

*Full Length Research Paper*

# Protective effect of shikimic acid on acetic acid induced colitis in rats

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The aim of the present study was to investigate the effect of shikimic acid (SA), an active component extracted from Chinese star anise, on acetic acid (AA)-induced colitis in rats. Male Sprague-Dawley rats were divided into five groups: control, AA, AA+sulfasalazine (SASP; 500 mg/kg), and AA + SA at doses of 200 and 400 mg/kg. Colitis was induced in all groups except the control group by AA. The AA group received saline; treatment groups received SA or SASP, respectively. The activities of myeloperoxidase (MPO), superoxide dismutase (SOD) and inducible nitric oxide synthase (iNOS), and the levels of malondialdehyde (MDA) and nitric oxide (NO) were measured from colonic samples. In AA-induced model, SA significantly ameliorated colon damage, inhibited the increase of MPO and iNOS activities, depressed MDA and NO levels, and enhanced SOD activity. The findings of this study demonstrate the protective effect of SA in AA-induced colitis, probably due to an antioxidant action.

**Key words:** Shikimic acid, ulcerative colitis, acetic acid, antioxidation.

## INTRODUCTION

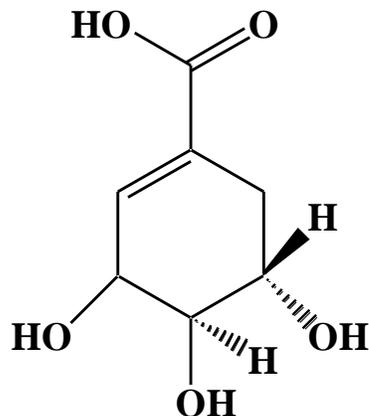
Ulcerative colitis (UC) is a representative type of inflammatory bowel disease. Although the pathogenesis of UC still remains unknown, clinical observations have found that increased platelet number and platelet activation are notable characteristics of UC (Pamuk et al., 2006; Kayo et al., 2006; Kapsoritakis et al., 2001). Platelets can activate neutrophils and affect inflammatory status (Bazzoni et al., 1991). These activated neutrophils produce and release several toxic reagents, such as reactive oxygen species and protease, which can cause tissue damage. Among them, reactive oxygen species (ROS), such as the superoxide anion, hydroxyl radicals,

and nitric oxide (NO), play an important role (Simmonds and Rampton, 1993). Activated neutrophils are major components of active lesions in UC. Activated platelets and platelet-neutrophil cross-talking in UC have evoked great interest in researchers (Collins et al., 1997; Suzuki et al., 2001; Danese et al., 2004). More and more studies suggested that the role of platelets might represent a previously unrecognized component of UC pathogenesis, and that antiplatelet drugs may provide new therapeutic possibilities in the management of UC (Mannaioni et al., 1997; Collins and Rampton, 1995; Xu et al., 2002; Tyrrell et al., 1999).

Shikimic acid (3, 4, 5-trihydroxy-1-cyclohexene-1-carboxylic acid; SA) is isolated from Chinese star anise (*Illicium verum* Hook. fil.). Chinese star anise has long been used in Traditional Chinese Medicine, and its fruit is listed in Chinese Pharmacopoeia (2010 edition) and used for treating vomiting, stomach aches, insomnia, skin inflammation and rheumatic pain (Itoigawa et al., 2004). Previous researches indicated that SA had analgesic and anti-inflammatory effects, inhibited platelet aggregation and blood coagulation, and antagonized focal cerebral

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**Abbreviations:** SA, Shikimic acid; AA, acetic acid; SASP, sulfasalazine; UC, ulcerative colitis; CMDI, colon mucosal damage index; MPO, myeloperoxidase; MDA, malondialdehyde; SOD, superoxide dismutase; NO, nitric oxide; iNOS, inducible nitric oxide synthase.



**Figure 1.** Chemical structure of shikimic acid.

ischemia injury (Fang et al., 1989; Ma et al., 1999; Ma et al., 2000). The present study determined whether SA exerts protective effects on experimental UC induced by acetic acid (AA) in rats, and further explored whether its mechanism is associated with an antioxidant action.

