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• *BASIC RESEARCH* •

Amelioration of experimental colitis by *Astragalus membranaceus* through anti-oxidation and inhibition of adhesion molecule synthesis

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Abstract

AIM: To investigate the protective effects of *Astragalus membranaceus* (Am) against hapten-induced colitis in male Sprague-Dawley rats as well as its underlying mechanism.

METHODS: Experimental colitis was induced in rats by enema administration of 2,4-dinitrobenzene sulfonic acid (DNBS). Rats were either pretreated with Am extract (2 or 4 g/kg, p.o. once daily) starting from 10 d before DNBS enema, or received Am post-treatment (2 or 4 g/kg, p.o. twice daily) on the three consecutive days following DNBS administration. Colonic lesion area and histological damage were determined, while the activities of myeloperoxidase (MPO) and xanthine oxidase, as well as reduced glutathione (GSH) content were measured in the excised colonic tissues. Besides, protein expression of inducible nitrite oxide synthase (iNOS), intercellular adhesion molecule-1 (ICAM-1) and P-selectin was also detected by Western blot analysis.

RESULTS: Our findings had shown that both macroscopic lesion area and histological colonic damage induced by DNBS were significantly reduced by both Am pre- and post-treatments. These were accompanied by attenuation of the elevated colonic MPO activity and downregulation of the iNOS, P-selectin, and ICAM-1 protein expression. Besides, deprivation of colonic GSH level under colitis condition was also preserved.

CONCLUSION: These results demonstrate that Am possesses both preventive and therapeutic potential in experimental colitis. The anti-inflammatory actions involve anti-oxidation along with inhibition of adhesion molecule synthesis in the colonic tissues.

Key words: IBD; *Astragalus membranaceus*; Reactive oxygen metabolites; Adhesion molecules

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INTRODUCTION

Inflammatory bowel disease (IBD) consists of a group of idiopathic diseases of the intestine characterized by chronic inflammation of the bowel with periods of exacerbation and remission[1]. Moreover, patients with IBD have a higher risk of developing colorectal cancer than the general population[2]. There are two major categories of IBD, ulcerative colitis (UC) and Crohn's disease (CD), which are both histologically and clinically dissimilar. Despite the fact that etiology of IBD still remains poorly understood, complex interactions among genetic, environmental and immunological have been implicated in the pathogenesis of IBD[3,4]. In fact, activation of the central immune cell populations will lead to the production of a wide variety of non-specific mediators of inflammation, including cytokines, chemokines, growth factors, arachidonic acid metabolites (e.g. prostaglandins and leukotrienes) and reactive oxygen metabolites (ROM)[3,5]. In the past few decades, new chemotherapeutic agents such as 5-aminosalicylic acid, corticosteroids, immunosuppressive and immunoregulatory agents, antibiotics and probiotics, anti-tumor necrosis factor (TNF)-α, anti-inflammatory cytokines, and anti-intercellular adhesion molecule (ICAM)-1 therapy, etc., had been developed to tackle with these problems. However, none of them appeared to have been successful in achieving complete remission or preventing relapse, while some may even cause serious side effects.

Neutrophil infiltration in the extravascular space involves a complex sequence of interaction between circulating neutrophils and the vascular endothelium, including rolling, adhesion and transendothelial migration. Myeloperoxidase (MPO) is an enzyme mainly found in azurophilic granules of neutrophils. It is a good marker of neutrophil infiltration in gastrointestinal tissues[6], as well as inflammation and tissue injury. Besides, significant amounts of superoxide can be generated by a variety of endogenous enzyme systems such as the peroxisomal enzyme xanthine oxidase (XO) following tissue ischemia. XO activity is often increased in intestinal inflammation through the induction by TNF-α and activated neutrophils[7]. It has been suggested that XO is a significant source of superoxide in the inflammation of IBD[8]. Nonetheless, the concentrations of endogenous antioxidants, such as reduced glutathione (GSH), alpha-tocopherol and

cysteine, are all significantly decreased in IBD patients. As a result, the balance between antioxidants and ROM becomes seriously impaired[9,10]. Most currently used therapeutic drugs for IBD, in particular sulfasalazine and its active moiety 5-aminosalicylic acid, control the disease partly by their property as potent ROM scavengers[11].

Nitric oxide (NO) will react with superoxide to form peroxynitrite, which is a highly cytotoxic oxidant[12]. Inducible nitrite oxide synthase (iNOS) is the inducible form of the enzyme responsible for the synthesis of pro-inflammatory NO, which is mainly induced in the infiltrated inflammatory cells, such as neutrophils, macrophages and mononuclear cells by stimulants such as cytokines[13]. Several studies had shown that NO production and iNOS activity were increased in colonic mucosa from UC and CD patients[14]. Immunohistochemical studies also indicated that iNOS was localized in neutrophils, macrophages and epithelial cells in the active area of the inflamed colon in UC patients[15]. Other than NO generated by the iNOS isozyme, leukocytes migration in IBD patients also requires the interaction between different cell adhesion molecules[16]. They are surface bound glycoprotein molecules on endothelial cells that are essential for the capture and migration of leukocytes. P-selectin and ICAM-1 are adhesion molecules which are essential for migration of leukocytes from the blood stream into the inflamed tissue. P-selectin captures circulating leukocytes in the early extravasation process, while ICAM-1 is involved further in the migration of leukocytes across the connective tissue in the lamina propria, and in the interaction between leukocytes and epithelial cells[17]. Furthermore, P-selectin is susceptible to be activated by endotoxin and cytokines[18]. Semiquantitative analyses have shown that P-selectin expression was significantly increased in both UC and CD patients, primarily expressed on venules and capillaries near the small lymphocyte aggregates[19,20]. Moreover, the elevated P-selectin and ICAM-1 levels could persist for more than 48 h in the colonic tissues after a single stimulus.

