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*Original Article*

# Effect of rhubarb polysaccharide combined with semen crotonis pulveratum on intestinal lymphocyte homing in rats with TNBS-induced colitis

Dan Li[1], Hairong Liu[2], Enze Wang[3], Fang Tang[1]

[1]*Department of Traditional Chinese Medicine, General Hospital of Tianjin Medical University, Tianjin, China;* [2]*Cangzhou Central Hospital, Cangzhou, China;* [3]*Tianjin Lerentang Pharmaceutical Factory, Tianjin, China*

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**Abstract:** This study aimed to explore the influence of the combination of rhubarb polysaccharide and semen crotonis pulveratum on lymphocyte homing in the intestinal mucosa of ulcerative colitis (UC) rats. A rat colitis model was induced by 2,4,6-trinitrobenzenesulfonic acid (TNBS). Rats were allocated to the control group, the model group, the salazosulfapyridine group, the semen crotonis pulveratum+ rhubarb polysaccharide high dose group (B+R80 group), the moderate group (B+R60 group), and the low group (B+R40 group) (all n=10). Flow cytometry detected CD3+, CD4+, CD8+ and the CD4+/CD8+ ratio in peripheral blood and the lamina propria of the intestinal mucosa. Mucosal addressin cell adhesion molecule-1 (MAdCAM-1) mRNA in intestinal mucosal tissues was monitored with RT-PCR. Lymphocyte function-related molecule (αLβ2) and intercellular adhesion molecule-1 (ICAM-1) were detected by immunohistochemistry in colon tissues. Compared with the model group, CD8+ levels in the peripheral blood from the B+R40, B+R60, and B+R80 groups increased ($P<0.05$), and the CD4+/CD8+ ratio decreased ($P<0.01$). CD4+ and CD4+/CD8+ ratio in the intestinal mucosa were increased ($P<0.01$), and the difference was most notable in the B+R40 group. The colon macroscopic damage index score decreased to different degrees ($P<0.05$), histopathologic score decreased ($P<0.01$), and the expression of MAdCAM-1 decreased. The expression of ICAM-1 was significantly decreased ($P<0.05$), but similar between groups ($P>0.05$). αLβ2 expression was significantly decreased in the B+R80 and B+R40 groups ($P<0.01$). Rhubarb polysaccharide and semen crotonis pulveratum in combination decreased damage to the intestinal mucosa by regulating homing of lymphocytes in UC rats to improve the immune function of the mucosa.

**Keywords:** Ulcerative colitis, lymphocyte homing, rhubarb polysaccharide, semen crotonis pulveratum

## Introduction

Ulcerative colitis (UC) is an idiopathic inflammation involving the rectum, colonic mucosa and submucosa [1]. Locally manifesting continuous, diffuse mucosa and submucosal inflammatory changes in the intestinal tract, generate into erosion and ulceration that can be observed by eye. There are many UC pathogenesis-related theories: genetic susceptibility and gene polymorphisms theory, environmental pathogenic theory, inflammatory factor and oxygen radical theory, and intestinal microecology theory [2]. Among the various theories, immune disorders are believed to be the main reason for UC pathogenesis, and abnormal lymphocyte homing is the pathological basis for immune disorder [3]. When UC occurs, lots of lymphocytes migrate into the area of inflammation, and more inflammatory factors and inflammatory mediators are activated, leading to an imbalance of the immune system in the intestinal mucosa. The intestinal epithelial barrier is damaged, finally resulting in intestinal mucosa injury.

The intestinal tract is the largest mucosal immune system. The directional migration of lymphocytes in the blood entering into peripheral organs or specific tissues is a very complicated process that is strictly regulated, and involves a series of interaction among addressins, such as chemotactic factors, homing receptors, and addressins. Lymphocytes are

organized into specific gut-associated lymphoid tissues such as Peyer's patches, mesenteric lymph nodes, and diffuse effector sites of the gut epithelium and lamina propria [4]. Homing receptors on the lymphocytes' surface specifically bind homing vascular addressins on the intestinal mucosa surface, and the lymphocytes then continuously migrate to inflammation sites. The lymphocyte homing receptor, includes L-selectin, α4β7 integrin, and lymphocyte function-related antigens (LFA, αLβ2). The corresponding ligands are vascular endothelial cell addressins in the organs and tissues, including mucosal vascular addressin cell adhesion molecule 1 (MadCAM-1), and intercellular adhesion molecule 1 (ICAM-1) [4]. This binding can make adhesion between endothelial cells and lymphocytes closer, and mediates lymphocytes entering into the inflammatory tissues. Therefore, specific recognition between the homing receptor and addressin is the molecular basis of lymphocyte selective homing [5-7]. Currently, therapies for UC involve blocking tumor necrosis factor-α as a method of blocking inflammation. However, this is not successful in many patients and other methods are also being investigated that target a decrease in adhesion, by correction of abnormal lymphocyte homing [8]. The search for alternative treatments for UC has led some to consider the use of methods established in traditional Chinese medicine [9, 10].