## MATERIALS AND METHODS

### Drugs and reagents

SA (purity > 98%; isolated from Chinese star anise and provided by the Department of Phytochemistry, Beijing University of Traditional Chinese Medicine), sulfasalazine (SASP; from Shanghai Sunve Pharmaceutical, China; 0.25 g/tablet), and MPO, malondialdehyde (MDA), superoxide dismutase (SOD), NO, and inducible nitric oxide synthase (iNOS) kits from Nanjing Jiancheng Biochemical Engineering, China. The chemical structure of SA is shown in Figure 1.

### Animals

Male Sprague-Dawley rats (220 to 240 g) were obtained from Beijing Vital River Laboratory Animals, China. The animals were housed under a 12-h light–dark cycle at a constant ambient temperature (22 to 25°C), with normal rat chow and water *ad libitum*. All rats were deprived of food for 48 h prior to the experimental procedure, but were allowed free access to tap water. The animals used in this study were handled and treated in accordance with the Animal Management Rules of the Health Ministry of the People's Republic of China (Document No. 55, 2001) for the experimental care and use of animals.

### Induction of colitis and experiment protocols

After 48 h of fasting, the rats were anesthetized with intraperitoneal pentobarbital sodium (35 mg/kg) before the induction of colitis. A polyethylene catheter (external diameter of 2 mm) was inserted into the anus and the tip was advanced to 8 cm proximal to the anus margin, through which 2.0 ml of 6% (v/v) AA in 0.9% saline was administered. The catheter was remained in the colon for 20 s, and before its withdrawal, 5.0 ml of saline was injected in order to

ensure that the AA spread completely within the colon. Control animals received only a vehicle (saline) enema (Millar et al., 1996). The rats were divided randomly into five groups: (1) control—no UC induced (p.o., saline,  $n = 10$ ), (2) AA (p. o., saline,  $n = 9$ ), (3) AA + SASP (p. o., 500 mg/kg SASP,  $n = 10$ ), (4) AA + 200 mg/kg SA (p.o.,  $n = 10$ ), and (5) AA + 400 mg/kg SA (p. o.,  $n = 9$ ). The treatments were given twice daily for 6 days starting 24 h after the induction of colitis.

### Evaluation of colon macroscopic damage

All the rats in each group were killed on the 7th day, and the entire colon was removed, opened longitudinally, and rinsed with saline solution. The length (cm) and weight (mg) of the colon were measured, from which weight/length (mg/cm) ratios obtained. The colon was examined visually immediately and the damage [colon mucosal damage index (CMDI)] in AA-induced colitis was scored on the following scale of 0 to 4 according to the criteria described by Millar et al (Millar et al., 1996): 0, no change; 1, mucosal erythema alone; 2, mild mucosal edema, slight bleeding, or small erosions; 3, moderate edema, slight bleeding, ulcers, or erosions; 4, severe ulceration, erosions, edema, and tissue necrosis. Each colon was observed and evaluated by two independent observers. After macroscopic examination, the intestinal segment from each rat was stored at -70°C for subsequent measurement of biochemical parameters.