Astragalus membranaceus (Am, Fisch.) Bge. *var mongholicus* (Bge.) Hsiao or (Fisch., Am), also called Huang-qi in Chinese, is the dried root of a perennial herbaceous plant. It is an important "tonifying" herb being prescribed to strengthen body defense against pathogenesis and to promote the discharge of pus as well as tissue restitution. Traditional herbal formulations containing Am can be used to treat chronic ulceration and sores[21], mellitus diabetes and albuminuria in chronic nephritis, etc.[22]. Rats injected with the herb extract (0.5 g/kg, i.p.) for 1 mo had not been developing abnormalities[23]. Two groups of bioactive chemicals have been isolated from Am, which are polysaccharides (i.e. astragalan I-III) and saponins (i.e. astragalosides I-VIII, acetylastragaloside I, isoastragalosides I-II and soyasaponin I), respectively. Besides, about 0.18-2.0% of *Astragalus* root consists of coumarin and flavonoid derivatives[24]. These compounds are capable of minimizing free radical damage to membranes by inhibiting lipid peroxidation[25] and by protecting the intestinal endothelium[26]. There had been a number of clinical studies on the treatment of IBD using herbal formulations with Am as leading drug, with results ranging from the relief of symptoms to the prevention of UC recurrence with increased serum superoxide dismutase activity (anonymous Chinese case reports). However, there is no experimental or clinical study about the sole effect of Am on IBD.

The present investigation aimed to determine the preventive and therapeutic effects of Am extract on experimental colitis induced by 2,4-dinitrobenzene sulfonic acid (DNBS), and to delineate the underlying anti-oxidation mechanisms and modulation of colonic synthesis of inflammatory mediators and adhesion molecules.

MATERIALS AND METHODS

Animals and treatments

Male Sprague-Dawley rats, weighing 180-200 g, were acclimatized under constant temperature (22±1 ℃) and humidity (40-45%) with 12-h light-dark cycles for 10 d before experimentation. Groups of six animals each were housed in rack-mounted wire cages. They were reared on a standard laboratory diet (Purina, USA), and given tap water *ad libitum*. Both body weight and food intake of the rats were measured daily throughout the entire experimental period. In addition, the appearance of stools (normal, loose/watery, or bloody) was also observed as part of index to determine disease activity.

Animals were anesthetized with a mixture of 75 mg/kg ketamine and 10 mg/kg xylazine (Alfasan, the Netherlands) intraperitoneally before colitis induction. Following this, 250 μL of 500 mL/L ethanol containing 30 mg of DNBS dihydrate (Aldrich, USA) was slowly infused into the lumen of the colon through a siliconized catheter (12 cm long, 2-mm external diameter) inserting to an 8-cm position proximal to the anus. Animals of the normal untreated group (normal) received enema saline administration. The entire infusion process took approximately 10 s to complete, with the catheter remaining inside the colonic lumen for 5 min before the withdrawal in order to prevent possible leakage.

The concentrated powder of Am was obtained from Purapharm (Hong Kong) and was used throughout the entire experiment. The herbal compound was extracted from the dried roots of Am (Fisch.) Bge. var. *mongholicus* (Bge.) Hsiao, and was manufactured under Good Manufacturing Practice standard. A pilot study was undertaken to determine the dose-response relationship of the herbal extract prior to the actual investigation. It was found that 2 and 4 g/kg are being optimal pharmacological doses for the anti-inflammatory action against experimental colitis formation. Groups of rats received oral pre-treatment of the concentrated Am extract (2 or 4 g/kg in 10 mL/kg tap water) once daily for 10 d prior to DNBS induction. Alternatively, other groups of animals received oral Am treatment (2 or 4 g/kg in 10 mL/kg tap water) twice daily on the three consecutive days following DNBS induction. Control animals (control) received oral administration of normal drinking water instead. All animals were killed by intraperitoneal injection of 100 mg/kg of ketamine, followed by cervical dislocation, at 4 d after colitis induction. This time was chosen on the basis that development of acute inflammation arrives at peak level in DNBS-evoked colitis, whereas active tissue repair and regeneration begins afterwards[27].

Assessment of macroscopic colonic lesion area and inflammation

The entire segment of the colon from the rectum to the cecocolic junction was removed, opened and rinsed thoroughly with normal saline. The isolated colon was examined for the presence of gross macroscopic damage and inflammation. Lesion area was recorded by tracing onto a plastic transparency, and measured by using 1-mm^2 grids. Macroscopic lesion area was represented in square millimeter. Other disease activity index was also measured, including the ratio of colon weight to body weight, which was used as a parameter to assess the degree of tissue edema and reflects the severity of colonic inflammation. Moreover, a 6-8-mm sample block of the inflamed colonic tissue with the full thickness was excised from a region of grossly visible damage for histological analysis, while the remaining colonic segment was pulverized and stored at -80 ℃ for subsequent assays.

Histological study of the colonic tissue

After 48 h of 10% buffered formalin fixation, the excised tissue block was processed for histological analysis. After fixation, tissues were dehydrated by a 4-h successive immersion in ethanol and cleared with xylene for 45 min. The specimens were then immersed in melted paraffin at 80 ℃ for 3½ h for embedment into a cast. Six-micrometer sections were sliced with a microtome (Leica RM2135, Germany) and prepared on Vectabond-coated glass slides. The slides were then processed for subsequent staining. After hydration, the processed sections were immersed into periodic acid for 5 min. The sections were then dipped in Schiff's reagent for 5 s. After rinsing in running tap water and washed with distilled water, the tissue sections were dipped into Harris' hematoxylin for 3 s and rinsed in running tap water (for a few seconds) and washed with distilled water. Following hematoxylin counter-staining, tissues on slides were dehydrated and finally mounted in Permount (Sigma, USA). Colonic tissues were scored for histological damage using the criteria of Wallace and Keenan: 0=intact tissue with no apparent damage; 1 = damage limited to surface epithelium; 2 = focal ulceration limited to mucosa; 3=focal, transmural inflammation and ulceration; 4 = extensive transmural ulceration and inflammation bordered by normal mucosa; 5 = extensive transmural ulceration and inflammation involving entire section[28].

