminobenzidine. Sections prepared by substituting PBS for the primary antibody served as the negative control. iNOS, COX-2 or NF-κB p65 negatively expressed cells manifested as blue-stained nuclei while the positive cells as brown or dark brown cytoplasm and/or cell membrane. Expressions of these target proteins were semiquantitated respectively with automatic image analyzer (Nikon, Japan) and HPIAS-2000 image analyzing program, in which the average value of positive cell's absorbance (A) in ten randomly selected high power fields (400×) for each section was used for the comparison of the target protein expressions.

Statistical analysis

Experimental results were expressed as $\bar{x} \pm s$. Statistical comparisons between groups were made by ANOVA followed

by Student's *t* test. *P* value less than 0.05 was considered statistically significant.

RESULTS

Inflammatory changes of the colonic mucosa

CMDI, HS and MPO activities were the main parameters for evaluating the degree of colonic inflammation in inflammatory bowel disease. Compared with the normal control, these parameters were all significantly increased in the colonic mucosa of the model control induced by acetic acid ($P < 0.01$). After the model rats were treated with SF (400, 800 mg·kg⁻¹) or 5-ASA (100 mg·kg⁻¹) as described in the experimental protocol, these elevated parameters were significantly ameliorated ($P < 0.05$ or 0.01) as shown in Table 1, in which a therapeutic dose protocol of 800 mg·kg⁻¹ SF was observed as effective as 100 mg·kg⁻¹ 5-ASA in the treatment of this rat model of colitis.

Table 1 Effects of sodium ferulate on CMDI, HS and MPO activity in the colon tissue of acetic acid-induced rats colitis ($n=8$, $\bar{x} \pm s$)

Group	Dose (mg·kg ⁻¹)	CMDI	HS	MPO (U·g ⁻¹)
Normal	-	0.0±0.0	0.7±1.1	24.8±11.5
Model	-	2.9±0.6 ^d	4.3±0.9 ^d	98.1±26.9 ^d
5-ASA	100	1.6±0.9 ^b	3.4±0.5 ^a	29.6±10.8 ^b
SF	200	2.0±0.8 ^a	3.6±1.2	79.5±38.4
SF	400	1.8±0.8 ^b	3.3±0.9 ^a	63.8±30.5 ^a
SF	800	1.6±0.9 ^b	3.1±1.0 ^a	36.2±14.2 ^b

^a $P < 0.05$, ^b $P < 0.01$ vs model group; ^d $P < 0.01$ vs normal group.

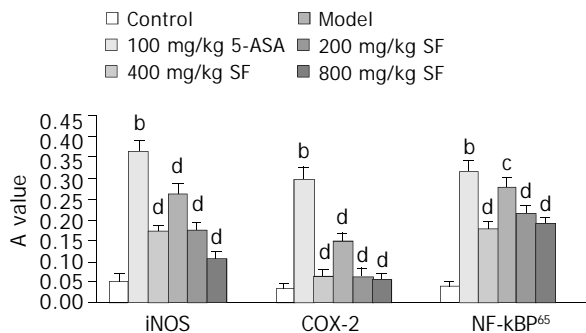


Figure 1 Effects of SF on the expressions of iNOS, COX-2 and NF- κ B P65 in the colonic tissue of rats colitis induced by acetic acid ($n=8$, $\bar{x} \pm s$). ^b $P < 0.01$, vs control group; ^c $P < 0.05$, ^d $P < 0.01$, vs model group.

Colonic oxidative alterations

Prominent oxidative stress in colonic mucosa was induced by enema of acetic acid in the model controls as shown by the significant elevation of colonic MDA and NO contents and decrease of colonic SOD activities (Table 2). These oxidative abnormalities in colonic mucosa were obviously ameliorated by the treatment with SF at the dose protocols of 200, 400, 800 mg·kg⁻¹, which manifested as the significant reduction of colonic MDA content and the increase of SOD activity compared with the model control rats ($P < 0.05-0.01$). Furthermore, a significant improvement for the elevated colonic NO content was also noted in animals treated with SF at the dose protocol of 400 or 800 mg·kg⁻¹ ($P < 0.05-0.01$) as shown in Table 2.