### Tissue preparation for MDA, NO, SOD, and iNOS determination

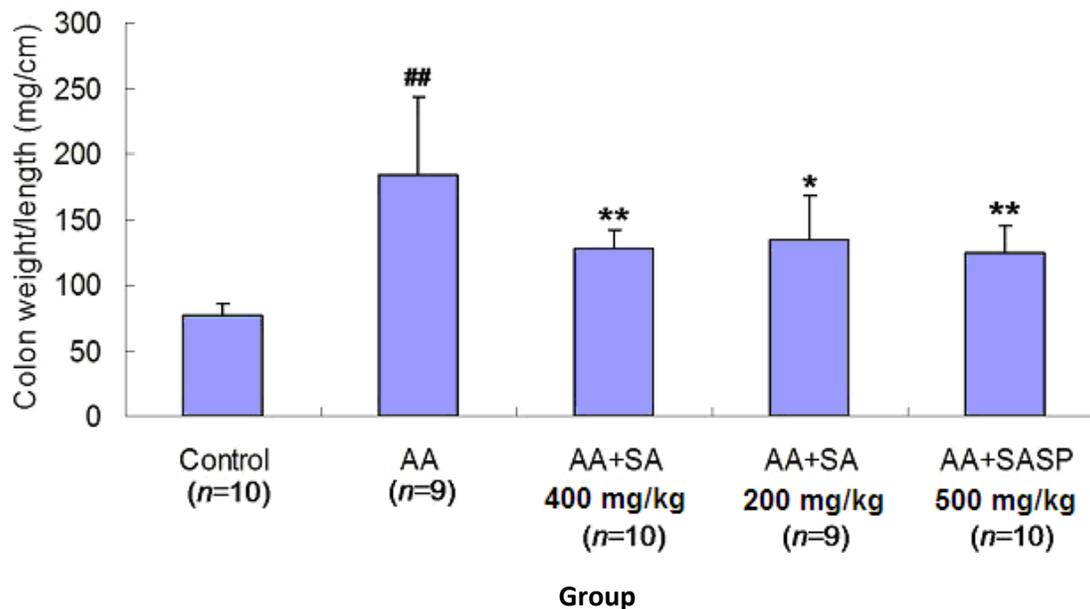
Colonic samples obtained from each group were homogenized with 10 volumes of ice-cold 50 mM phosphate buffer (pH 7.4) and centrifuged at 4000 g for 30 min at 4°C, and the protein concentration of the supernatant was determined according to the Bradford method. This supernatant was used to assay MDA and NO levels, and SOD and iNOS activities.

### Measurement of MPO activity

MPO activity of colonic samples was determined as an index of the accumulation of neutrophils. The tissue samples (0.5 g) were homogenized in 10 volumes of ice-cold phosphate buffer (50 mM  $K_2HPO_4$ , pH 6.0) containing hexadecyltrimethylammonium bromide [HETAB; 0.5% (w/v)]. The homogenate was centrifuged at 10000 g for 10 min at 4°C, and the supernatant was discarded. The pellet was then rehomogenized with an equivalent volume of 50 mM  $K_2HPO_4$  containing 0.5% (w/v) HETAB and 10 mM ethylenediaminetetraacetic acid (EDTA). MPO activity was assessed by measuring the hydrogen-peroxide-dependent oxidation of o-dianisidine dihydrochloride. One enzyme unit was defined as the amount of enzyme producing one absorbance change per minute at 460 nm and 37°C. Enzyme activity was calculated as U/g tissue (Bozkurt et al., 2003).

### Measurement of MDA Level

MDA levels in the colonic samples were determined by the method of Ohkawa et al. (1979). In brief, 0.1 ml of tissue homogenate, 500  $\mu$ l of thiobabutaric acid reagent (3.7 g/L in 0.25 M HCl), and 1.5 ml of 15% trichloroacetic acid (in 0.25 M HCl) were combined in a 10-ml screw-cap centrifuge tube, mixed, and heated for 40 min in boiling water. After cooling in an ice bath, 3 ml of n-butanol was added to the aforementioned solution, which was then mixed and centrifuged at 4000 g for 10 min, and the chromogen was extracted. The absorbance of the organic phase was determined



**Figure 2.** Effects of SA on colonic weight/length ratios. Each column represented as the mean $\pm$ SD. ## $P$ <0.01, compared to the control group; \* $P$ <0.05, \*\* $P$ <0.01, compared to the AA group. AA, Acetic acid; SA, shikimic acid; SASP, sulfasalazine.

spectrophotometrically at 532 nm. MDA levels are expressed as nmol/mg protein.

#### Measurement of SOD activity

SOD activity of colonic samples was measured according to the method of Fridovich (1983). This method employs xanthine and xanthine oxidase to generate superoxide radicals which can react with p-iodonitrotetrazolium violet (INT) to form a red formazan dye, the intensity of which was measured spectrophotometrically at 505 nm. The assay medium contained 0.01 M phosphate buffer, 3-cyclohexylamino-1-propanesulfonic acid (CAPS) buffer solution (50 mM CAPS and 0.94 mM EDTA) at pH 10.2, the substrate solution (0.05 mM xanthine and 0.025 mM INT) and 80  $\mu$ l of xanthine oxidase. SOD activity is expressed as U/mg protein.