Measurement of MPO activity in the colonic tissue

MPO activity is used as a quantitative index of inflammation and a marker of neutrophil infiltration in the tissue[29]. MPO activity was measured by a modified method described by Suzuki and co-workers[30]. Pre-weighed colonic tissues were homogenized with a homogenizer (IKA Labortechnik T25 basic, Germany) in ice-cold 50 mmol/L PBS (pH 6) containing 0.5% hexadecyl-trimethylammonium bromide and 0.336% EDTA (9 μL/mg tissue) for 30 s. The homogenized samples were freeze-thawed and sonicated for 20 s thrice, and then centrifuged at 30 000 *g* (Beckman coulter, Avanti™ J-25I, USA) for 20 min at 4 ℃. Following this, 20 μL of resulting supernatant with dilution by 80 mmol/L PBS (pH 5.4) was mixed with 7.5 μL of 2 mmol/L 3, 3', 5, 5'-tetramethylbenzidine dissolved in 80 mL/L *N*,*N*-dimethylformamide and 2.5 μL of 0.3 mol/L hydrogen peroxide. The final mixture was incubated at 25 ℃ for 25 min. Finally, 125 μL of 0.5 mmol/L sulfonic acid was added to stop the reaction. The end-point absorbance of the mixture was measured at 450 nm in a spectrophotometer (Packard, Fusion™, Canada) using horseradish peroxidase (HRP, Sigma, UK) as standard. The final value of MPO activity was represented as unit per milligram protein.

Measurement of XO activity in the colonic tissue

One unit of XO activity is defined as that could convert 1 μmoL of xanthine to uric acid per min at pH 7.5 and 25 ℃. The XO activity assay was modified from the method of Parks and co-workers[31]. Colonic tissues were homogenized in the 50 mmol/L sodium phosphate buffer (pH 7; 3 μL/mg tissue) containing 0.1 mmol/L EDTA-2Na, 10 mmol/L dithioerythritol and 1 mmol/L phenylmethyl sulfonyl fluoride (PMSF) for 30 s. The mixture was centrifuged at 20 000 *g* (Beckman coulter, Avanti™ J-25I, USA) for 15 min. Then, 12 μL of the supernatant was mixed with 1 mmol/L xanthine (Sigma, UK), 2 mmol/L EDTA-2Na, 200 μg/mL of bovine saline albumin (BSA) and 50 mmol/L of $NaPO_4$ buffer. The mixture was incubated at 25 ℃ for 5 min. Finally, the reaction was terminated by the addition of 400 μL of 0.5 mol/L HCl. XO activity was assessed spectrophotometrically by measuring the absorbance at 295 nm (Packard, Fusion™, Canada). The final values of XO were expressed as milliunit per milligram protein.

Measurement of GSH level in the colonic tissue

The amount of GSH in colonic tissues was determined following the procedures of Cho's research team[32]. Colonic tissues were homogenized in ice-cold 125 mmol/L sodium phosphate buffer with 6.3 mmol/L EDTA (pH 7.5, 3 μL/mg tissue) for 30 s. The crude homogenate was centrifuged (Beckman coulter, Avanti™ J-25I, USA) at 30 000 *g* at 4 ℃ for 30 min. Then, 200 μL of 40 g/L sulfosalicylic acid was added to 100 μL of supernatant and allowed to stand on ice for 5 min to precipitate protein. The mixture was centrifuged again at 5 000 r/min (Eppendorf centrifuge, model 5417R, Germany) at 4 ℃ for 10 min. Subsequently, 100 μL of the de-proteinized supernatant was mixed well with 300 μL of 125 mmol/L sodium phosphate buffer (pH 8) and 2 μL of 10 mmol/L 5,5' dithiobis-(2-nitrobenzoic acid). The solution was allowed to stand at room temperature for 15 min to develop a yellow color. The absorbance was then read against the reagent blank at 412 nm in a spectrophotometer (Packard, Fusion™, Canada). A standard curve of reduced GSH (Sigma, UK) was used for the calculation of the concentration of GSH in the colonic tissues. The final values were expressed as nanomole per milligram protein.

Measurement of protein concentration in the colonic tissue

Total protein contents of the colonic samples were determined for a series of Western blot analyses. Colonic tissues were weighed and homogenized for 30 s at 4 ℃ in RIPA lysis buffer (50 mmol/L Tris, 150 mmol/L NaCl, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 2 mmol/L EDTA, 1% Triton X-100 and 10% glycerol)

with 1 mmol/L PMSF, aprotinin (5 mg/mL), and pepstatin A (5 mg/mL) added freshly. The sample was then centrifuged at 13 000 *g* for 20 min and the resulting supernatant was collected. Five microliters of tissue sample supernatant was diluted with normal saline. Seventy-five microliters of a 1 000× diluted sample was mixed with 75 μL of Coomassie Plus Reagent (Pierce, UK). The mixture was then incubated at room temperature for 10 min. The samples' absorbance was measured at 595 nm by a spectrophotometer (Packard, Fusion™, Canada). The stock concentration of the standard protein BSA was made up to 100 μg/mL. A standard curve in the range of 0-25 μg protein was used for the calculation of the protein concentration present in the colonic tissue. The results are used to quantify the protein sample for electrophoresis in Western blotting and act as a unit of the biochemical assays.

Analysis of the protein expressions of iNOS, P-selectin, and ICAM-1 in the colonic tissue

The protein samples (ICAM-1: 50 μg of total protein; P-selectin and iNOS: 200 μg of total protein) were denatured and separated on 10% SDS-polyacrylamide gel electrophoresis, and then transferred to nitrocellulose membrane (Bio-Rad, USA). The membranes were blocked in blocking solution (5% skim milk powder in TBST containing 0.2 mol/L Tris, 1.37 mol/L NaCl, 1 mol/L HCl, and 0.1% Tween 20) for 30 min at room temperature. Subsequently, it was incubated in a 4 ℃ cold room overnight with the primary antibodies (anti-ICAM-1, anti-P-selectin, anti-iNOS; all in the dilution of 1:1 000) diluted in blocking solution. Membranes were washed six times and incubated with the secondary antibody (HRP-goat anti-rabbit IgG (H+L) conjugate; 1:5 000) in blocking solution for 45 min at room temperature. All antibodies were obtained from Santa Cruz, USA. Protein blots were developed by a chemiluminescence ECL Western blotting detection system (Amersham, USA). The protein bands were visualized by autoradiography using X-ray film (Fuji, Japan) and analyzed by the Bio-Rad Image Analysis System (USA).

