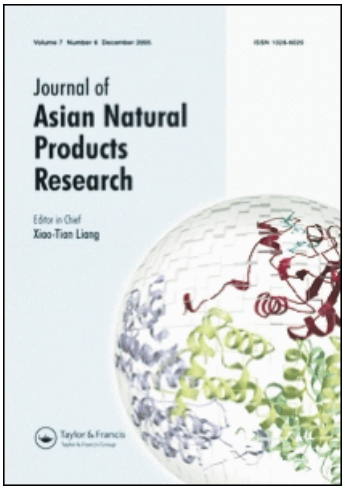

Journal of Asian Natural Products Research
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713454007

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Online publication date: 08 November 2010

To cite this Article Zhao, Wen-Chang , Song, Li-Jun and Deng, Hong-Zhu(2010) 'Effect of sophoridine on dextran sulfate sodium-induced colitis in C57BL/6 mice', Journal of Asian Natural Products Research, 12: 11, 925 — 933
To link to this Article: DOI: 10.1080/10286020.2010.505188
URL: http://dx.doi.org/10.1080/10286020.2010.505188

Journal of Asian Natural Products Research
Vol. 12, No. 11, November 2010, 925–933

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(*Received 26 March 2010; final version received 28 June 2010*)

Sophoridine (SRI), one of the quinolizidine alkaloids, is a new anticancer drug with noticeable antitumor action and lower toxicity. To our knowledge, there is no report about its effect on colitis. Repeated colitis was induced by administration of four cycles of 4% DSS. The severity of colitis was assessed on the basis of clinical signs, colon length and histology scores. Moreover, cecum secretory immunoglobulin A (sIgA) and plasma haptoglobin (HP) were analyzed by enzyme-linked immunosorbent assay and ICAM-1, and macrophage migration inhibitory factor (MIF) gene expression was analyzed by quantitative reverse transcriptase real-time polymerase chain reaction using SYBR Green I. SRI administration significantly attenuated the damage and caused substantial reduction of the rise in plasma HP, and maintained the level of cecum sIgA. SRI inhibited the ICAM-1 gene expression and had no effect on MIF gene expression. In conclusion, for the first time, the activity of SRI on DSS-induced colitis mice was investigated, which suggests that SRI could be an attractive therapeutic option in the treatment of inflammatory bowel disease.

Keywords: sophoridine; inflammatory bowel disease; dextran sulfate sodium

1. Introduction

Inflammatory bowel disease (IBD), ulcerative colitis (UC), and Crohn's disease are chronic immuno-inflammatory disorders of the gastrointestinal tract, in which rich intestinal antigen and bacterial metabolites such as LPS and DAP lead to an inappropriate and exaggerate mucosal immune response [1,2]. The dysregulated mucosal immune response associated with subsequent activation of these cells, such as lymphocytes and macrophages, causes a self-augmenting cycle of proinflammatory cytokine production, cell recruitment, and inflammation [3–5]. As we know, most current therapies in clinic for IBD are glucocorticosteroids, 5-aminosalicylate, immunosuppressive agents, anti-TNFα monoclonal antibody, etc. However, there are some patients with IBD, who are refractory even to the combined use of these agents or steroid-resistant UC. Furthermore, one of the most serious complications of IBD is colorectal cancer. Therefore, the ever-increasing incidence rate of IBD during recent decades, combined with the limited efficacy and potential adverse effects of current treatments, explains the real need for seeking more specific and selective methods for treating these diseases.

Sophoridine (SRI) extracted from the Chinese herb *Sophora alopecuroides* L. is one of the main alkaloids, which has

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ISSN 1028-6020 print/ISSN 1477-2213 online

DOI: 10.1080/10286020.2010.505188
http://www.informaworld.com

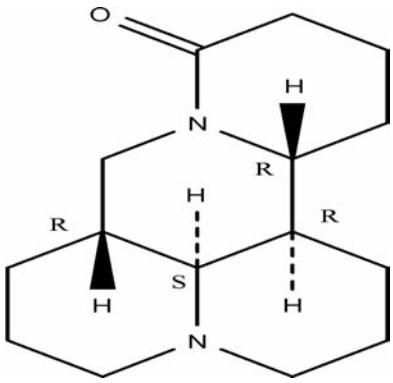

Figure 1. Chemical structure of SRI.