The Chinese rhubarb plant, *Rheum officinale*, possesses many medicinal effects in traditional Chinese medicine, such as bowel-relaxing, clearing heat and detoxifying, blood cooling, stasis-expelling, and it is generally used for constipation, blood heat, blood stasis, and damp-heat [11]. Tannins in *Rheum officinale* inhibit the purgative action of rhubarb anthraquinone. Therefore, *Rheum officinale* possesses double effects of purgative and antidiarrheal actions [12]. Chinese language reports suggest it also maintains the integrity of cellular structure, protects the gastrointestinal mucous membrane, and resists injury of the gastrointestinal mucous membrane [13, 14]. Rhubarb polysaccharide is one of the water-soluble components in *Rheum officinale*, possessing various bioactivities. It promotes immune system recovery balance or strengthening by stimulating mature, differentiation, and reproduction of immune cells. Rhubarb extract improves mucosal integrity and reduces ileal inflammation in rats with 5-fluorouracil-induced intestinal mucositis [15], and protects against radiation-induced intestinal mucosal injury in rats [16].

Semen crotonis pulveratum is a processed form of croton plant seeds. Its use in traditional Chinese medicine includes drastically purging cold and eliminating phlegm, treating constipation, ascites bulging, and throat impediments. Our previous study indicated that a small dose of semen crotonis pulveratum could treat diarrhea by improving absorption function of intestinal tract and reducing intestinal motility [17]. Another study found that it inhibits inflammation in UC in rats, it increased circulating Treg cells, altered cytokine production and decreased cyclooxygenase-2 (COX-2) and ICAM-1 expression [18].

The combination of *Rheum officinale* and semen crotonis pulveratum has traditionally been used to treat diarrhea. In a previous study, we found that this combination had a similar antidiarrheal effect to a small-dose of semen crotonis pulveratum, and was superior to single small-dose of semen crotonis pulveratum in adjusting serum electrolyte concentration, and improving activity of Na+-K+-ATPase in colon epithelial cells [19].

There are many different animal models for studying immunopathogenesis of the bowel [20]. A rat model of inflammatory intestinal disease induced by 2,4,6-trinitrobenzenesulfonic acid (TNBS) has been widely studied [21] Cumming School of Medicine, University of Calgary, Calgary, Alberta, Canada. We hypothesized that rhubarb polysaccharide in combination with semen crotonis pulveratum might affect immune function and intestinal lymphocyte homing in the intestinal mucosa. So, the aim of this study was to investigate the effect of rhubarb polysaccharide combined with semen crotonis pulveratum at different doses on a TNBS rat model. T lymphocyte subsets in peripheral blood and intestinal mucosa were observed, as well as MAdCAM-1 mRNA, αLβ2, ICAM-1 expression patterns and the structural damage to the intestinal mucosa tissues.

## Materials and methods

### *Experimental animals*

The experiment was approved by the Ethics Committee of Tianjin Medical University. Wistar

rats (n=60) were purchased from Academy of Military Medical Sciences aged 6-8 months with body weight 200±20 g. The rats were fed at the Institute of Radiation Medicine, Chinese Academy of Medical Sciences, and acclimated for 3 days.

*Establishment of rat UC model induced by TNBS*

The model was built as previously reported [22]. The rats were labeled with picric acid 24 hours before modeling using a random number table. The rats were then divided into the normal control group (N group), the model control group (C group), the salazosulfapyridine (SASP) group (Y group), the semen crotonis pulveratum+ rhubarb polysaccharide high dose group (B+R80 group), the moderate group (B+R60 group), and the low group (B+R40 group) (n=10). All the rats were fasted but allowed free access to water. The rats were given 10% chloral hydrate by intraperitoneal injection (3 mL/kg), and inversely placed in a fixator. A modified 8FR catheter (the catheter was cut 12 cm along the its end and the end of a 5 mL syringe whose metal needle was pulled out was sealed to the catheter with tape, a position 8 cm away from the end of catheter was marked and the stability of the catheter was validated) was slowly inserted into the rectum at 8 cm. Except for rats in the N group, the rats in other groups were slowly administrated with a TNBS enema (volume (mL)=0.8 mL × body weight (g) ÷ 200 g), followed by 0.5 mL air. The rats in the N group were given saline based on the same equation. The rats were placed upturned for 5 minutes to prevent the enema from flowing out, and then returned to their cage in a low-head-high-bottom position for observation. After recovering consciousness, they were housed together according to their grouping and returned to a normal feeding and drinking regime.

*Drug administration and sample collection*

Sulfasalazine tablets were purchased from Tianjin Medical University General Hospital, manufactured by Xinyitianping Pharmaceutical Co., Ltd, Batch No. 09150313. Rhubarb polysaccharide (RHP) and semen crotonis pulveratum were provided by Tianjin Lerentang Pharmaceutical Factory. The dose of rhubarb polysaccharide referred to previous references [23, 24]. 80 mg, 60 mg, and 40 mg of rhubarb polysaccharide was suspended in 2 mL CMS, respectively, to prepare high, moderate and low dose solution, and was given to rats at 400 mg/(kg×d), 300 mg/(kg×d), 200 mg/(kg×d). The dose of semen crotonis pulveratum referred to the previous experiments [25, 26] at concentration of $1.67\times10^{-1}$ mg/mL prepared with distilled water. The dose of salazosulfapyridine (SASP) referred to reference [27]: 100 mg drug powder was suspended into 2 mL 0.5% CMS solution. Before preparation, the coating of the tablets was completely scraped with a knife, and ground in a mortar. The ground powder was sieved with an 80-mesh sieve. The powder was further ground, and then sieved with 180 mesh until the drug was ground into very fine powder. The drug was given at 500 mg/(kg×d).