Statistical analysis

Results were expressed as mean±SE. Differences between two groups were examined using the one-way analysis of variance (ANOVA) followed by the Dunnett's test. A *P*-value of less than 0.05 was considered significantly different.

RESULTS

Alleviation of DNBS-induced colitis by Am extract

Gross macroscopic observation had demonstrated that administration of DNBS resulted in mucosal lesion formation and inflammation in the distal colonic wall. There was also a drastic drop in body weight of the DNBS-induced rats. The animals also appeared to have diarrhea, soft stool, and rectal bleeding (in the first 2 d after colitis induction). Besides, ratio of the distal colon weight to animal body weight was also markedly increased due to tissue edema. Hyperemia of mesenteric arteries and fibrinous adhesion to other bowel tissues were frequently observed. Figure 1A shows that there was a significant reduction in macroscopic lesion area by both Am pre- and post-treatments in a dose-dependent manner. Microscopically, colonic tissues in all Am-treated groups had demonstrated significant reduction in histological damage scores (Figure 1B), as represented by well-organized mucosal architecture and less infiltrated leukocytes in the mucosal and submucosal layers of the inflamed colon. This was in contrast with the extensive morphological disorientation and generalized glandular disruption in the colonic tissues of the DNBS control group. These implicated that colonic protection produced by Am extract can be achieved at the histological level.

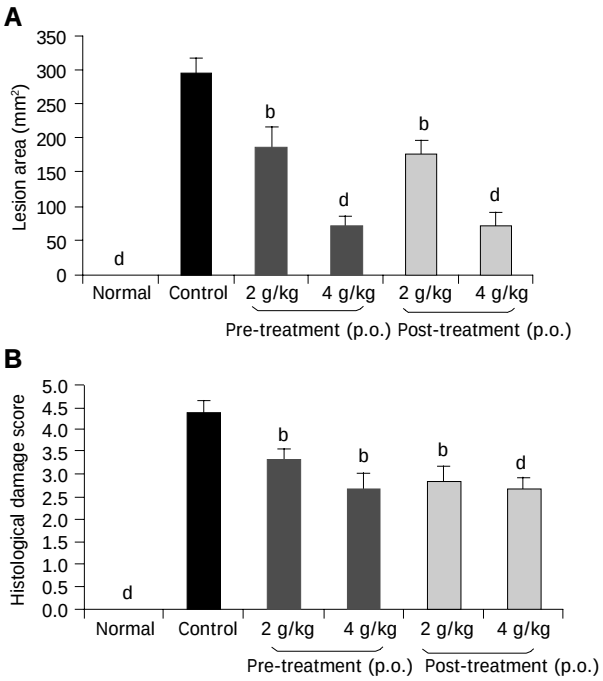

Figure 1 A: Effect of oral *Astragalus* extract (Am) treatment (2 or 4 g/kg) on DNBS-induced lesion formation in rat colon. Animals were either pre-treated with Am once daily for 10 d from 1 d after DNBS induction, or received Am post-treatment twice daily on the three consecutive days following DNBS induction. Control animals (control) received oral administration of normal drinking water only. Animals without colitis induction or drug treatment were regarded as normal animals (normal). All animals were killed 4 d after DNBS administration. Values are mean±SE (n = 6). ^{b}P<0.01, ^{d}P<0.001 *vs* control; B: Effect of oral *Astragalus* extract (Am) treatment (2 or 4 g/kg) on DNBS-induced histological damage score in rat colon. Animals were either pre-treated with Am once daily for 10 d from 1 d after DNBS induction, or received Am post-treatment twice daily on the three consecutive days following DNBS induction. Control animals (control) received oral administration of normal drinking water only. Animals without colitis induction or drug treatment were regarded as normal animals (normal). All animals were killed 4 d after DNBS administration. Values are mean±SE (n = 6). ^{b}P<0.01, ^{d}P<0.001 *vs* control.

Effects of Am extract on colonic activities of the oxidative enzymes MPO and XO and on content of the anti-oxidative enzyme GSH in inflamed colonic tissue

MPO activity was dramatically increased in the colonic tissue after DNBS administration (Figure 2A). Both Am pre- and post-treatments significantly reduced the elevated colonic MPO activity. The attenuating effect of Am on colonic MPO activity was consistent with its colonic protective actions both macroscopically and histologically (Figures 1A and B). Besides the MPO pathway, ROM can also be

generated through XO-catalyzed reactions. Nevertheless, there was no significant change in colonic XO activity after DNBS induction of colonic damages. Likewise, Am treatments also caused no alteration in XO activity. Therefore, it appeared that XO has no involvement in the pathogenesis of DNBS-induced colitis as well as in the mode of action of Am extract.

Figure 2B shows the effect of Am extract on the depletion of one of the key components of antioxidant defenses in the intestine, GSH. GSH concentration in the colonic tissue was dramatically decreased after DNBS enema. Pre- and post-treatment of Am (4 g/kg) significantly preserved the deprivation of the GSH level under colitis condition. The preservation of GSH in the colon by Am treatments could indeed strengthen the colonic defensive mechanism and thus reduce the susceptibility to tissue injury.

Effects of Am extract on protein expression of the pro-inflammatory enzyme iNOS, and adhesion molecules ICAM-1 and P-selectin in inflamed colon

There was a significant upregulation of colonic iNOS protein expression following DNBS administration. Such overexpression was attenuated by both Am pre- and post-treatments (Figure 3A). Similarly, the protein expression of both P-selectin and ICAM-1 was also dramatically upregulated under colitis condition (Figures 3B and C). Although there was a dose-dependent diminution of the P-selectin overexpression in all Am treatment groups (Figure 3B), only pre-treatment with 4 g/kg of Am was capable of inhibiting the elevated ICAM-1 protein expression in the colitic tissue, with no significant effect observed in other treatment groups (Figure 3C).