pleiotropic pharmacological effects including antitumor, immunomodulation, and central nervous system sedation, and so on. Its chemical structure is shown in Figure 1 [6,7]. Recently, our previous work indicated that total alkaloids of *S. alopecuroides* L. (containing 43.8% SRI determined by HPLC) could yield a protective effect against colitis in 2,4,6-trinitrobenzene sulfonic acid/ethanol-inducing rat via antioxidation [8]. For further research work, we hypothesize that SRI might exert its beneficial effect on colitis.

Therefore, the main objective of our work was to investigate the anti-inflammatory effect of SRI on a DSS-induced colitis model in C57BL/6 mice, which produces inflammation limited to the colonic mucosa that is more closely related to human UC.

2. Results and discussion

2.1 General observations

Previous experiment demonstrated that in DSS-induced colitis, the severity of inflammation is DSS load-dependent, and that a critical DSS load greater than or equal to 30 mg/g body weight is required to reliably induce colitis in the C57BL/6 strain [9]. In our experiment, the treated mice loaded DSS no less than 40 mg/g (body weight per day), data were not shown, and there were no mice death during the experiment. As shown in Figure 2, the percent weight change of DSS-induced colitis had obviously decreased and colitis gave rise to obvious bloody stools, perianal bleeding, and weak strength after the second DSS cycle. The inflammatory changes of the intestinal tract were associated with a significant augment ($p < 0.01$) of weight/length of the mice colon, as shown in Table 1. The colon of DSS-treated mice appeared edematous, and the intestine wall was thick, flare, and became shortened depicting fibrotic thickening of tissue, and its content was soft, non-forming.

SRI treatment in DSS mice significantly reduced the loss in body weight, the stool consistency scores, and fecal occult blood score of macroscopic damage, as shown in Figure 2. No significant increase in the weight/length of the mice colon and spleen index was found in the SRI group compared to the normal control group ($p > 0.05$). Also, there was no significant difference in food and water intake between groups during the whole experiment.

2.2 Histopathological examinations

On histological examination of the colon from normal mice, the histological features of the colon were typical of a normal structure. In DSS-treated mice, the inflammation extended through the mucosa, muscularis mucosae, and submucosa. Extensive granulation tissue with the presence of fibroblasts and lymphocytes, leukocytes, and diffuse inflammatory infiltrates was apparent. In some sections of ulcerated areas, necrotic tissue adjacent to surface cells could be observed. The mucosa adjacent to ulcers showed grossly elongated crypts. Furthermore, severe and extensive denudation of the surface epithelium (erosions) and mucodepletion of glands appeared. After administration of SRI, the colonic histopathology was