On the 4th day after modeling, the rats were administrated the appropriate drug for their group by gavage (dose mL=2 mL × body weight (g)÷200 g) for 7 days. The rats in N groups were given carboxy methyl starch sodium (CMS) solution with same volume for 7 days. At 7 days after administration, the rats were fasted but allowed water for 24 hours, and then anesthetized with 10% chloral hydrate (3 mL/kg) via intraperitoneal injection. The anesthetized rats were placed on the platform in supine position, and skin preservation was conducted on one side of groin. The colon tissue was dissociated, and the rats were dissected along the mesentery. The tissues were washed in saline at 4°C 3 times, and excess water was absorbed by filter paper. The occurrence of colon injury was observed, recorded, scored, and photographed. Colon tissue ulcer lesion (1 cm) were removed and fixed in 10% neutral formaldehyde. The remaining tissue was placed in a filter paper, and one end was fixed. The intestinal mucosal tissue was slightly scraped. After weighing, 100 mg of the tissue were homogenized. The remaining tissue was marked, placed into liquid nitrogen immediately, and transferred into -80°C for storage.

*Colon macroscopic damage index (CMDI) score*

Referring Tsune et al.'s standard [27], the score was estimated by eye. The score was the sum of adhesion, ulcer formation and inflammation condition as shown in **Table 1.**

**Table 1.** CMDI scoring standard

| Macroscopic expression | Score |
|---|---|
| Adhesion | |
| No | 0 |
| Light (easily peeling between colon and other tissues | 1 |
| Severe | 2 |
| Ulcer formation and inflammation condition | |
| No | 0 |
| Local hemorrhage, no ulceration | 1 |
| One ulcer without hemorrhage or bowel wall thickening | 2 |
| Two ulcers with inflammation | 3 |
| More than two ulcers with inflammation | 4 |
| More than two ulcers and/or inflammation >1 cm | 5 |
| Ulcers and/or inflammation >2 cm, 1 point increase for 1 cm increase in lesion range | 6-8 |

**Table 2.** Histopathologic (HS) score standard

| Item | 0 | 1 | 2 | 3 |
|---|---|---|---|---|
| Epithelial damage and ulcer formation | No | Erosion | Dot ulcer | Flake ulcer |
| Ulcer depth | No | Submucosa | Muscular layer | Serosal layer |
| Inflammatory cell infiltration | No | Infiltration cell counting <10% in each high-magnification view | Infiltration cell counting 10~25% in each high-magnification view | Infiltration cell counting 25~35% in each high-magnification view |
| Infiltration depth | No | Submucosa | Muscular layer | Serosal layer |

*Colon histopathologic score*

After fixing in 10% neutral formaldehyde solution, the colon tissues were embedded in paraffin, sliced into 5 μm slices, stained with hematoxylin and eosin (H&E), observed under optical microscope, and scored. The scoring standard is shown in **Table 2**.

*Lymphocytes in peripheral blood and intestinal mucosa detected by flow cytometry*

Fluorescein isothiocyanate (FITC) labeled mouse anti-rat CD4 monoclonal antibody (BD, USA), allophycocyanin (APC) labeled mouse anti-rat CD3 monoclonal antibody (BD, USA), and R-phycoerythrin (PE) labeled mouse anti-rat CD8 monoclonal antibody (BD, USA) were added at the suggested concentrations respectively into a flow cytometry tube, and mixed well. Whole blood was processed for anticoagulation, and 50 μL was added into each tube ($1\times10^6$). The blood was completely mixed, and placed at room temperature avoiding light for 15 minutes. 100 μL red blood cell lysis buffer was added to each tube, and then with distilled water 2 mL at room temperature avoiding light for 5 minutes. The blood was centrifuged at 1300 rpm for 5 minutes at 4°C. The supernatant was discarded, and 2 mL PBS was used to wash the precipitate until no red could be seen. Then, the tube was centrifuged at 1300 rpm for 5 minutes at 4°C. The supernatant was discarded, and PBS 400 μL was added to resuspend the pellet, after which the sample was detected within 1 hour.

Intestinal mucosal tissue was placed in a soft tube and pre-cooled PBS was added. The tissue was repeatedly sucked and blown using a 1 mL syringe equipped with a G12 needle. The tissue suspension was filtered through a 100 mesh filter, and the filtrate was collected and filtered through a 200 mesh filter to prepare a single cell suspension. The suspension was centrifuged at 2000 rpm for 10 minutes at 4°C. The supernatant was discarded, and the precipitate was mixed with pre-cooled PBS. The cell density was adjusted at $1\times10^6$/mL after counting. The antibody was diluted according to the instruction, and each tube was added with CD4-FITC, CD3-APC, and CD8-PE antibody (1T each). After mixing, the single cell suspension was added into the loading tube for flow cytometry. The cells were completely mixed, and placed at 4°C for 30 minutes. Then, the tube was centrifuged at 800 rpm for 5 minutes at 4°C. The supernatant was discarded, and the