DISCUSSION

In the present study, DNBS was administrated intracolonically to stimulate cell-mediated immunity in rats[33]. This hapten molecule produces acute and chronic colonic inflammation as well as ulceration in rats[34]. Besides, the histopathologic

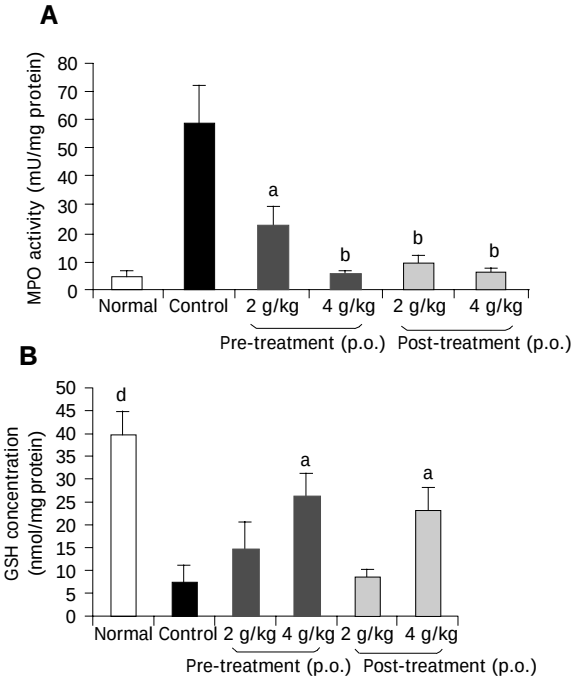

Figure 2 A: Effect of oral *Astragalus* extract (Am) treatment (2 or 4 g/kg) on MPO activity in rat colon. Animals were either pre-treated with Am once daily for 10 d from 1 d after DNBS induction, or received Am post-treatment twice daily on the three consecutive days following DNBS induction. Control animals (control) received oral administration of normal drinking water only. Animals without colitis induction or drug treatment were regarded as normal animals (normal). All animals were killed 4 d after DNBS administration. Values are mean±SE (n = 6). ^{a}P<0.05, ^{b}P<0.01 *vs* control; B: Effect of oral *Astragalus* extract (Am) treatment (2 or 4 g/kg) on GSH concentration in rat colon. Animals were either pre-treated with Am once daily for 10 d from 1 d after DNBS induction, or received Am post-treatment twice daily on the three consecutive days following DNBS induction. Control animals (control) received oral administration of normal drinking water only. Animals without colitis induction or drug treatment were regarded as normal animals (normal). All animals were killed 4 d after DNBS administration. Values are mean±SE (n = 6). ^{a}P<0.05, ^{d}P<0.001 *vs* control.

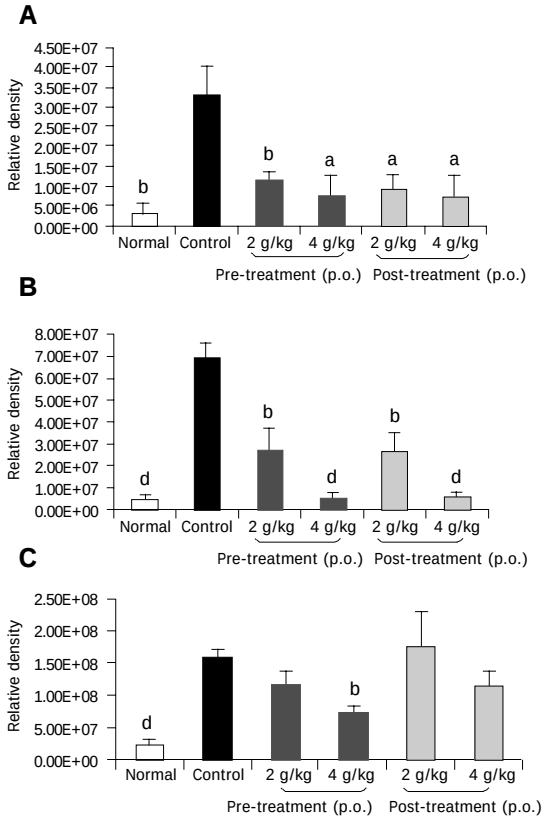

Figure 3 A: Effect of oral *Astragalus* extract (Am) treatment (2 or 4 g/kg) on iNOS protein expression in rat colon. Animals were either pre-treated with Am once daily for 10 d from 1 d after DNBS induction, or received Am post-treatment twice daily on the three consecutive days following DNBS induction. Control animals (control) received oral administration of normal drinking water only. Animals without colitis induction or drug treatment were regarded as normal animals (normal). All animals were killed 4 d after DNBS administration. Values are mean±SE (n = 6). ^{a}P<0.05, ^{b}P<0.01 *vs* control; B: Effect of oral *Astragalus* extract (Am) treatment (2 or 4 g/kg) on P-selectin protein expression in rat colon. Animals were either pre-treated with Am once daily for 10 d from 1 d after DNBS induction, or received Am post-treatment twice daily on the three consecutive days following DNBS induction. Control animals (control) received oral administration of normal drinking water only. Animals without colitis induction or drug treatment were regarded as normal animals (normal). All animals were killed 4 d after DNBS administration. Values are means±SE (n = 6). ^{b}P<0.01, ^{d}P<0.001 *vs* control; C: Effect of oral *Astragalus* extract (Am) treatment (2 or 4 g/kg) on ICAM-1 protein expression in rat colon. Animals were either pre-treated with Am once daily for 10 d from 1 d after DNBS induction, or received Am post-treatment twice daily on the three consecutive days following DNBS induction. Control animals (control) received oral administration of normal drinking water only. Animals without colitis induction or drug treatment were regarded as normal animals (normal). All animals were killed 4 d after DNBS administration. Values are mean±SE (n = 6). ^{b}P<0.01, ^{d}P<0.001 *vs* control.

conditions have more features in common with human UC than with CD. The use of natural immunomodulatory and anti-oxidative products provides an innovative and relatively non-toxic/less toxic alternative to modulate inflammatory disorders, including IBD. Am had exhibited marked antioxidant and immunomodulatory properties in both clinical and experimental studies. However, there is a lack of information regarding its effect on intestinal inflammatory disorders and the associated mechanism of action. The current study is the first one of its kind to demonstrate the effect of Am on experimental colitis. Our findings had indicated that DNBS could lead to a considerable degree of inflammation and tissue injury in the rat colon, with the concomitant infiltration of neutrophils into the colonic tissues. These were reflected by significant colonic lesion formation, high score in histological damage grading, as well as elevated MPO activity in the DNBS-induced animals. Such detrimental effects were alleviated by either pre- or post-treatment of oral Am.