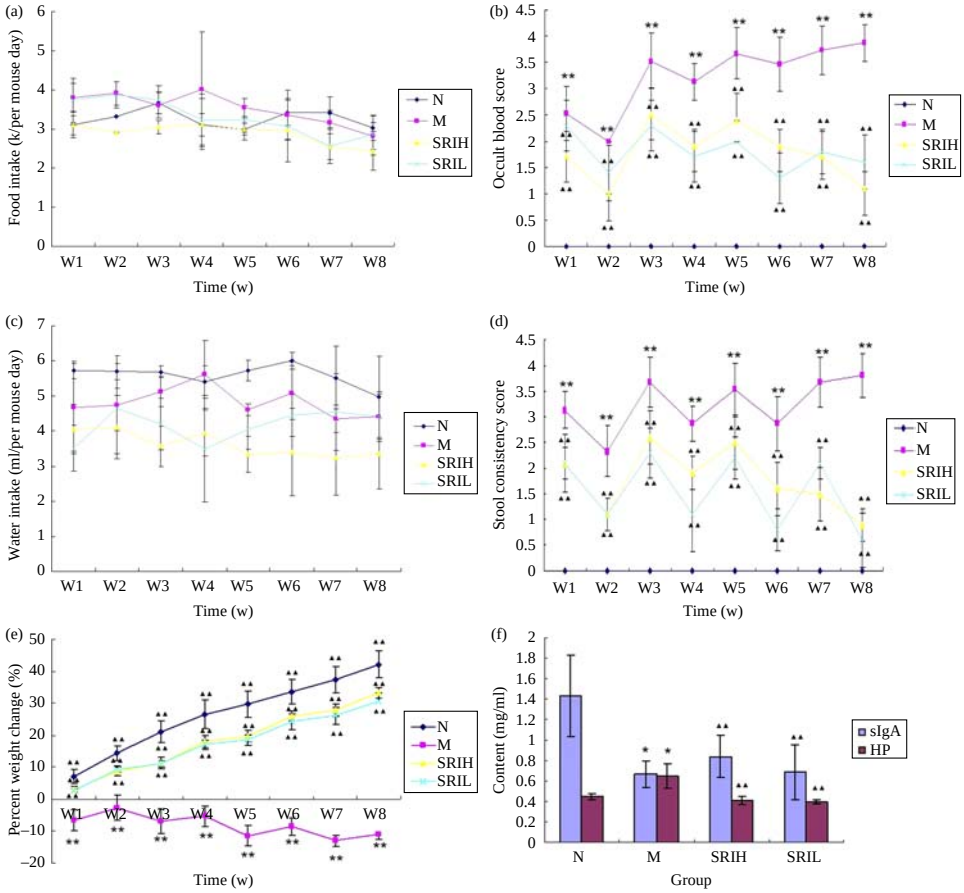

Figure 2. Effect of SRI on DSS-induced colitis C57BL/6 mice. (a) Time course of food intake. Weekly changes in food intake. (b) Weekly changes in occult blood scores. (c) Time course of water intake. Weekly changes in water intake. (d) Weekly changes in stool consistency scores. (e) Time course of percent body weight change. Weekly changes in body weight. Body weight change was calculated by (weight at weekX − week0/weight at week0) × 100. (f) Effect of SRI on the cecum sIgA and plasma HP content. Data are expressed as mean ± SD. (**) $p < 0.01$ vs. normal. (*) $p < 0.05$ vs. normal; (▲▲) $p < 0.01$ vs. DSS control. (▲) $p < 0.05$ vs. DSS control.

dramatically reduced, and there was an attenuation of morphological signs of cell damage. The colonic mucosa showed ulcers in the process of healing, evolving to a more chronic inflammatory infiltrate, with mononuclear predominance and initiation of a repair process. The mice of 25 mg/kg SRI group showed less eosinophilic infiltrate and mucous damage, as shown in Figure 3.

2.3 Scanning electron microscopy observations

Scanning electron microscopy (SEM) observations of the colonic mucosa in normal mice showed well-defined concave grooves, and regular-shaped crypt openings containing mucin-like material. Mice that underwent the four cycles of DSS administration showed widened grooves, dilatations of glandular crypts losing their

Table 1. The effect of SRI on clinical variables of C57BL/6 mice with chronic colitis induced by four-cycle DSS exposure.

Groups	*n*	Histological grading	Spleen index (g/g)	Ratio of colon weight and colon length (g/cm)
Normal	11	0.00 ± 0.000	0.0023 ± 0.00032	0.027 ± 0.0036
DSS	15	13.47 ± 0.834**	0.0089 ± 0.0032**	0.038 ± 0.0027**
SRIL	10	7.92 ± 1.165$^{▲▲}$	0.0029 ± 0.00032$^{▲▲}$	0.024 ± 0.0029$^{▲▲}$
SRIH	10	8.01 ± 0.769$^{▲▲}$	0.0031 ± 0.00044$^{▲▲}$	0.024 ± 0.0019$^{▲▲}$

Notes: Colonic parameters such as histological grading, spleen index (g/g), ratio of colon weight and colon length (g/cm) in the DDS group were significantly different from normal controls. Especially, there was statistical significance between SRIL, SRIH vs. DSS group. Data are expressed as mean ± SD. (**) $p < 0.01$ vs. normal. (▲▲) $p < 0.01$ vs. DSS control.

regular shape by assuming fissure-like aspects, petal-like swelling, and depletion of goblet cells, leaving an irregular crater-like area. A widely damaged surface epithelium with a dishomogeneous distribution of microvilli characterized the inflamed mucosa (Figure 4). After SRI treatment, the SEM appearance of the whole colonic mucosa showed the surface epithelium with a dishomogeneous distribution of microvilli, and some small lymphocyte-like cells were observed.

