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Research Article
Astragaloside IV Attenuates Trinitrobenzene Sulphonic Acid (TNBS)-Induced Colitis via Improving Mucosal Barrier Function: Role of Goblet Cells

[1,2]Kai-hong Zang, [3]Hong-yan Qin, [1,2]Hai-Jing Duan, [1]Qing-lin Ma and [1,2]Yuan Ren

[1]College of pharmacy, Gansu University of Traditional Chinese Medicine, 730000 Lanzhou, China
[2]Gansu Key Laboratory of Pharmacology and Toxicology of Traditional Chinese Medicine, Dingxi East Road, Chengguan District, 730000, Lanzhou, China
[3]Department of Pharmacy, First Hospital of Lanzhou University, 730000 Lanzhou, China

Abstract

Background and Objective: Astragaloside IV, the main bioactive ingredient of Radix Astragali showed anti-inflammation and would healing effect in practice. This study aimed to investigate the therapeutic effect of astragaloside IV (AS-IV) on experimental colitis as well as its role in mucosal healing and barrier function. **Materials and Methods:** TNBS-induced colitis rats were orally treated with AS-IV at the dose of 10, 20 and 40 mg kg^{-1} per day for 8 consecutive days. After drug treatment, histological damage score and myeloperoxidase activity of the colon tissue were detected, mucosal barrier function was evaluated by measuring the serum level of D-lactate and diamine oxidase and colonic goblet cells and the mRNA expression of Mucin were also evaluated by immuno-histochemistry and RT-PCR, respectively. The proteins and genes in Wnt and Notch signaling were further investigated by Western blot and RT-PCR to identify the effect of AS-IV on the differentiation of goblet cells. **Results:** Histological scores, myeloperoxidase activity and serum level of D-lactate and diamine oxidase in colitis rats were significantly increased, while mRNA expression of Muc-2 and Muc-3 were significantly decreased. AS-IV administration significantly reduced histological scores, myeloperoxidase activity and the level of D-lactate and diamine oxidase in colitis rats and the expression of Muc-2 and Muc-3 were markedly increased. Moreover, the protein expression in Wnt signaling, i.e., Lrp5, Lrp6 and β-catenin, in the colon of colitis rats was significantly elevated, but the genes expression in Notch signaling, i.e., Rath1, Gfi1 and Klf4, in colitis rats were markedly decreased, and these alteration of Wnt and Notch signaling in colitis rats were markedly reversed by AS-IV administration. **Conclusion:** AS-IV attenuates colon inflammation in colitis rats via improving mucosal barrier function, the regulatory effect on the proliferation and differentiation of goblet cells may contribute to the therapeutic role of AS-IV in colitis.

Key words: Astragaloside IV, colitis, mucosal barrier function, goblet cells, differentiation

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Corresponding Author: Yuan Ren, College of pharmacy, Gansu University of Traditional Chinese Medicine, Dingxi East Road, Chengguan District, 73000 Lanzhou, Gansu Province, People's Republic of China Tel: (+86) 0931-8762653

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Ulcerative Colitis (UC), one of the major sub-types of inflammatory bowel disease (IBD), is characterized by chronic, recurrent intestinal disorders, such as diarrhea, mucilage or blood-pus stools. Nowadays, the pathogenesis of UC has not been clearly identified, but it is believed to have correlation with genetic susceptibility, immune imbalance, intestinal infection and alterations in commensal microbiota[1,2]. It is reported that about 50% of UC patients were subjected to repeated symptoms exacerbation every few weeks or months[3], which significantly reduced the quality of life of patients and caused a huge financial burden on the healthcare system. Currently, the treatment of UC is mainly focused on maintaining symptom-free remission.

Intestinal barrier consists of epithelial layer, tight junctions and mucus layer. The mucus layer remain permeable to allow the passing through of small absorptive molecules and also restrain the access of bacteria and bacterial compounds[4]. Among the pathogenic factors of UC, the disturbed epithelial barrier function as well as the increased intestinal permeability have been considered as the key factors in the development of colitis[5]. Goblet cells are the most abundant secretory epithelial cells in the gut. The main function of goblet cells is mucins secretion, which is critical for the formation of intestinal mucus barrier[6]. It is reported that depleted goblet cells as well as impaired mucins production were both associated with the disturbed epithelial barrier function of IBD[7], indicating the important role of goblet cells in the pathogenesis of UC. Results from clinical studies showed that achievement of mucosal healing in UC patients could predict both long-term remission and low risk for surgery[8,9], improved function of goblet cells was accompanied with attenuated colonic inflammation[10]. It is well accepted that interventions for mucosal healing and epithelial barrier promoting could limit inflammation and prevent future injury[11]. Therefore, strengthening epithelial resistance to noxious stimuli and enhancing intestinal repair could be effective approaches for the treatment of UC.