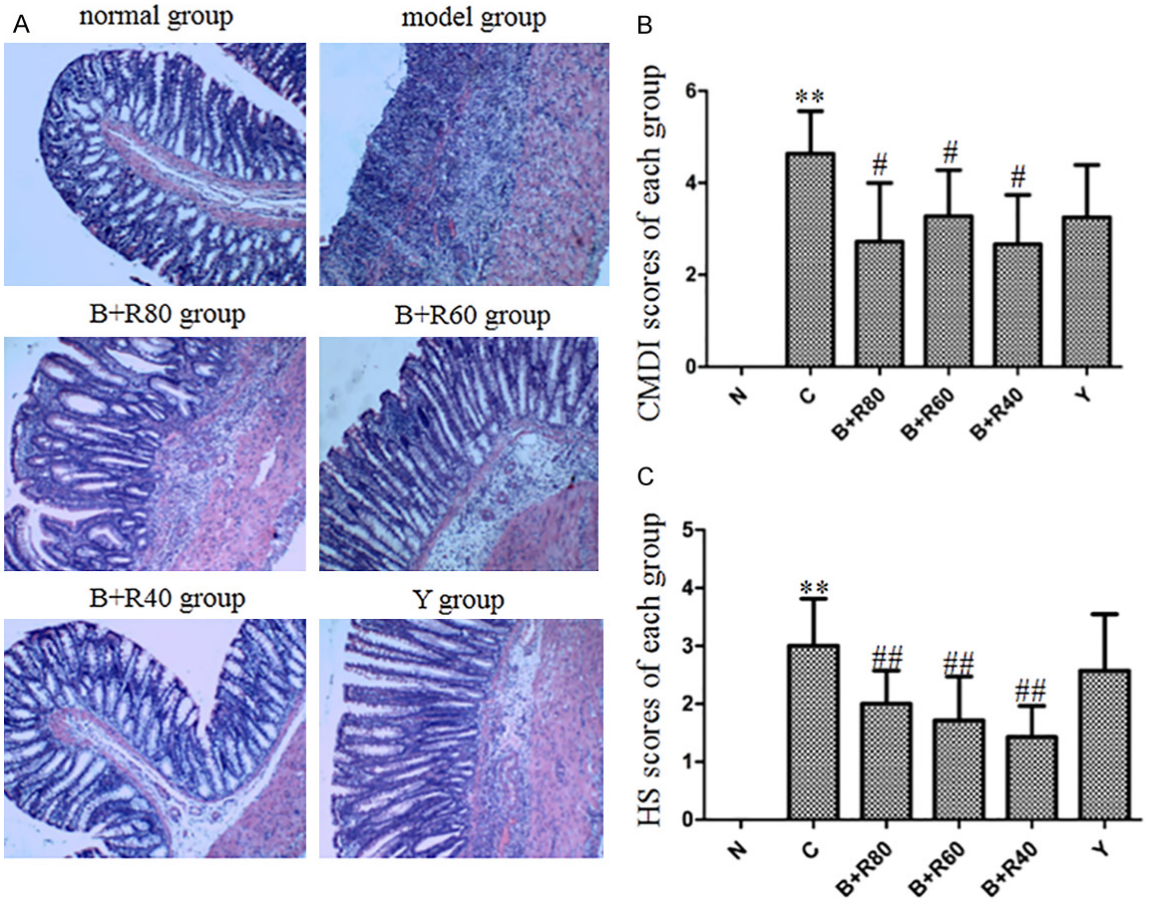


**Figure 1.** Damage to the colon of rats in the different groups. A: Representative images of the micrographs for the normal control group, the model group, the combination dose groups (B+R40, B+R60, B+R80), and the positive control group (Y). B: CMDI scores for each group. C: HS scores for each group. ***P*<0.01 vs. normal control group. ##*P*<0.01, #*P*<0.05 vs. model group.

pre-cooled PBS 400 μL was added into the tube, which was detected within 1 hour.

*MAdCAM-1 expression in colon tissue detected by reverse transcription PCR*

Total RNA was extracted from colon tissues with a Trizol total RNA extraction kit (Invitrogen, USA). Reverse transcription was performed with the reverse transcription kit (Transgene, China). Amplification used the amplification kit (Transgene, China). GAPDH was used as the internal control. The primer sequences were as following: MAdCAM-1 forward 5'-GAAATCCACCAGAACCCAGA-3' and reverse 5'-AGTAGGCAAGGAAGGCGA-3'; GAPDH forward 5'-GGTGCTGAGTATGTCATGGA-3' and reverse 5'-CTTCTGAGGCAGTGATGG-3' were synthesized by Sangon Biotech (Shanghai) Co., Ltd. The reaction conditions were the following: 94°C for 3 minutes, 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute, 72°C for 5 minutes. A total of 35 cycles were used for each PCR amplification run. Goldview II nucleic acid staining agent (Beijing Solarbio Science & Technology Co., Ltd.) was used to visualize the amplified DNA on an agarose gel using an imager. The obtained image was firstly analyzed by Image J, and the gray values of each sample and internal reference bands were calculated, based on which the ratio was calculated (relative expression of target mRNA=grey value of sample band/gray value of GAPDH band).