The main pathological feature of IBD is an infiltration of polymorphonuclear neutrophils and mononuclear cells into the intestinal tissues. Neutrophils and monocytes migration is in turn triggered by chemotactic bacterial cell wall products and locally produced cytokines[35,36]. The neutrophil-generated ROM such as superoxide anions, hydrogen peroxide, *N*-chlorinated derivatives, as well as the release of granule enzymes[37] are responsible for the endothelial cell damage. Sedghi and co-workers reported the increase of chemiluminescence in inflamed colonic mucosa and suggested that neutrophils are the likely cellular sources of ROM in the inflamed colon in UC patients[38]. MPO activity was significantly increased and correlated with laboratory parameters and endoscopic grade of inflammation in stool from IBD patients[39]. Besides, MPO conversion of hydrogen peroxide to hypochlorus acid seems closely related to ROM-mediated tissue injury[38]. ROM can be generated through two pathways. The first one is via NADPH oxidase activation in the infiltrated neutrophils, which induces the release of large amount of ROM and oxidants derived from MPO[37]. Alternatively, the other pathway acts through XO induction after periods of hypoxia[40]. In this study, MPO-catalyzed reaction was found to be activated after DNBS administration and attenuated by Am treatments. However, induced production of ROM and reduction of oxidative damage caused by Am did not seem to act through the XO pathway. This result is consistent with a clinical study illustrating that colonic XO activity was not elevated in UC patients[41]. In fact, it had been suggested that XO may not be a major source of superoxide and ROM production in UC[42]. The data presented here actually implicates that the anti-oxidative effect of Am mainly involves the modulation of MPO in the inflamed colon.

The imbalance of ROM generation and antioxidant defense in the colon is an important contributor to the initiation and maintenance of inflammation in IBD[43]. Reduced colonic GSH level is crucial in inducing inflammatory changes. The decrease in colonic GSH concentration following colitis induction could be due to over-production of ROM that deplete GSH by inhibiting the synthetic enzymes for GSH production in the colonic tissue[44]. In the present study, depletion of GSH was significantly preserved by Am, with concurrent attenuation of the elevated MPO activity. This finding implicates that Am could maintain the free radical-scavenging ability of the colonic defense system. Consequently, this diminishes the burden of colonic ROM accumulation (e.g. superoxide), and could further improve the imbalance between ROM and endogenous antioxidants. It is implicit that Am exhibits anti-oxidative activities in several ways, including the inhibition of oxidative stress due to lipid peroxidation and protein oxidative modification, as well as direct removal of free radicals[45]. Among different Am constituents, the total flavonoids appear to possess the strongest free radical-scavenging potential, while total saponins and polysaccharides are somewhat weaker free radical-scavenging agents. In addition, *Astragalus* polysaccharides could modify the increase in malondialdehyde content and diminution of GSH level in the liver of *E-coli* endotoxin-induced rats, and is capable of antagonizing the reduction of GSH peroxidase and superoxide dismutase activities in spleen, thymus and intestinal lymph nodes of traumatic rats[46].

Our findings had demonstrated that iNOS protein expression was significantly upregulated in the colonic tissue after DNBS enema, which was attenuated by oral Am administration. iNOS synthesis can be induced by pro-inflammatory cytokines or bacterial toxins in a variety of cells such as neutrophils, macrophages, endothelial cells and enterocytes, etc.[47]. Therefore, the reduction of neutrophil infiltration, as indicated by the decrease of MPO activity during the anti-inflammatory action of Am, may contribute at least partly to the alleviating effect of Am on iNOS expression in the colonic tissue. Elevated iNOS expression will result in the production of a huge amount of colonic inflammatory NO radicals. Increased production of NO as a result of the overexpression of iNOS protein and mRNA had been demonstrated in affected areas of the intestine in patients suffering from UC or CD[14]. Overwhelming production of iNOS-produced NO causes mucosal vasodilation and modulation of intestinal water and electrolyte transport, which together increase vascular permeability[48]. One of the underlying mechanisms of NO in inducing colonic toxicity involves the interaction with superoxide to produce peroxynitrite, a potent oxidant that can initiate membrane lipid peroxidation, sulfhydryl oxidation of proteins and nitration of acromatic amino acids[29]. In turn, peroxynitrite was found to inhibit superoxide production in a dose-dependent manner[49]. Together with our findings concerning GSH level, Am treatment could reduce superoxide and NO production by preservation of GSH and attenuating iNOS protein expression. As a result, these actions could reduce peroxynitrite formation and in turn diminish the damaging effect of DNBS-induced colonic injury. In addition, it is possible that hydroxyl radicals could be formed by the interaction of NO and hydrogen peroxide[50], which was prevented by Am through inhibition of iNOS synthesis.

Gene expression of cell adhesion molecules is induced by pro-inflammatory cytokines and lipopolysaccharide, such as the cytokines TNF-α, interleukin (IL)-1 and interferon (IFN)-γ[51]. P-selectin was considerably increased in the

colonic endothelium early after colitis induction, which could last for at least 3 wk[52]. In this study, protein expression of P-selectin and ICAM-1 was significantly upregulated after DNBS induction, leading to continuous influx of leukocytes to maintain the local inflammation. Both Am pre-treatment and post-treatment significantly downregulated the protein expression of P-selectin, while ICAM-1 expression was only significantly inhibited in the 4 g/kg Am pre-treatment group. In general, these findings suggest that Am could reduce neutrophil infiltration into colonic tissues through the attenuation of P-selectin and ICAM-1 synthesis evoked by DNBS. Moreover, the subsequent attenuation of MPO activity could further lead to reduction of iNOS synthesis and ROM production. In the study of McCafferty's group, increased P-selectin expression was observed 3 d after induction of acetic acid induced colitis[53]. Moreover, Jones and co-workers reported that there was local upregulation of ICAM-1 in UC and CD patients[54]. ICAM-1 will be manifested during the early inflammatory status of the intestine, while P-selectin expression can only be detectable in a slightly later stage. It is imminent that ICAM-1 plays a pivotal role in determining the initial inflammatory events, while P-selectin will be manifested later in time and is responsible for the maintenance of such responses. Am pretreatment actually prepared the colonic mucosa for subsequent insult by suppressing ICAM-1 synthesis. Nevertheless, after colitis was induced, Am could no longer be capable of inhibiting ICAM-1 production as the inflammatory reactions had already been started.