Effects of SF on the colonic PGE₂, TXB₂ levels

After the rats were insulted by enema of acetic acid, their

colonic contents of PGE₂ and TXB₂ were significantly increased compared with the normal control ($P < 0.01$). A significant amelioration of PGE₂ contents was observed in animals treated with SF at all dose protocols ($P < 0.05-0.01$). However, significant reduction of colonic TXB₂ contents compared with the model control was only seen in rats treated with SF at the dose protocol of 400 and 800 mg·kg⁻¹ ($P < 0.05-0.01$) as shown in Table 3.

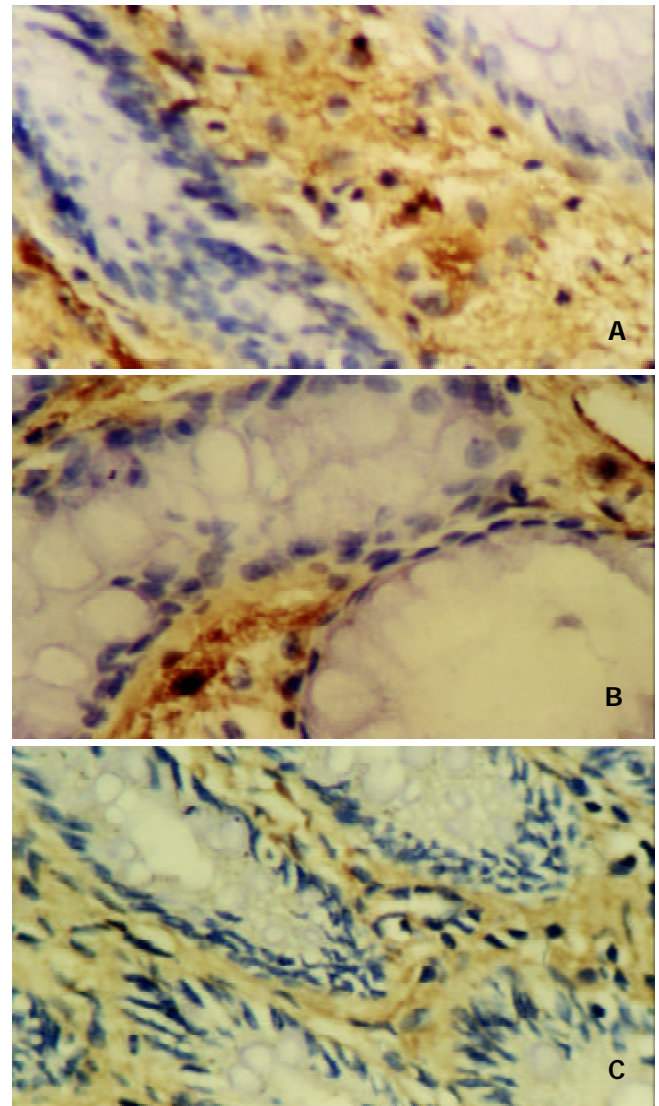


Figure 2 Expressions of iNOS (A), COX-2 (B) and NF- κ B p65 (C) in the inflammatory areas of colonic tissue from rats with colitis induced by acetic acid enema, respectively. The strongly positive signal were found. SP stain $\times 400$.

Table 2 Effects of sodium ferulate on SOD activity, MDA and NO contents in the colon tissue of acetic acid-induced rats colitis ($n=8$, $\bar{x} \pm s$)

Group	Dose (mg·kg ⁻¹)	SOD (kU·g ⁻¹)	MDA (nmol·g ⁻¹)	NO (nmol·g ⁻¹)
Normal	-	36.14±1.91	9.21±3.85	176±45
Model	-	28.33±1.17 ^d	57.53±12.36 ^d	331±92 ^d
5-ASA	100	32.74±3.52 ^b	31.85±11.72 ^b	244±51 ^a
SF	200	31.63±3.83 ^a	43.25±13.47 ^a	256±54
SF	400	32.15±4.26 ^a	41.84±10.62 ^a	247±42 ^a
SF	800	33.24±3.69 ^b	37.34±8.58 ^b	216±33 ^b

^a $P < 0.05$, ^b $P < 0.01$, vs model group; ^d $P < 0.01$ vs normal group.