Radix astragali (Huangqi) is a well-known herb for reinforcing Qi (the vital energy) in Traditional Chinese Medicine. *Radix astragali* has been shown to have several bioactivities, such as wound healing, anti-inflammatory and anti-bacteria[12]. *Radix astragali* contains a variety of single compounds, including polysaccharides, flavonoids and saponins. Astragaloside IV (AS-IV), a tetracyclic triterpenoid saponin, has been identified as one of the bioactive ingredients in *Radix astragali*. The pharmacological activities of AS-IV involve anti-oxidation and anti-inflammation. Previous studies showed that AS-IV attenuated ethanol-induced gastric mucosal damage in rats, also promoted would healing in diabetes model[13]. Recently, AS-IV was reported to attenuate colitis in a rat model via regulating energy metabolism and promoting mucosal healing[14]. However, till date, the mechanism about the effect of AS-IV on mucosal barrier function has not been identified. The present study aimed to investigate the effect and the underlying mechanism of AS-IV on mucosal barrier by assessing epithelial barrier function and the effect of AS-IV on the proliferation and differentiation of goblet cells was also evaluated in trinitrobenzene sulfonic acid (TNBS)-induced colitis model.

MATERIALS AND METHODS

Materials: About 2,4,6-trinitrobenzenesulfonic acid (TNBS), hexadecyltrimethylammonium bromide and o-dianisidine dihydrochloride were purchased from Sigma-Aldrich (St., Louis, USA). Chloral hydrate was purchased from Bangjing Scientific Supplies (Shanghai, China). AS-IV (98% purity) was purchased from Langze Pharmaceutical Technology (Lanjin, China), salicylazosulfapyridine (SASP) were purchased from Zhongxi Pharmaceutical Factory (Shanghai, China). D-lactate (DLA) and diamine oxidase (DAO) ELISA kits were purchased from Yuanmu Technology (Shanghai, China). All the reagents and solvents were of analytical grade.

Animals and colitis induction: Sprague-dawley male rats (aged 6 weeks with body weight around 220 g) were obtained from the Laboratory Animal Center of Gansu University of Chinese Medicine. Rats were maintained at 25 with light-dark cycle and free access to food and water. All the rats were allowed to adapt the environment for at least 1 week before experiment. The animal studies were carried out in accordance with the proposals of the Committee on the Ethics of Animal Experiments of Gansu University of Chinese Medicine (Approval ID 2016-022). Colitis was induced as previous reports. Briefly, rats were fasted overnight and then deeply anesthetized with chloral hydrate (350 mg kg^{-1}, i.p). A plastic catheter was placed in the colon at a depth of 8 cm from anus, TNBS solution (2.5 mg in 50% ethanol, 0.8 mL per rat) was instilled. The rats in control group were administered with 0.8 mL saline. After that, rats were left on a warm mound of bedding in a head-down position until regain consciousness. The rats that showed soft or diarrhea stool with the body weight decrease were selected as the colitis rats.

Study design and sample collection: The colitis rats were randomly divided into 5 groups. Group 1 (n = 8) was set as the colitis model group, rats in this group were orally treated with

water. Group 2 (n = 8) was set as the positive control, rats in this group were orally treated with SASP (300 mg kg^{-1}). Rats in group 3, 4 and 5 (n = 8 per group) were orally given AS-IV at the dose of 10, 20 and 40 mg kg^{-1}, respectively. Group 6 (n = 10) was set as the normal control, rats in this group were orally treated with water. Drugs were given to rats at 24 h post TNBS and administered once daily for consecutive 8 days. At 8 days post drugs treatment, the rats were anesthetized, blood sample was collected from abdominal aorta. The proximal colon was harvested and divided into 2 parts, one was fixed in 4% formalin for hematoxylin and eosin (H and E) staining and Periodic acid-Schiff (PAS) staining, the other was frozen at -80 for Western-blot, RT-PCR and myeloperoxidase (MPO) activity assay.