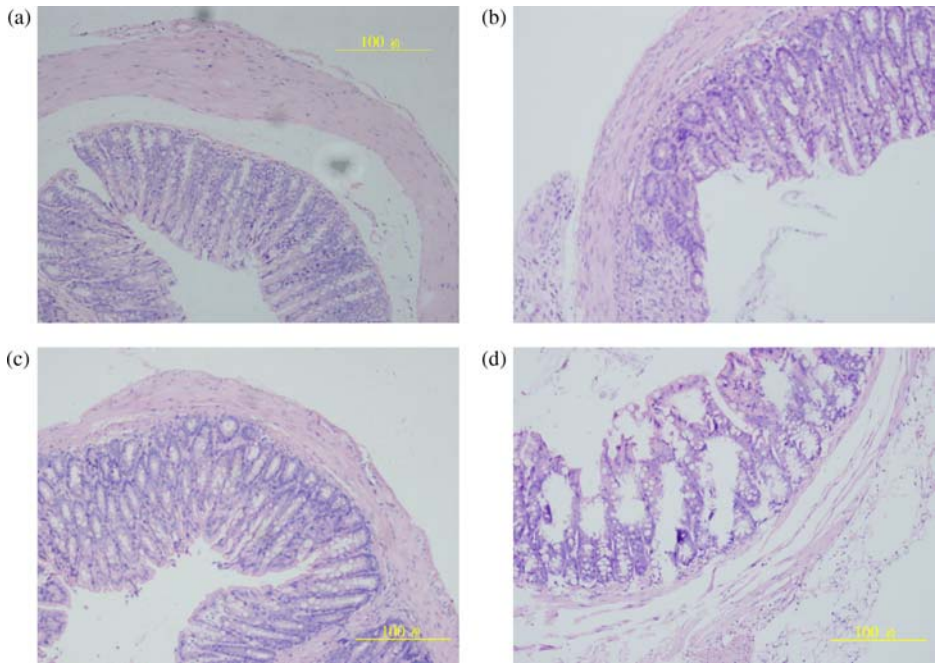

Figure 3. Representative micrographs showing the histopathology of DSS colitis mice stained with hematoxylin and eosin. Original magnification 200 ×. (a) Noncolitic group showing the normal histology of the mice colon. (b) DSS control group showing epithelial injury, focal loss of epithelial glands, extensive intestinal ulceration with abundant inflammatory infiltrate in the lamina and submucosa, mucosa depletion, and edema. (c, d) SRIH and SRIL showed an attenuation of morphological signs of cell damage, epithelial regeneration, and decrease in inflammatory cell infiltration.