Table 3 Effects of sodium ferulate on the content of PGE₂ and TXB₂ in the colon tissue of acetic acid-induced rats colitis (n=8, $\bar{x}\pm s$)

Group	Dose (mg·kg ⁻¹)	PGE ₂ (ng·g ⁻¹)	TXB ₂ (ng·g ⁻¹)
Normal	-	42.8±32.8	8.83±3.75
Model	-	186.2±96.2 ^d	34.26±13.51 ^d
5-ASA	100	67.0±37.7 ^b	18.53±14.26 ^a
SF	200	90.7±52.3 ^a	23.21±12.46
SF	400	77.2±26.9 ^b	18.07±14.83 ^a
SF	800	58.4±23.9 ^b	15.52±8.62 ^b

^a $P < 0.05$, ^b $P < 0.01$ vs model group; ^d $P < 0.01$ vs normal group.

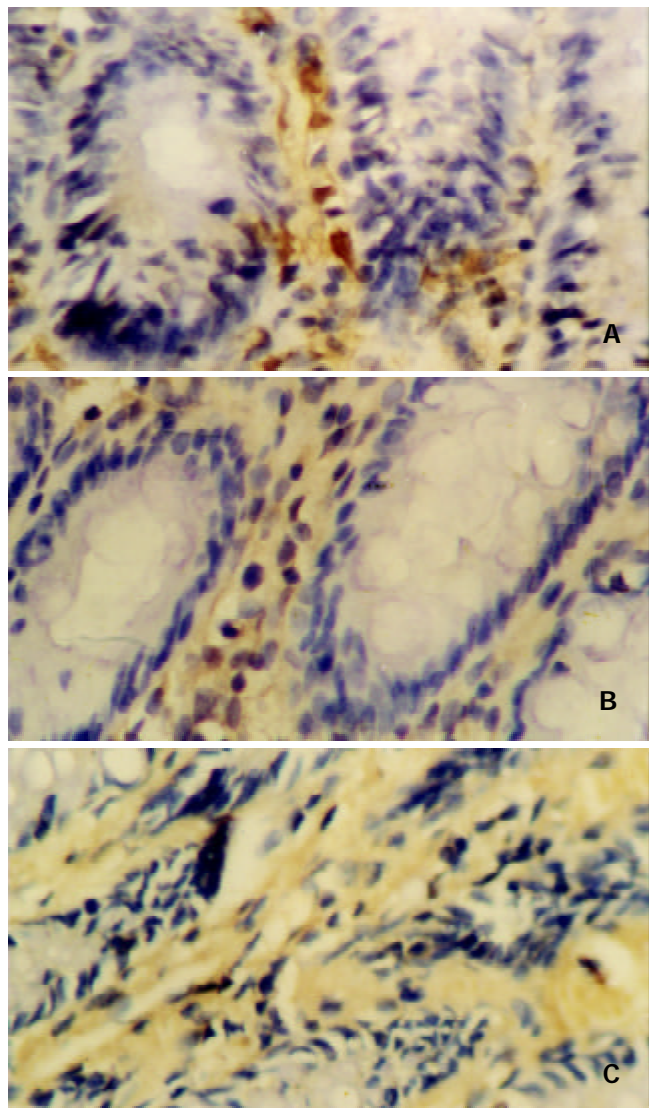


Figure 3 Expressions of iNOS (A), COX-2 (B) and NF-κB p65 (C) were significantly decreased in the colonic tissue from rats with colitis induced by acetic acid enema after treated with 800 mg·kg⁻¹ ASP, respectively. The weakly positive staining could be found. SP stain ×400.

SF reduces the expression of colonic NOS, COX-2 and NF-κB p65

As shown in Figure 1, 2 and 3, the expressions of colonic NOS, COX-2 and NF-κB p65 proteins were observed to be significantly increased in animals clastered with acetic acid compared with the normal controls ($P < 0.01$), which were ameliorated significantly in a dose-dependent manner as the animals treated with SF at the given doses ($P < 0.05-0.01$).

DISCUSSION

Induction of colitis by acetic acid (AA) in rats is the classical method to produce an experimental model of inflammatory bowel disease (IBD). Several major causative factors in the initiation of human IBD such as exorbitant oxidative stress, enhanced vasopermeability, prolonged neutrophils infiltration and increased production of inflammatory mediators were all involved in the induction of this animal model^[19,20,24]. It is therefore acknowledged nowadays that this experimental model is suitable for the investigation of IBD pathogenesis and evaluation the therapeutic agents of this disease.