#### Measurement of NO level

Levels of NO were determined with the Griess reagent according to the method described by Green et al. (1982). Briefly, 100  $\mu$ l of supernatant was mixed with an equal volume of fresh Griess reagent [0.1% (w/v) *N*-1-naphthylethylenediamine dihydrochloride and 1% (w/v) sulfanilamide in 2.5% (v/v) phosphoric acid], incubated for 10 min at room temperature, and then the absorbance of the mixture was determined spectrophotometrically at 540 nm.

#### Measurement of iNOS activity

iNOS activity was measured according to the method of Ryoyama et al. (1993), whereby L-arginine and molecular oxygen were catalyzed by NOS to generate NO. The rate of NO production by NOS in 1 min was determined with the Griess reaction. Briefly, 100  $\mu$ l of supernatant was mixed with 400  $\mu$ l of buffer solution (10 mM L-arginine, 0.2 mM nicotinamide adenine dinucleotide

phosphate, 0.1 mM dithiothreitol, and 1 mM EDTA) at pH 7.4 and incubated for 15 min at 37°C, after which 0.05 ml of 1 M HCl was added and the solution mixed. A 100- $\mu$ l aliquot of this solution was mixed with 100  $\mu$ l of fresh Griess reagent and then incubated for 10 min at room temperature. The absorbance of the final solution was determined spectrophotometrically at 540 nm. iNOS activity is expressed as U/mg protein.

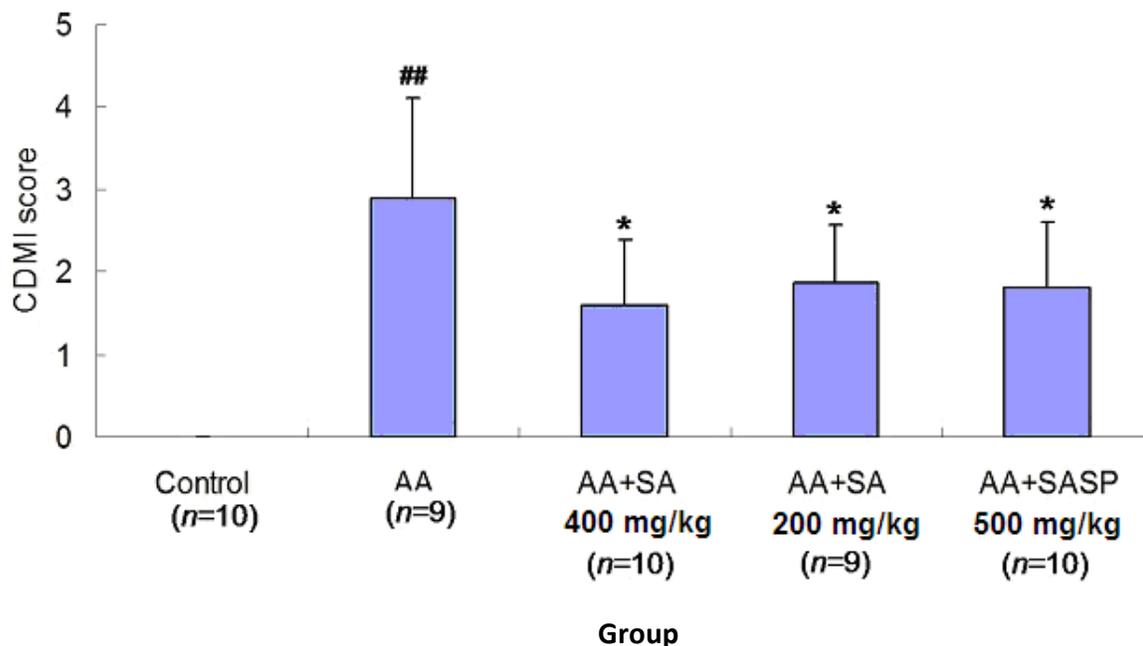
#### Statistical analysis

All statistical analyses were performed with SPSS version 11.5 for Windows. Macroscopic sore was compared by the Kruskal–Wallis non-parametric test and other parameters were compared by one-way ANOVA followed by the least significant difference (LSD) test. All results were expressed as mean  $\pm$  SD. Statistical significance was set at  $P$  < 0.05.