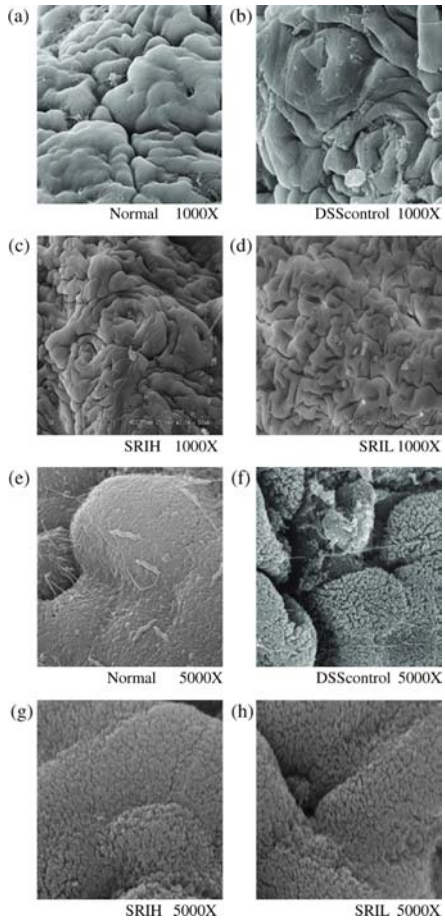

Figure 4. SEM. (a, e) Control mice: the microphotograph showed a normal aspect of the colonic mucosa. (b, f) Colonic mucosa of DSS mice: focal alteration of epithelial cells and slight dilatation of apex of crypts, dilatation of glandular crypts, and depletion of goblet cells were visible. The damaged colonic mucosal surface fissure-like aspects, petal-like swelling, and a complete subversion of its architecture were observed. (c, d, g, h) SRIH and SRIL showed the surface epithelium with a dishomogeneous distribution of microvilli, and some small lymphocyte-like cells were observed. Original magnification 1000 × for micrographs in a–d, 5000 × for micrographs in e–h.

2.4 Changes in plasma haptoglobin and cecum secretory immunoglobulin A for SRI-treated mice

As shown in Figure 2, the important content increase in plasma haptoglobin (HP) and decrease in cecum secretory immunoglobulin A (sIgA) characterized the colitis caused by DSS, which was consistent with the histological findings. Moreover, after treatment with SRI, the data clearly indicated a significant reverse alteration.

2.5 SRI treatment suppresses colon tissue ICAM-1 gene expression for DSS-exposed mice

The levels of the gene expression were then analyzed by quantitative reverse transcriptase real-time polymerase chain reaction (qRT-PCR). The amplification kinetics of the gene ICAM-1, macrophage migration inhibitory factor (MIF), and GAPDH assays were approximately equal, and gel electrophoresis of PCR products showed a single band. Therefore, the comparative Ct ($\Delta\Delta$Ct) method for the determination of gene expression was employed. As shown in Figure 5, the ICAM-1 gene expression fold change of DSS-treated mice was 1.57 ± 0.55 vs. sham 0.32 ± 0.26. Exposure of the colon to DSS caused strong expression of the gene ICAM-1; in contrast, SRIH and SRIL downregulated the ICAM-1 expression in the treated groups vs. DSS control (0.32 ± 0.24, 0.55 ± 0.43). No significant change of the gene MIF expression was observed in SRIH and SRIL.

2.6 Discussion

SRI, one of the quinolizidine alkaloids, is a new anticancer drug with noticeable antitumor action and lower toxicity. To our knowledge, there is no report about its effect on IBD. For the first time, its anti-inflammatory effect on DSS-induced colitis was investigated. The results demonstrated an improvement of DSS-induced colitis in mice treated with SRI as reflected in the experimental data by means of a macroscopic and histological disease score. SRI significantly mitigated the

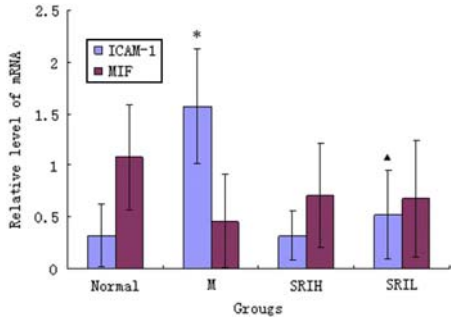

Figure 5. Examination of ICAM-1 and MIF gene expression with SYBR Green I real-time RT-PCR. GAPDH was used as an internal control. DSS upregulated the ICAM-1 gene expression; in contrast, SRIH and SRIL inhibited its upregulation. There was no apparent change in the MIF gene expression for SRIH and SRIL. Data are expressed as mean ± SD. (**) $p < 0.01$ vs. normal. (*) $p < 0.05$ vs. normal; (▲▲) $p < 0.01$ vs. DSS control. (▲) $p < 0.05$ vs. DSS control.

appearance of diarrhea and the disruption of colonic architecture. Moreover, there was a remission of morphological signs on cell damage, and the colonic mucosa showed ulcers in the process of healing, and an evolution to a more chronic inflammatory infiltrate, with mononuclear predominance and initiation of a repair process. As we know, the protective effect of mucus as an active barrier may be attributed largely to its viscous and gel-forming properties that are derived from mucin glycoprotein constituents [10]. As sIgA is also a component of mucin, the experiment showed that SRI protected the crypt gland and mucous architecture, and maintained the cecum sIgA level. All these observations may have significance on the beneficial effect of SRI on colitis.