*αLβ2 and ICAM-1 expression in colon tissue detected by immunohistochemical staining*

The fresh intestinal mucosal tissues were peeled as soon as possible after death, fixed in paraformaldehyde, followed by paraffin embed-

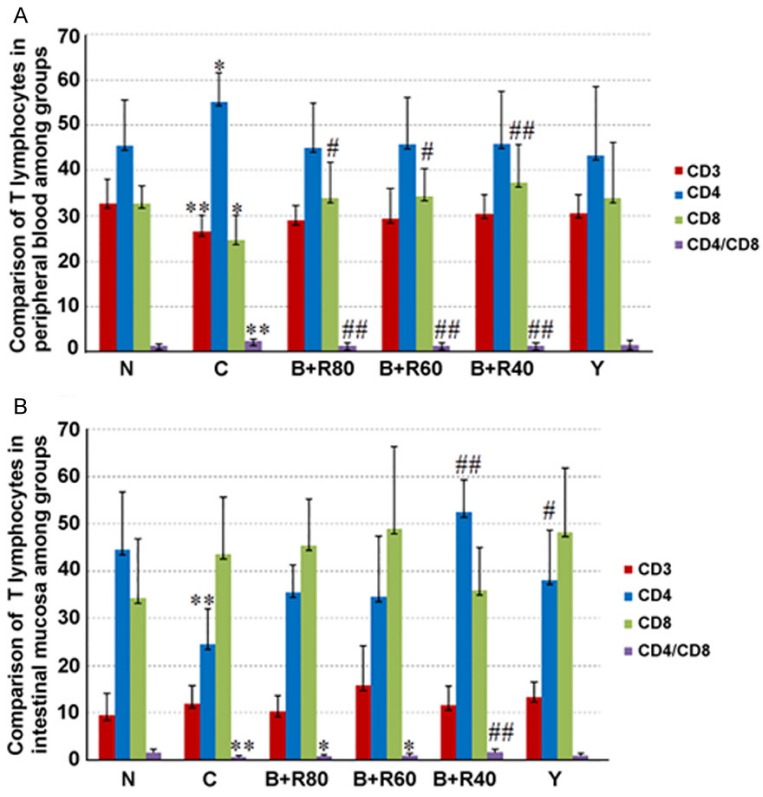


**Figure 2.** T-lymphocytes in the peripheral blood and intestinal mucosa. A: Graph representing the different lymphocyte levels in the peripheral blood of the normal control group, the model group, the combination dose groups (B+R40, B+R60, B+R80), and the positive control group (Y). B: Graph representing the different lymphocyte levels in the intestinal mucosa in each of the groups. ***P*<0.01, **P*<0.05 vs. normal control group. ##*P*<0.01, #*P*<0.05 vs. model group.

ding and sectioning, and sliced to a thickness of 3-5 μm. An immunohistochemical test kit (ZSGB-Bio, China, Lot No.: SP-9000, Batch No.: WK160327) was used to detect protein expression with rabbit anti-rat CD18 primary antibody (BEIJING BIOSYNTHESIS BIOTECHNOLOGY CO., LTD., Lot No.: bs-0503R, Batch No.: AB080-11569) and rabbit anti-rat CD54 primary antibody (BEIJING BIOSYNTHESIS BIOTECHNOLOGY CO., LTD., Lot No.: bs-0608R, Batch No.: AE090704). With the concentrated DAB kit (ZSGB-Bio, China, Lot No.: ZLI-9017) to stain nuclei. Primary antibody working solution was added in the experimental group, and PBS was added into the control group. Then, they were placed in a humid box overnight at 4°C. They were washed with PBS for 5 minutes 3 times, then reagent B (biotinylated secondary antibody working solution) was added, incubated for 20 minutes at 37°C, and washed with PBS for 5 minutes 3 times. Then, horseradish peroxidase labeled streptomycin working solution was added, incubated at 37°C for 20 minutes, and washed with PBS for 5 minutes 3 times. The tissue slides were processed for dehydration, permeabilization and mounting, prior to observation under an optical microscope.

A double blind method was used to observe the slices. The regions with uniform staining were chosen. Three visual fields were randomly selected in each slice, and 5 slices were observed in each group. Image-Pro Plus 6.0 was used to calculate the integrated optical density (IOD) of positive cells in each visual field. The average value was used as expression level of each slice.

*Statistical analysis*

SPSS 19.0 (IBN Corp., USA) was used to analyze the data. Measurement data are expressed as mean ± SD. One-way analysis of variance and independent-samples T test were used. Least significant difference (LSD) test was used for pairwise comparison of groups, if equal variance. If not, the comparison was analyzed by Tamhane's T2 test. P<0.05 was termed as statistical significance.

## Results

*Comparison of CMDI and HS scores among groups*

Damage to the colon was measured with CMDI and HS. Compared with normal control group, the CMDI and HS scores in the model group were significantly increased (P<0.01) suggesting that the model had been successful. Compared with the model group, after drug intervention, CMDI scores were significantly decreased to different degrees (P<0.05). HS scores were also significantly decreased (P< 0.01). Comparison among the three groups