In conclusion, the results of the present study have shown that Am have both preventive and therapeutic potential in experimental colitis by its modulation of inflammatory mediators and anti-oxidation. It could significantly inhibit P-selectin and ICAM-1 protein expression, attenuate neutrophil infiltration and iNOS synthesis, as well as preserve the deprivation of GSH in colonic tissue following DNBS challenge. Our findings indeed open up a new avenue in deriving new chemotherapeutic strategies against IBD.

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REFERENCES

1 **Knigge KL.** Inflammatory bowel disease. *Clin Cornerstone* 2002; **4**: 49-60
2 **Farrell RJ,** Peppercorn MA. Ulcerative colitis. *Lancet* 2003; **359**: 331-340
3 **Fiocchi C.** Inflammatory bowel disease: etiology and pathogenesis. *Gastroenterology* 1998; **115**: 182-205
4 **Papadakis KA,** Targan SR. Current theories on the cause of inflammatory bowel disease. *Gastroenterol Clin N Am* 1999; **28**: 283-296
5 **Swidsinski A,** Ladhoff A, Pernthaler A, Swidsinski S, Loening–Baucke V, Ortner M, Weber J, Hoffmann U, Schreiber S, Dietel M, Lochs H. Mucosal flora in inflammatory bowel disease. *Gastroenterology* 2002; **122**: 44-54
6 **Morris GP,** Beck PL, Herridge MS, Depew WT, Szewezuk MR, Wallace JL. Hapten-induced model of chronic inflammation and ulceration in the rat colon. *Gastroenterology* 1989; **96**: 795-803
7 **Freidl HP,** Till GO, Ryan US, Ward PA. Mediator induced activation of xanthine oxidase in endothelial cells. *FASEB J* 1989; **3**: 2512-2518
8 **Simmonds NJ,** Rampton DS. Inflammatory bowel disease – a radical view. *Gut* 1993; **34**: 865-868
9 **Ramakrishna BS,** Varghese R, Jayakumar S, Mathan M, Balasubramanian KA. Circulating antioxidants in ulcerative colitis and their relationship to disease severity and activity. *J Gastroenterol Hepatol* 1997; **12**: 490-494
10 **Sturniolo GC,** Mestriner C, Lecis PE, D'Odorico A, Venturi C, Irato P, D'Inca R. Altered plasma and mucosal concentrations of trace elements and antioxidants in active ulcerative colitis. *Scand J Gastroenterol* 1998; **33**: 644-649
11 **Miles AM,** Grisham MB. Antioxidant properties of aminosalicylates. *Methods Enzymol* 1994; **234**: 555-572
12 **Beckman JS,** Koppenol WH. Nitric oxide, superoxide and peroxynitrite: the good, the bad and the ugly. *Am J Physiol* 1996; **271**: C1424-C1437
13 **Valentine JF,** Tannahill CL, Stevenot SA, Sallustio JE, Nick HS, Eaker EY. Colitis and interleukin 1â up-regulated inducible nitric oxide synthase and superoxide dismutase in rat myenteric neurons. *Gastroenterology* 1996; **111**: 56-64
14 **Rachmilewitz D,** Stamler JS, Bachwich D, Karmeli F, Ackerman Z, Podolsky DK. Enhanced colonic nitric oxide generation and nitric oxide synthase activity in ulcerative colitis and Crohn's disease. *Gut* 1995; **36**: 718-723
15 **Ikeda I,** Kasajima T, Ishiyama S, Shimojo T, Takeo Y, Nishikawa T, Kameoka S, Hiroe M, Mitsuuaga A. Distribution of inducible nitric oxide synthase in ulcerative colitis. *Am J Gastroenterol* 1997; **92**: 1339-1341
16 **Meenan J,** Mevissen M, Monajemi H, Radema SA, Soule HR, Moyle M, Tytgat GNJ, van Deventer SJH. Mechanisms underlying neutrophil adhesion to apical epithelial membranes. *Gut* 1996; **38**: 201-205
17 **Vainer B,** Nielsen OH. Changed colonic profile of P-selectin, platelet-endothelial cell adhesion molecules-1 (PECAM-1), intercellular adhesion molecule-1 (ICAM-1), ICAM-2, and ICAM-3 in inflammatory bowel disease. *Clin Exp Immunol* 2000; **121**: 242-247
18 **Ala A,** Dhillon AP, Hodgson HJ. Role of cell adhesion molecules in leukocyte recruitment in the liver and gut. *Int J Exp Pathol* 2002; **84**: 1-16
19 **Nakamura S,** Ohtani H, Watanabe Y, Fukushima K, Matsumoto T, Kitano A, Kobayashi K, Nagura G. *In situ* expression of the cell adhesion molecules in inflammatory bowel disease: evidence of immunologic activation of vascular endothelial cells. *Lab Invest* 1993; **69**: 77-85
20 **Schürmann GM,** Bishop AE, Facer P, Vecchio M, Lee JCW, Rampton DS, Polak JM. Increased expression of cell adhesion molecule P-selectin in active inflammatory bowel disease. *Gut* 1995; **36**: 411-418
21 **Bensky D,** Gamble A. Chinese herbal medicine: materia medica. Revised ed. Seattle: *Eastland Press* 1993
22 Pharmacopoeia Commission of the People's Republic of China. Pharmacopoeia of the People's Republic of China, vol. I. English ed. Beijing: *Chemical Industry Press* 2000
23 **Chang H,** But P. Pharmacology and applications of Chinese materia medica, vol. 2. Singapore: *World Scientific* 1987
24 **Huang KC.** The pharmacology of Chinese herbs. Boca Raton: *CRC Press* 1999
25 **Toda S,** Yase Y, Shirataki Y. Inhibitory effects of Astragali Radix, crude drug in oriental medicines on lipid peroxidation and protein oxidative modification of mouse brain homogenate by copper. *Phytother Res* 2000; **14**: 294-296
26 **Hei ZQ,** Zhang JJ, Lin SQ, Liu KX, Chen BX. Effects of Astragalus membranaceus injection on nitric oxide and endothelin concentration of intestinal mucosa after hemorrhage shock-reperfusion in rats. *Zhongguo Zhongyao Zazhi* 2004; **29**: 444-447
27 **Ko JKS,** Sham NF, Guo X, Cho CH. Beneficial intervention of

experimental colitis by passive cigarette smoking through the modulation of cytokines in rats. *J Invest Med* 2001; **49**: 21-29