## RESULTS

### Effects of SA on macroscopic scoring

All rats were found to be sick after rectal injection of AA, and have diarrhea with bloody stool and abdominal distention. AA caused severe macroscopic edematous inflammation in the colon, as assessed by the high score of CMDI and the increased colon weight/length ratios. SA (200 and 400 mg/kg) and SASP (500 mg/kg) ameliorated the UC induced by AA significantly, in which a therapeutic dose protocol of 400 mg/kg SA was observed as effective as 500 mg/kg SASP in the treatment of this rat model of colitis (Figures 2 and 3).



**Figure 3.** Effects of SA on colonic CMDI score. Each column represented as the mean $\pm$ SD. ## $P$ <0.01, compared to the control group; \* $P$ <0.05, compared to the AA group. AA, Acetic acid; SA, shikimic acid; SASP, sulfasalazine; CMDI, colon mucosal damage index.

### Effect of SA on neutrophil infiltration in the colon tissues

The colonic inflammation was monitored biochemically by measuring the level of MPO activity in the colonic tissue taken from the site of inflammation. MPO activities were estimated in the colonic tissue as a marker of granulocyte and monocyte influx into the tissue. In AA-treated rats, the histological inflammatory effect was mirrored by a sevenfold increase in mucosal MPO activity. SA or SASP produced a significant reduction in MPO activity; however, none of these treatments returned the MPO activity to control values (Figure 4).

### Effect of SA on MDA and NO levels, SOD and iNOS activities in colon tissues

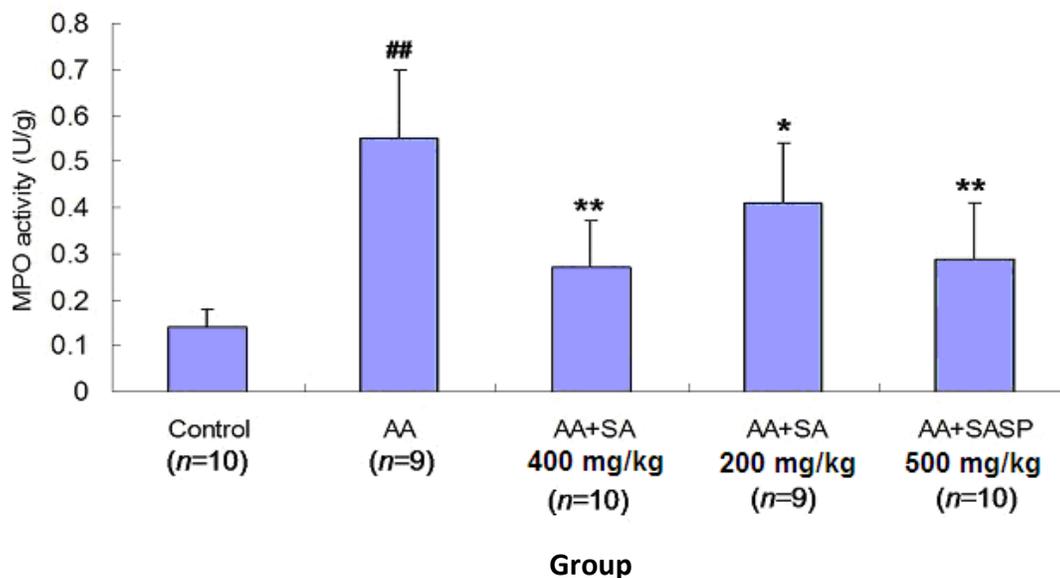
Oxidative damage is an important cause of damage to the colonic mucous membrane of UC rats. In the AA group, MDA and NO levels, and iNOS activity were significantly increased, while SOD activity was significantly decreased compared with the control group. Treatment with SASP significantly reversed the changes in these four parameters induced by AA. SA (200 and 400 mg/kg) reduced levels of MDA and increased the activity of SOD. Similarly, NO levels and iNOS activity were significantly decreased after treatment with SA (400 mg/kg; Figure 5 to 8).