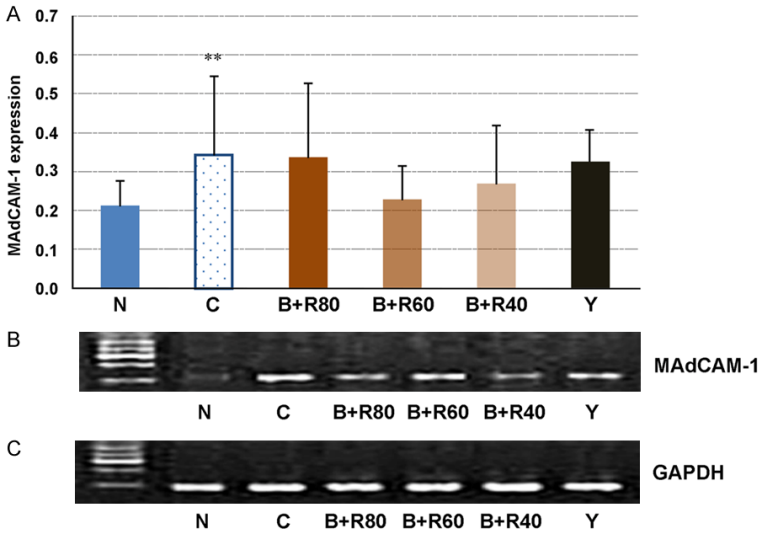


**Figure 3.** MAdCAM-1 mRNA levels in the intestinal mucosa. A: Graph representing the quantified MAdCAM-1 mRNA levels in the intestinal mucosa of the normal control group, the model group, the combination dose groups (B+R40, B+R60, B+R80), and the positive control group (Y). B: Agarose gel image showing the DNA bands after RT-PCR of the MAdCAM-1 mRNA from intestinal mucosa of the different groups. C: Agarose gel image of the GAPDH loading control. ***P*<0.01 vs. normal control group.

that received different doses of rhubarb polysaccharide indicated that there was no significant dose-effect relationship, but CMDI and HS scores in the B+R40 group showed the most decrease (**Figure 1**). These results show that combination treatment decreased damage to the colon.

*Comparison of T lymphocytes among groups*

Compared with the normal control group, the model group peripheral blood CD3+ proportion was significantly decreased (P<0.01), and CD4+ and CD4+/CD8+ ratio were significantly increased (P<0.05, P<0.01). CD8+ T lymphocyte proportion significantly was decreased (P<0.05) (**Figure 2A**). The CD4+ level and CD4+/CD8+ ratio of the intestinal mucosa were also significantly decreased (P<0.01) (**Figure 2B**). Compared with the model group, after drug intervention, CD8+ levels in the peripheral blood of the 3 combination dose groups were significantly increased (P<0.05, P<0.01), especially in the B+R40 dose group. CD4+ showed a slight decrease, and CD4+/CD8+ ratio was significantly decreased (P<0.01), which was similar to the Y group who had received the established salazosulfapyridine UC treatment regimen (P>0.05) (**Figure 2A**). Compared with the T lymphocyte subset level of the intestinal mucosa in the model group, CD4+ levels (P<0.01, P<0.05) and CD4+/CD8+ ratio (P<0.01, P>0.05) in the B+R40 and Y groups were significantly increased. The CD4+/CD8+ ratio in the B+R80 and B+R60 dose groups increased, but the difference with the control group remained significant (P<0.05) (**Figure 2B**). These results suggest that lymphocytes that are recruited to inflammation in the model group were decreased when the animals received the combination treatment, and the treatment with low dose rhubarb polysaccharide was the most effective.

*Comparison of MAdCAM-1 expression among groups*

The results of RT-PCR indicated that compared with the normal control group, MAdCAM-1 mRNA levels in the model group significantly increased (P<0.01). This showed that as expected the rat model increased MAdCAM-1 mRNA levels, indicating that the model group was expressing the ligand for lymphocyte homing in their tissue. After drug intervention, MAdCAM-1 expression levels in the 3 combination dose groups decreased compared to the model group, and those of B+R60 and B+R40 were close to the levels of the normal control group (P>0.05). Comparison among the 3 rhubarb polysaccharide dose groups indicated that B+R60 and B+R40 were superior to the B+R80 group. The 3 groups also had significantly down-regulated MAdCAM-1 expression compared with Y group (**Figure 3A**) showing that the combination dose has a greater influence on the mRNA levels than standard treatment. The gel electrophoretograms of MAdCAM-1 and internal reference are illustrated in **Figure 3B**, **3C**. These results show that the combination treatment decreases expression of MAdCAM-1 mRNA and so these tissues are likely to have lower expression of the ligand for recruiting lymphocytes and in this aspect the combination treatment might be better than standard treatment.