Accumulating evidence indicated that emigration of leukocytes to the site of inflammation is related to ICAM-1, and enhanced colonic mucosal endothelial cell ICAM-1 expression is an early event in the inflammatory cascade of acute colitis [2,11–13]. The results showed that ICAM-1 gene expression was corrected with the development of colonic inflammation and, moreover, SRI administration was able to diminish ICAM-1 gene expression.

MIF is a ubiquitously expressed cytokine with a variety of mitogenic and proinflammatory functions [14,15]. Plasma concentrations of MIF in patients with active Crohn's disease were six-fold higher than in healthy individuals, and the development of chronic colitis is dependent on the cytokine MIF [16]. The data indicated that SRI had no apparent effect on MIF gene expression in DSS-treated mice.

In conclusion, the present study demonstrated that SRI attenuated intestinal inflammation in the experimental colitis model, which was associated with the downregulation of ICAM-1 gene expression. Therefore, SRI could be a potential therapeutic agent for the treatment of IBD.

3. Methods

3.1 Animals and chemicals

SRI (98.5% purity determined by HPLC area normalization method, batch No. 070802) was purchased from YanChi Pharmaceutical company (Ningxia, China). DSS (molecular weight: 5000) was obtained from Amersham Biosciences Pharmacia Biotech (Uppsala, Sweden); hematoxylin and eosin were purchased from Sigma Chemical Co. (St Louis, MO, USA); RNAlater solution was from Ambion company (Austin, TX, USA); total RNA extract reagent was purchased from Invitrogen (Carlsbad, CA, USA); and SYBR Prime Script™ RT-PCR kits were purchased from TAKARA Biotechnology Co., Ltd (Dalin, China). Enzyme-linked immunosorbent assay sIgA and haptoglobin kit were purchased from Nanjing Jianchen Bioscientific Company (Nanjing, China). All other reagents were of analytical grade.

3.2 Animal care

Female C57BL/6 mice, 6 weeks of age, obtained from the animal center of Southern Medical University were housed five or six per cage, and kept at the animal house facilities with a room temperature of 20 ± 2°C, 50% humidity and 12:12 h light–dark cycles, fed a standard pellet diet, and were administered tap water *ad libitum*. The Animal Research Board Committee of Southern Medical University approved the studies. Mice were acclimatized for 2 weeks before the experiment. Body weights were measured every week for the whole duration of the experiment.

3.3 Animal experiment

After a 14-day acclimation period, a total of 46 mice were randomly divided into four groups. Group 1 ($n = 11$ mice) was administered water as a normal control. Groups 2–4 received four cycles of DSS administration (each cycle: 4% DSS dissolved in distilled water for 7 days and then distilled water for 7 days). This schedule was used to simulate the cycle of acute flare-ups alternating with periods of disease inactivity observed in human UC patients [17,18]. Group 2 ($n = 15$ mice) was administered distilled water by oral gavage, 10 ml/kg, once daily for the whole experimental period. Groups 3 and 4 ($n = 10$) were administered SRI solution by oral gavage once daily at the dose of 25 mg/kg (low-dose group of SRI, SRIL) and 50 mg/kg (high-dose group of SRI, SRIH), respectively. Mice were weighed every week for the determination of percent weight change. This was calculated as: % weight change = (weight at day X − day 0/weight at day 0) × 100. Mice were monitored clinically for rectal bleeding, diarrhea, and general signs of morbidity, including hunched posture and failure to groom. Drinking water, food consumption, occult and rectal bleeding, and stool consistency were monitored daily during DSS administration. Occult blood in the feces was evaluated using the orthotolidine methods.

3.3.1 General assessment of colitis

The mice were killed by dislocation after 56 days of experiments. Some colon samples were stored in RNAlater solution for 24 h, and then stored at −20°C for PCR. The colon was resected between the ileocecal junction and the proximal rectum, close to its passage under the pelvisternum. The colon was placed onto a non-absorbent surface and measured with a ruler, taking care not to stretch the tissue. Colon length was measured as an indication of colonic inflammation.