### DISCUSSION

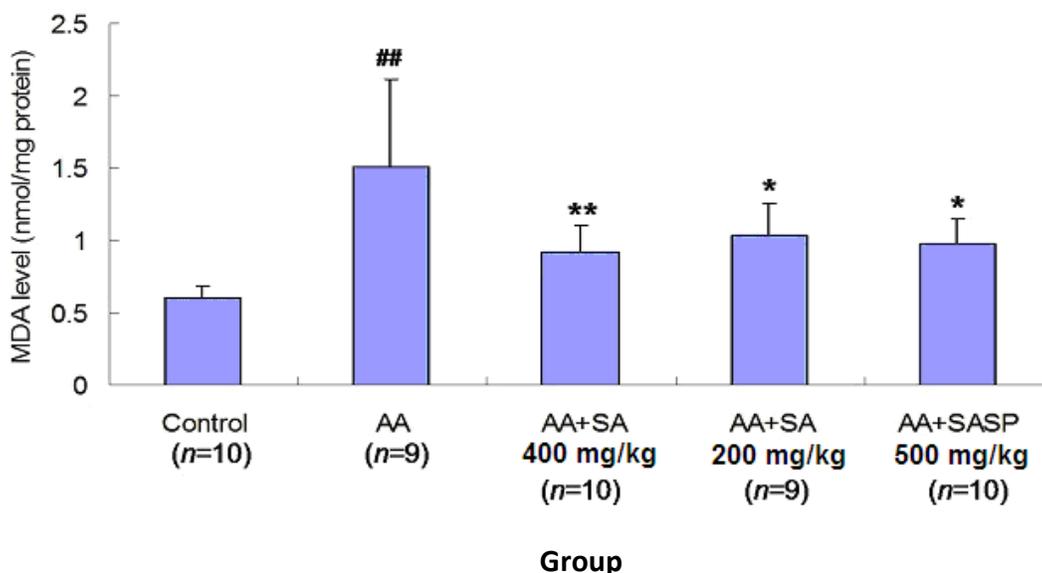
Since the pathogenesis and pathological changes associated with AA-induced acute mucosal inflammatory injury in the distal colon are similar to those in human UC, an acid-induced model is commonly used to investigate the pathogenesis of ulcerative colitis, and to screen for and evaluate new drugs with which to treat it (Millar et al., 1996).

The results of the present study demonstrated that treatment of AA-induced colitis rats with SA markedly improved their colonic damage. Moreover, all parameters indicating the presence of oxidative injury were inhibited by SA, suggesting that SA may have a potent anti-inflammatory effect on the inflamed colonic tissue. Changes in macroscopic CMDI score and colon weight/length ratios showed that the effect of SA may improve the symptoms of colitis as well as protect the colon tissue. SA may also exert its beneficial activities in the intestine, where the concentration of SA is highest in the body (Chong et al., 2001).

MPO is an enzyme found predominantly in neutrophils and has been used as a quantitative index of inflammation due to the correlation between MPO activity and histological analysis of neutrophil infiltration in colon (Cetinkaya et al., 2006; Krawisz et al., 1984). Therefore, MPO activity has been widely used to detect and follow intestinal inflammatory processes, and a reduction in the activity of this enzyme can be interpreted as a



**Figure 4.** Effects of SA on colonic MPO activity in UC rats. Each column represented as the mean±SD. <sup>##</sup> $P<0.01$ , compared to the control group; <sup>\*</sup> $P<0.05$ , <sup>\*\*</sup> $P<0.01$ , compared to the AA group. AA, Acetic acid; SA, shikimic acid; SASP, sulfasalazine; MPO, myeloperoxidase.