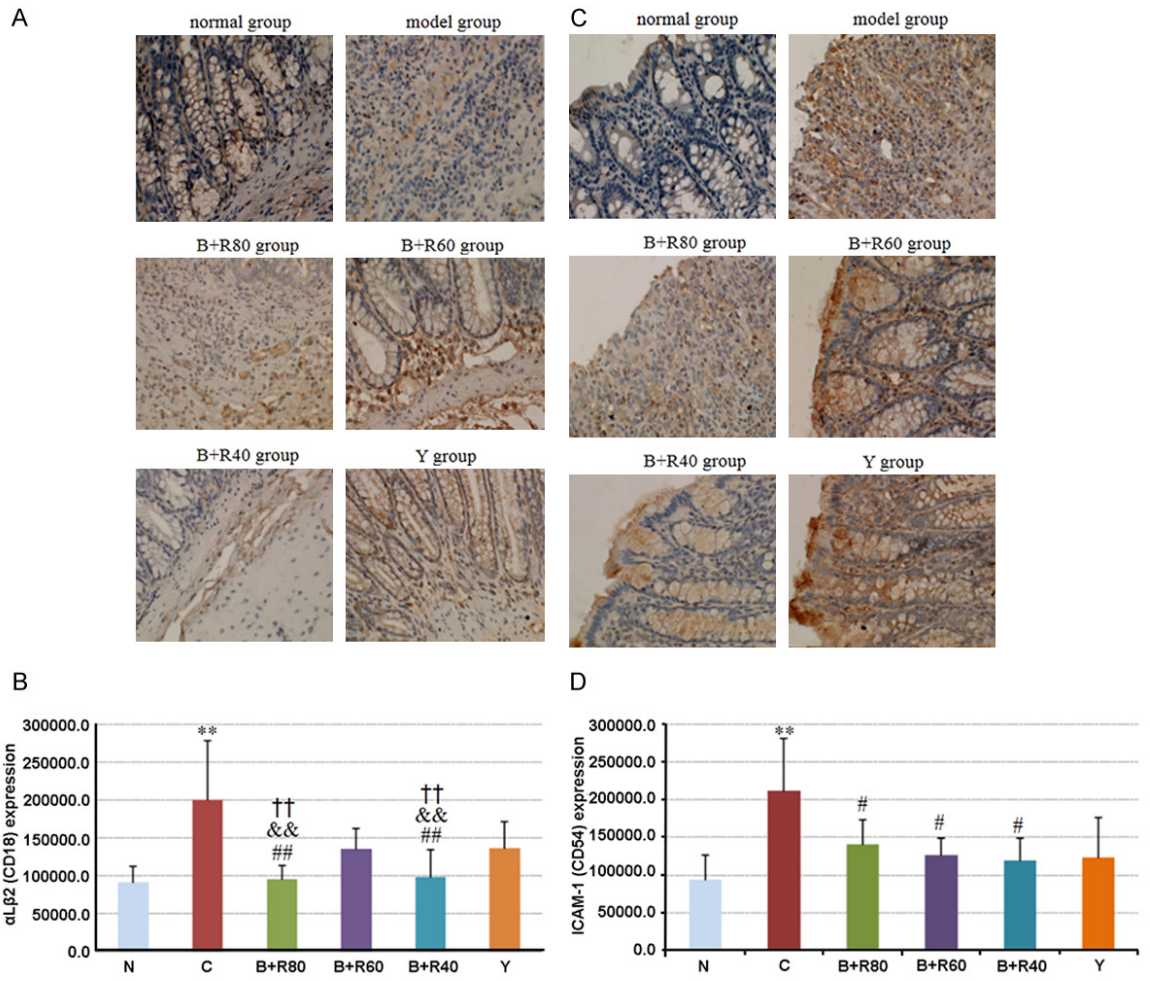


**Figure 4.** Immunohistochemical staining of αLβ2 and ICAM-1 in the different groups. A: Representative images of the micrographs probed with αLβ2 antibodies for the normal control group, the model group, the combination dose groups (B+R40, B+R60, B+R80), and the positive control group (Y). B: Quantified expression levels of αLβ2 for each group. C: Representative images of the micrographs probed with ICAM-1 antibodies for the normal control group, the model group, the combination dose groups (B+R40, B+R60, B+R80), and the positive control group (Y). D: Quantified expression levels of ICAM-1 for each group. ***P*<0.01 vs. normal control group. ##*P*<0.01, #*P*<0.05 vs. model group. &&*P*<0.01 vs. B+R60. ††*P*<0.01 vs. B+R60.

*Comparison of αLβ2 and ICAM-1 expression among groups*

Epression of αLβ2 is mainly distributed in the epithelial basement membrane of UC colon tissues or collagen of connective tissues [28]. Positive staining was mainly located in the cell membrane, and DAB staining showed pale brown granules. αLβ2 was expressed in the colon intestinal mucosa at low levels, but staining in the model group showed a strong positive signal (**Figure 4A**). ICAM-1 is mainly expressed in vascular endothelial cells, leukocytes and the top of epithelial membrane. Positive staining was mainly in the cell plasma and/or cell membrane. DAB staining showed as pale brown, and ICAM-1 expression level was low in the colon tissues of the normal control group or not expressed at all. The staining of colon tissue in the model rats was strong (**Figure 4C**). After drug therapy, the staining degrees of αLβ2 and ICAM-1 were alleviated to different degrees.

Compared with the normal control group, αLβ2 and ICAM-1 positive expression in the colon tissues was significantly higher in the model group (P<0.01). Compared with model group, the ICAM-1 positive expression in the 3 combination dose groups was significantly decreased (P<0.05) (**Figure 4D**). Positive αLβ2 expression

in B+R80 and B+R40 groups was significantly decreased (P<0.01) and was close to the normal control group. However, the B+R60 group was not significantly different to the model group (P>0.05) (**Figure 4B**). Comparison among the 3 combination dose groups indicated that the ICAM-1 expression was not significantly different between them (**Figure 4D**). For the down-regulation of αLβ2 positive cell expression, B+R80 and B+R40 groups were superior to that of the B+R60 group (P<0.01) (**Figure 4B**). Compared with positive control drug group (Y group), there was no significant difference in ICAM-1 expression for any of the 3 combination dose groups (P>0.05) (**Figure 4D**). For αLβ2 expression, the down-regulation effect of B+R80 and B+R40 groups was superior to the Y group (P<0.01) (**Figure 4B**).