3.3.2 Histological score

The colon was fixed with 10% neutral buffered formalin. The tissues were prepared for routine processing. Serial tissue sections (3 μm) were made and mounted on glass slides and were routinely stained with hematoxylin and eosin for histopathological analysis. Sections were coded with an accession number and reviewed by a pathologist without access to the code. Each section was scored for lesions based on severity, ulceration, hyperplasia, and crypt alteration [19].

3.3.3 Scanning electron microscopy

Specimens from all groups were also processed by using a standard technique for SEM. Fragments of 2 mm thick from the colon were fixed with glutaraldehyde in 0.1 M Sorensen's phosphate buffer (pH 7.4) for 24 h at room temperature. Samples were then postfixed in a phosphate-buffered 2% osmium tetroxide solution at 4°C for 1 h. The samples were glued onto stubs, coated with gold in a SCD040 balzer sputterer, and observed using a Philips 505 SEM at 10–30 kV.

3.3.4 Estimation of cecum sIgA and plasma HP

Cecum content was collected rapidly and suspended in two-fold saline, and the supernatant was collected by centrifugation. Content of cecum sIgA was determined by mouse sIgA double antibody sandwich enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's protocol. The standard curve was constructed and calculated, and then correlated and regressed. For the estimation of plasma HP, blood was collected into a heparin tube via removing mice eyeball. Care was taken to minimize hemolysis, and stored at -20°C. Then, the plasma HP concentrations in the plasma were determined by mouse HP double antibody sandwich ELISA kit according to the manufacturer's protocol. The HP concentrations in the plasma were determined as described for sIgA.

3.3.5 RNA isolation and qRT-PCR using SYBR Green I fluorescent dye

The RNAlater-preserved colon tissues were homogenized and extracted using trizol reagent according to the kit's instructions. Extracted RNA was dissolved in 20 μl free RNAase water and validated with agarose gel electrophoresis. Total RNA of 2 μg from each sample were converted into cDNA. Relative gene expression quantitation for ICAM-1, MIF, with GAPDH as an internal reference gene, was carried out using Mx3005 real-time PCR amplification detection system in triplicates, based on the SYBR-Green method. Primer sequences were as follows: ICAM-1, sense 5′-CAACTGGAAGCTGTTTGAGCTGAG-3′ and reverse 5′-AGGGTGAGGTCCTTGCCTACTTG-3′; MIF, sense 5′-CTGCACAGCATCGGCAAGA-3′ and reverse 5′- TTGGCAGCGTTCATGTCGTAA -3′; GAPDH, sense 5′-AAATGGTGAAGGTCGGTGTG-3′ and reverse 5′-TGAAGGGGTCGTTGATGG-3′. The PCR reaction mixture consisted of 0.1 μmol/l of each primer, 1 × SYBR Premix EX Taq (Perfect Real Time) premix reagent, and 50 ng of cDNA to a final volume of 20 μl. Cycling conditions were 95°C for 10 min, followed by 40 cycles at premature 95°C for 5 s, annealing temperature was 59°C for 30 s. PCR specificity was confirmed by dissociation curve analysis and gel electrophoresis. The relative induction of gene mRNA expression, comparative ΔΔCt was calculated using the ΔΔCt method as follows: ΔΔCt = (Ct,target − Ct,GAPDH)treatment − (Ct,target − Ct, GAPDH)nontreatment, and the final data were derived from $2^{-(\text{average } \Delta\Delta Ct)}$. GAPDH was used as an internal control.

3.3.6 Statistical analysis

Data were expressed as mean ± SD, and were analyzed using SPSS 13.0 statistical analysis software. Body weights were compared with repeated measure variance analysis and one-way ANOVA. Values were taken to be statistically significant at $p < 0.05$.

Acknowledgements

This work was financially supported by the Guangzhou Science and Technology Committee Grants for Significant Projects. We thank Dr Qin Fan for molecular biology analyses.

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