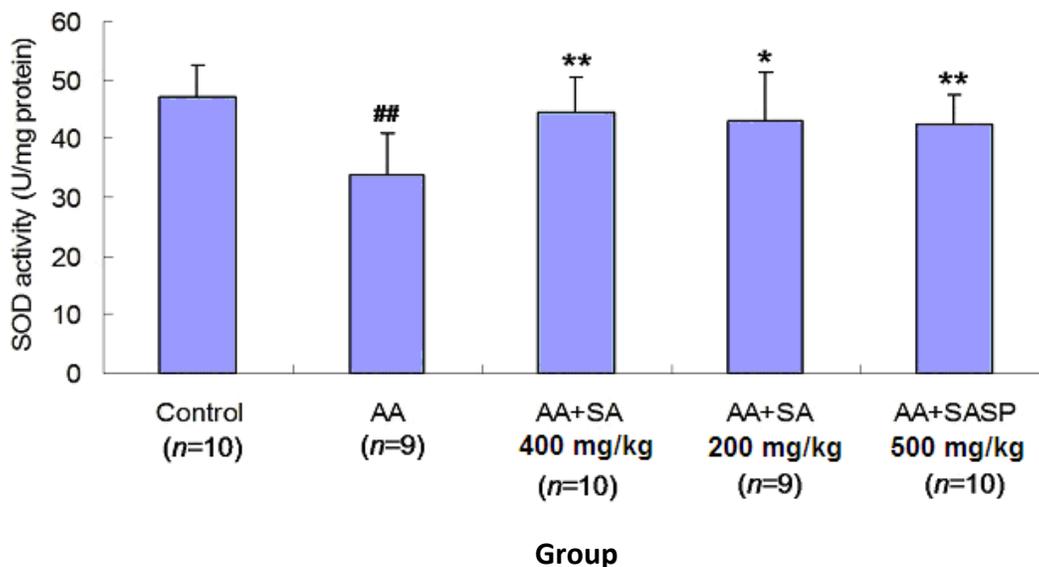


**Figure 5.** Effects of SA on colonic MDA level in UC rats. Each column represented as the mean±SD. <sup>##</sup> $P<0.01$ , compared to the control group; <sup>\*</sup> $P<0.05$ , <sup>\*\*</sup> $P<0.01$ , compared to the AA group. AA, Acetic acid; SA, shikimic acid; SASP, sulfasalazine; MDA.

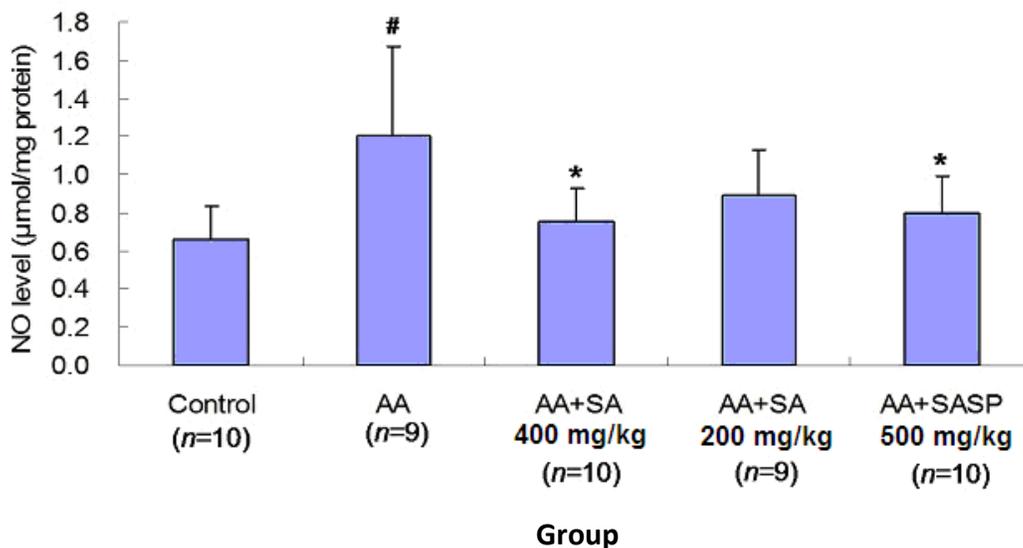
manifestation of the anti-inflammatory activity of a given drug. In our study, MPO activity increased in colitis groups, SA could inhibit MPO activity in AA-induced colitis rats. These findings suggested that SA exerted a strong anti-inflammatory effect.

The MDA level was used as an index of oxidative

status in UC. MDA is a product of lipoperoxidative processes that take place as a consequence of the colonic oxidative insult (Pavlick et al., 2002; Loguercio et al., 1996). Many studies have found that toxic colitis injury can increase MDA levels in rats, and conversely that various agents used in the treatment of the disease



**Figure 6.** Effects of SA on colonic SOD activity in UC rats. Each column represented as the mean±SD. ## $P<0.01$ , compared to the control group; \* $P<0.05$ , \*\* $P<0.01$ , compared to the AA group. AA, Acetic acid; SA, shikimic acid; SASP, sulfasalazine.