## Discussion

Currently, the pharmacology of semen crotonis pulveratum and *Rheum officinale* in combination has not been reported, but previous study suggests that this combination might have a beneficial effect for treating UC. To investigate this, we designed a study using a rat model of inflammatory intestinal disease. T lymphocyte subsets in peripheral blood and intestinal mucosa were observed, as well as MAdCAM-1 mRNA, αLβ2, ICAM-1 expression patterns and the structural damage to the intestinal mucosa tissues. The results show that model rats receiving combination therapy with rhubarb polysaccharide and semen crotonis pulveratum had less damage to the intestinal mucosa, and this was accompanied by decreased lymphocyte recruitment to from the blood to the mucosa, which reflected the decreased integrin and ligand expression. These results suggest that the combination treatment might relieve the symptoms of UC by regulating lymphocyte homing.

Compared with the normal control group, the CMDI and HS scores in the TNBS model groups were significantly increased, so the model was apparently successfully established. However, the general condition and colon general scores of all four drug administration groups were significantly decreased. The combination of semen crotonis pulveratum and rhubarb polysaccharide effectively improved the symptoms of UC rats and injury of the colon intestinal mucosa. Furthermore, each of the 3 doses had similar effectiveness compared with the positive control group treated with standard UC treatment (SASP).

In this study, we used the T lymphocyte subset amount changes in peripheral blood and intestinal mucosa as quantitative indices to evaluate intestinal mucosa injury degree, lymphocyte homing status, and intervention efficacy. The results showed that CD4+ and CD4+/CD8+ ratio in peripheral blood were increased and CD8+ was decreased, suggesting T lymphocyte system was in an active state. A high degree of lymphocyte homing, release of inflammatory factors and inflammatory media made the model rats produce a strong inflammatory response. Decreased CD4+ level and CD4+/CD8+ ratio in the intestinal mucosa and increased CD8+ suggested that the colon mucosa was severely injured, as cytotoxic T lymphocytes were significantly increased [29]. In this study the combination of rhubarb polysaccharide and semen crotonis pulveratum effectively improved CD8+ levels in the peripheral blood of UC rats, decreased CD4+ level and CD4+T/CD8+ ratio. It further increased the mucosal CD4+ level and CD4+/CD8+ ratio and activated the T cell immune response to improve immune function, protect, and intervene mucosal barrier injury, playing a similar role to SASP. The combination of rhubarb polysaccharide with low dose and semen crotonis pulveratum had the most effect on improving the inflammatory response.

MAdCAM-1 is the ligand of integrin α4β7. The specific binding of MAdCAM-1 with α4β7 is very important for intestinal lymphocyte homing. As expression of this ligand is considered important for UC, irritable bowel disease, and Crohn's disease [30], previous studies have considered methods of reducing MAdCAM-1 expression as therapy for these conditions. For example antisense therapy [31] and immunotherapy [32] have shown promise. The results of this study showed that MAdCAM-1 mRNA was increased in the rats of model group compared to normal controls. Showing that the injury degree of colon tissues had a positive correlation with MAdCAM-1 expression. After therapy of rhubarb polysaccharide and semen crotonis pulveratum, expression of MAdCAM-1 mRNA in the colon mucosal tissues decreased, suggesting that expression of MAdCAM-1 on the surface of endothelial cells of colon mucosal tissues was

downregulated. This would be expected to decrease T-lymphocytes entering into intestinal inflammatory sites, and reduce the UC inflammatory response.

During the occurrence and development of UC, infiltration of many inflammatory cells, increased release of inflammatory factors, and inflammatory media stimulates over-expression of ICAM-1 in the endothelial cells of the intestinal mucosa [33]. Simultaneously, it causes activation of LFA-1 (αLβ2), and further binds with LFA-1 and ICAM-1, making lymphocytes adhere to endothelial cells, and attracting more lymphocytes aggregation into inflammatory sites. These lymphocytes continuously release lots of inflammatory medium resulting in damage to intestinal mucosal tissue [34]. Therefore, ICAM-1 and LFA-1 play critical roles in immune cell adhesion, directed migration and tissue damage during UC. The results of this study indicate that expression of LFA-1 and ICAM-1 in the model group was increased, which might be one of the reasons for abnormal homing of inflammatory cells into inflammatory tissues and promotion of the UC inflammatory response amplification and migration. Rhubarb polysaccharide and semen crotonis pulveratum downregulated LFA-1 and ICAM-1 expression in the colon tissues, showing that combination treatment could block cell adhesion and activation and reduce lymphocyte over-homing in intestinal mucosal tissues.

This study shows that the combination method might be promising for treatment of UC. However, there are still some limitations. There were no results showing whole MAdCAM-1, αLβ2, and ICAM-1 mRNA and protein. Whether the cytokines generation is increased or whether the pro-inflammatory factors are expressed was not detected. Additionally, there is no clear dose-effect relationship between rhubarb polysaccharide and semen crotonis pulveratum, which needs further exploration.

In conclusion, combination use of rhubarb polysaccharide and semen crotonis pulveratum improved immune function of mucosa. The degree of injured intestinal mucosa was decreased by promoting regulation of lymphocyte homing. This suggests that the combination of rhubarb polysaccharide and semen crotonis pulveratum might be an effective treatment for UC after further study.

## Disclosure of conflict of interest

None.

**Address correspondence to:** Dr. Fang Tang, Department of Traditional Chinese Medicine, General Hospital of Tianjin Medical University, 154 Anshan Road, Heping District, Tianjin 300052, China. Tel: +86-13821185980; Fax: +86-13821185980; E-mail: zhongyi3599@126.com

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