28 **Wallace JL,** Keenan CM. An orally active inhibitor of leukotriene synthesis accelerates healing in a rat model of colitis. *Am J Physiol* 1990; **258**: G527-G534

29 **Rachmilewitz D,** Stamler JS, Karmeli F, Mullins ME, Singel DJ, Loscalzo J, Podolsky DK. Peroynitrite-induced rat colitis: a new model of colonic inflammation. *Gastroenterology* 1993; **105**: 1681-1688

30 **Suzuki K,** Ota H, Sasagawa S, Sakatani T, Fujikura T. Assay method for myeloperoxidase in human polymorphonuclear leukocytes. *Anal Biochem* 1983; **132**: 345-352

31 **Parks DA,** Williams TK, Beckman JS. Conversion of xanthine dehydrogenase to oxidase in ischemic rat intestine: a reevaluation. *Am J Physiol* 1988; **254**: G768-G774

32 **Cho CH,** Pfeiffer CJ, Misra HP. Ulcerative mechanism of ethanol and the action of sulphanilyl fluoride on the rat stomach *in-vivo*. *J Pharm Pharmacol* 1991; **43**: 495-498

33 **Kim HS,** Berstad A. Experimental colitis in animal models. *Scand J Gastroenterol* 1992; **27**: 529-537

34 **Hawkins JV,** Emmel EL, Feuer JJ, Nedelman MA, Harvey CJ, Kennedy AR, Lichtenstein GR, Billings PC. Protease activity in a hapten-induced model of ulcerative colitis in rats. *Dig Dis Sci* 1997; **42**: 1969-1980

35 **Grisham MB,** Granger DN. Neutrophil-mediated mucosal injury: role of reactive oxygen metabolites. *Dig Dis Sci* 1988; **33**: 6S-15S

36 **Riddel RH.** Pathology of idiopathic inflammatory bowel disease. In Kirsner JB, Shorter RG, eds. Inflammatory bowel disease. Philadelphia: *Lea and Febiger* 1988: 329-350

37 **Weiss SJ.** Tissue destruction by neutrophils. *N Engl J Med* 1989; **320**: 365-376

38 **Sedghi S,** Fields JZ, Klamut M, Urban G, Durkin M, Winship D, Fretland D, Olyaee M, Keshavarzian A. Increased production of luminal enhanced chemiluminescence by the inflamed colonic mucosa in patients with ulcerative colitis. *Gut* 1993; **34**: 1191-1197

39 **Saiki T.** Myeloperoxidase concentrations in the stool as a new parameter of inflammatory bowel disease. *Kurume Med J* 1998; **45**: 69-73

40 **Kooij A.** A re-evaluation of the tissue distribution and physiology of xanthine oxidoreductase. *Histochem J* 1994; **26**: 889-915

41 **Reynolds PD,** Rhenius ST, Hunter JO. Xanthine oxidase activity is not increased in the colonic mucosa of ulcerative colitis. *Aliment Pharmacol Ther* 1996; **10**: 737-741

42 **Kruidenier L,** Kuiper I, Lamers CB, Verspaget HW. Intestinal oxidative damage in inflammatory bowel disease: semi-quantification, localization, and association with mucosal antioxidants. *J Pathol* 2003; **201**: 28-36

43 **Buffinton GD,** Doe WF. Depleted mucosal antioxidant defences in inflammatory bowel disease. *Free Radic Biol Med* 1995; **19**: 911-918

44 **Sido B,** Hack V, Hochlehnert A, Lipps H, Herfarth C, Droge W. Impairment of intestinal glutathione synthesis in patients with inflammatory bowel disease. *Gut* 1998; **42**: 485-492

45 **Toda S,** Shirataki Y. Inhibitory effects of Astragali Radix, crude drug in oriental medicines on lipid peroxidation and protein oxidative modification by copper. *J Ethnopharmacol* 1999; **68**: 331-333

46 **Wang LX,** Han ZW. The effect of Astragalus polysaccharide on endotoxin-induced toxicity in mice. *Yaoxue Xuebao* 1992; **27**: 5-9

47 **Albina JE.** On the expression of nitric oxide synthase by human macrophages. Why no NO? *J Leuko Biol* 1995; **58**: 643-649

48 **Gaginella TS,** Kachur JF, Tamai H, Keshavarzian A. Reactive oxygen and nitrogen metabolites as mediators of secretory diarrhea. *Gastroenterology* 1995; **109**: 2019-2028

49 **Lee CI,** Liu XP, Zweier JL. Regulation of xanthine oxidase by nitric oxide and peroxynitrite. *J Biol Chem* 2000; **275**: 9369-9376

50 **Nappi AJ,** Vass E. Hydroxyl radical formation resulting from the interaction of nitric oxide and hydrogen peroxide. *Biochim Biophy Acta* 1998; **1380**: 55-63

51 **Lidington EA,** Moyes DL, McCormack AM, Rose ML. A comparison of primary endothelial cell lines for studies of immune interactions. *Trans Immunol* 1999; **7**: 239-246

52 **Sans M,** Salas A, Soriano A, Prats N, Gironella M, Pizcueta P, Elena M, Anderson D, Piqué P, Panés J. Differential role of selectins in experimental colitis. *Gastroenterology* 2001; **120**: 1162-1172

53 **McCafferty DM,** Smith CW, Granger DN, Kubes P. Intestinal inflammation in adhesion molecule-deficient mice: an assessment of P-selectin alone and in combination with ICAM-1 or E-selectin. *J Leuko Biol* 1999; **66**: 67-74

54 **Jones SC,** Banks RE, Haidar A, Gearing AJH, Hemingway IK, Ibbotson SH, Dixon MF, Axon ATR. Adhesion molecules in inflammatory bowel disease. *Gut* 1995; **36**: 724-730

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