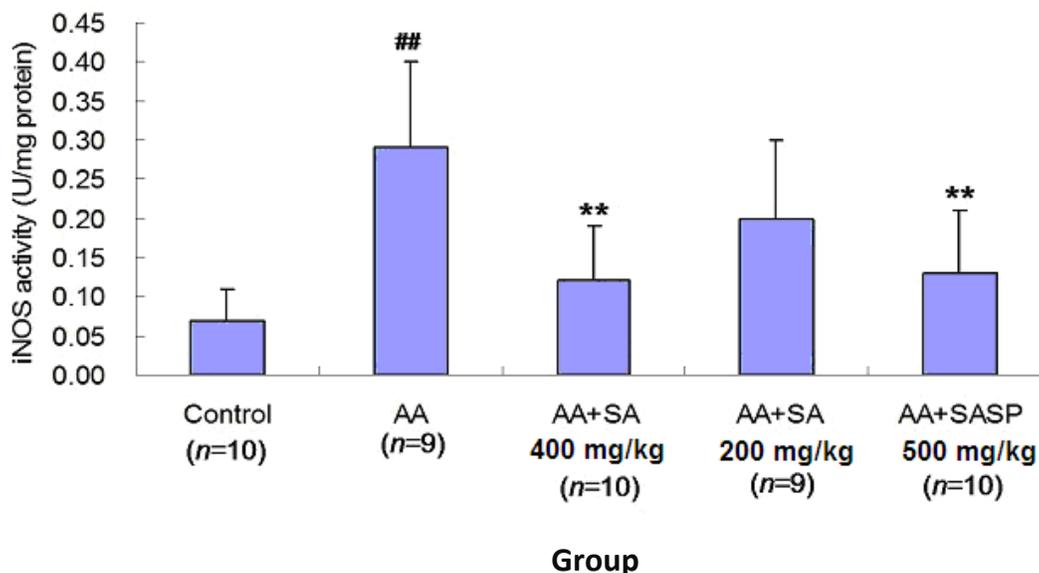
**Figure 7.** Effects of SA on colonic NO level in UC rats. Each column represented as the mean±SD. ## $P<0.01$ , compared to the control group; \* $P<0.05$ , compared to the AA group. AA, Acetic acid; SA, shikimic acid; SASP, sulfasalazine; NO, nitric oxide.

can decrease its level. Our results demonstrated that SA could markedly decrease the MDA level of colonic tissue with AA-induced colitis.

SOD is an enzymatic scavenger, which can convert superoxide anion to hydrogen peroxide. Thus, it can exert defensive effects against oxidative damage. Many experiments proved beneficial effects of SOD treatment against experimental colitis (Segui et al., 2004). The data

in this study showed that SA increased SOD activity in AA-induced colitis rats. Therefore, SA can strengthen enzymatic defensive system and reduce free radicals, and then alleviate inflammation.

In the pathological condition, NO which is mainly produced by the iNOS, is another free radical and a universal messenger (Kolios et al., 2004). A lot of experiments demonstrate that excess NO may exacerbate the



**Figure 8.** Effects of SA on colonic iNOS activity in UC rats. Each column represented as the mean $\pm$ SD. ## $P$ <0.01, compared to the control group; \*\* $P$ <0.01, compared to the AA group. AA, Acetic acid, SA, shikimic acid; SASP, sulfasalazine; iNOS, inducible nitric oxide synthase; UC, ulcerative colitis.

clinicopathological feature of ulcerative colitis by direct cytotoxicity, activation of neutrophils, vasodilatation and so on (Ribbons et al., 1995). In addition, NO can also interact with superoxide and form the highly toxic peroxynitrite radical, which reversibly increases the expression of iNOS by activating NF- $\kappa$ B, leading to the vicious cycle (Cooke and Davidge, 2002). Thus, the inhibition of overproduced NO offers the new way to treat inflammation. In the present study, we firstly discovered and authentically revealed that SA could not only inhibit NO production, but also inhibit iNOS activity of rat colon tissue.

In conclusion, the results in our experiments confirm that SA has protective effects on acute experimental colitis, and its anti-inflammatory effects seem to be relative to antioxidation. Thus, this compound may be a candidate agent for the development of novel anticolitis drug.

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