In the present study, we employed this rat model to make sure whether or not the ameliorative effects of SF on the experimental colitis existed as assessed by CMDI, HS and MPO activities that were usually regarded as the main parameters to evaluate the severity of colonic inflammation in inflammatory bowel disease^[19,20]. Compared with the normal control, these parameters were all significantly increased in the colonic mucosa of the model control animals induced by acetic acid ($P < 0.01$). However, these elevated parameters were significantly ameliorated ($P < 0.05$ or 0.01) as shown in Table 1 after the model rats were treated with SF (400, 800 mg·kg⁻¹) or 5-ASA (100 mg·kg⁻¹) as described in the experimental protocol, in which a therapeutic dose protocol of 800 mg·kg⁻¹ SF was observed as effective as 100 mg·kg⁻¹ 5-ASA in the treatment of this rat model of colitis. The results confirmed our previous speculation and strongly suggested that SF might serve as an alternative modality for the treatment of inflammatory bowel disease.

Although the pathogenesis is not known with certainty, some hypotheses have been proposed as the major causative factors contributing to inflammatory bowel disease. A large number of studies have revealed that the increase of oxidative stress and iNOS activity was a notable feature of IBD, which resulted in a pathological cascade of free radical reactions and further yielding more oxidative free radicals such as peroxynitrite (ONOO⁻) to impair the structure and function of cells^[2,25-27]. Meanwhile, excessive NO could dilate vasculature and enhance vasopermeability, as well as inactivate the activity of antioxidases such as SOD, CAT, and GSH-Px by means of reacting with hydrosulfide group (-SH) in the enzymes. Some oxidants have been known to modulate the expression of a variety of genes that are involved in the immune and inflammatory responses, which lead to the apoptosis of intestinal epithelial cells, cascades of inflammatory response and the disruption of integrity and function of the intestinal mucosa^[28-30].

Abnormal metabolism of arachidonic acid is another vital factor in the IBD pathogenesis. As the crucial synthetase in the arachidonic acid metabolic pathway, COX-2 could be activated to produce excessive PGE₂ and TXB₂, two important inflammatory mediators, in the inflammatory bowel disease, which contribute to the bowel hyperemia, edema and even dysfunction. In addition, TXB₂ could also induce platelet aggregation, vasoconstriction and microthrombosis, aggravating the inflammatory reaction^[31-35]. Administration of either COX-2 or thromboxane synthase inhibitors has been shown to be useful for the treatment of IBD^[32-34].

Recently, plenty of literatures reported that NF-κB played a critical role in the early transcriptional changes of various immunoregulatory genes^[28,36], whose activation and increased expression have been demonstrated to be involved in the pathogenesis of inflammatory bowel disease^[37]. Activated NF-κB could promote the production of various inflammatory factors, chemotaxins, cytokines, and adhesive factors, and the expression of iNOS and COX-2, which interact further with each other and lead to an uncontrollable cascade of inflammatory response^[38-41]. NF-κB could also activate anti-apoptotic genes

including TNF receptor related genes (TRAF1, TRAF2), Bcl-2 homologs (A1/Bfl-1, IEX-1L), and repress the apoptosis of some inflammatory cells such as neutrophils and activated macrophages, thereby elongating and worsening tissue inflammatory injury^[42-45]. In the NF- κ B family, p65 is the major functional subunit, and an antisense oligonucleotide to NF- κ B p65 has been shown to ameliorate inflammation even after induction of colitis^[1].

To elucidate the mechanisms underlying the therapeutic effects of SF on this rat model of colitis, we observed simultaneously the changes of these oxidative and inflammatory variables mentioned above in the colonic tissue after SF therapy. The results revealed that the SOD activity, MDA and NO levels, PGE₂ and TXB₂ contents, as well as the expressions of iNOS, COX-2 and NF- κ B p65 proteins in the colonic mucosa were significantly ameliorated in the rats treated locally with SF at the given dose protocols, especially at the 400 mg·kg⁻¹ and 800 mg·kg⁻¹ doses, compared with that in model control animals ($P < 0.05-0.01$). Taken together, these observations suggest the anti-oxidative stress and anti-inflammatory response are the fundamentals of SF action in the therapy of IBD, although the ameliorating effects of SF might be involved in multiple mechanisms.

In summary, the results of this study show that intracolonic treatment with SF at 400 mg·kg⁻¹ and 800 mg·kg⁻¹ dose protocols can ameliorate the pathological changes of experimental colitis in rats, which suggests that SF may serve as an effective therapeutic agent for the treatment of IBD.

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