H and E staining and PAS staining: The sections were stained with H and E and examined under light microscope (Nikon Inc., Japan). The severity of inflammation was graded using the macroscopic and histological scoring criteria which were reported previously[11,15]. For PAS staining, the staining procedures were conducted according to manufacturer's instruction (Maixin Biotech. Co. Ltd., Fuzhou, China). Five random fields at 200x magnifications were counted and quantified in each section using Image J NIH software (Wayne Rasband, NIH, USA.), the number of PAS-positive goblet cells was expressed per 10 crypt-villus axis.

MPO activity assay: The MPO activity was measured as previously described[15]. Briefly, the colon tissues were homogenized in 0.5% hexadecyltrimethylammonium bromide (0.5 mL per 50 mg of colon tissue), then centrifuged at 14 000 g at 4 for 15 min. The supernatant was mixed with potassium phosphate buffer (50 mmol, pH 6.0) with 0.0005% o-dianisidine dihydrochloride and 0.1% hydrogen peroxide. MPO activity was calculated from the rate of absorbance change during 1 min at 460 nm.

ELISA assay: Blood samples were centrifuged at 10,000 g for 10 min at 4 , serum was harvested and stored at -80 until detection. The level of D-lactate (DLA) and diamine oxidase (DAO) were determined using ELISA kits according to the manufacturer's protocol and instruction.

Western-blot analysis: The proteins were extracted from the colon samples. Tissue extracts containing 30 μg total proteins were subjected to 10% SDS-PAGE gel and then transferred electrophoretically to PVDF membranes. After blocking with 5% non-fat milk for 1 h at room temperature, the membranes were incubated with primary antibodies (anti-proliferating cell nuclear antigen (PCNA), Abcam, 1:1000, anti-Lrp5, Cell Signaling, 1:1000, anti-Lrp6, Cell Signaling, 1:1000, anti-β-catenin, cell signaling, 1:1000) overnight at 4 . Following secondary antibody incubation, the immunoreaction was detected using ECL western blotting kit (GE Healthcare Life Sciences, Amersham, UK). Optical density of each band was semi-quantified by Image J-software (NIH, USA).

Table 1: Primers sequence used for real-time PCR

Primers	Sequence (5'→3')
Muc-2	(F) ACCACCATTACCACCACCTCAG
	(R) CGATCACCACCATTGCCACTG
Muc-3	(F) CACAAAGGCAAGAGTCCAGA
	(R) ACTGTCCTTGGTGCTGCTGAATG
Rath1	(F) CTGTGCCGCCTTGAAGAAAC
	(R) GACCAAATGCCTTTGACACTACA
Gfi1	(F) GACGCTCCTAACCCCCAAAA
	(R) GTTCGAATGTTTGTGTGGCCT
Klf4	(F) CCCACACAGGTGAGAAACCT
	(R) ATGTGTAAGGCGAGGTGGTC
GAPDH	(F)TGCTGAGTATGTCGTGGAG
	(R)GTCTTCTGAGTGGCAGTGAT

F: Forward, R: Reverse

Quantitative real-time PCR: Total RNA of colon tissues was extracted using TianGen kit (Tiangen Biotech Beijing, China) and quantified using NanoDrop spectrophotometer. The cDNA was synthesized using 1 μg of RNA with an Omniscript reverse transcription (RT) kit (Tiangen Biotech Beijing, China). For qPCR reaction, 5 μL of a 1:5 dilution of cDNA was added to 10 μL Bio-Rad SYBR green supermix with primers (final concentration, 300 nM, final volume, 20 μL) and qPCR was carried out using a MJ MiniOpticon machine (Bio-Rad, USA). The primer Sequence used for this assay have been tabulated in Table 1.

Statistical analysis: Data are presented as Mean±SEM. Statistical analysis was conducted using SPSS 16.0. Differences among multiple groups were analyzed using one-way ANOVA followed by Student-Newman-Keuls (SNK) test. Differences were considered significant when $p<0.05$.

RESULTS

Effect of AS-IV on severity of colon inflammation: As shown in Fig. 1, the pathological score and MPO activity in the colon of colitis rats were significantly increased when compared to that in the normal control ($p<0.01$), SASP treatment significantly decreased both pathological score and MPO

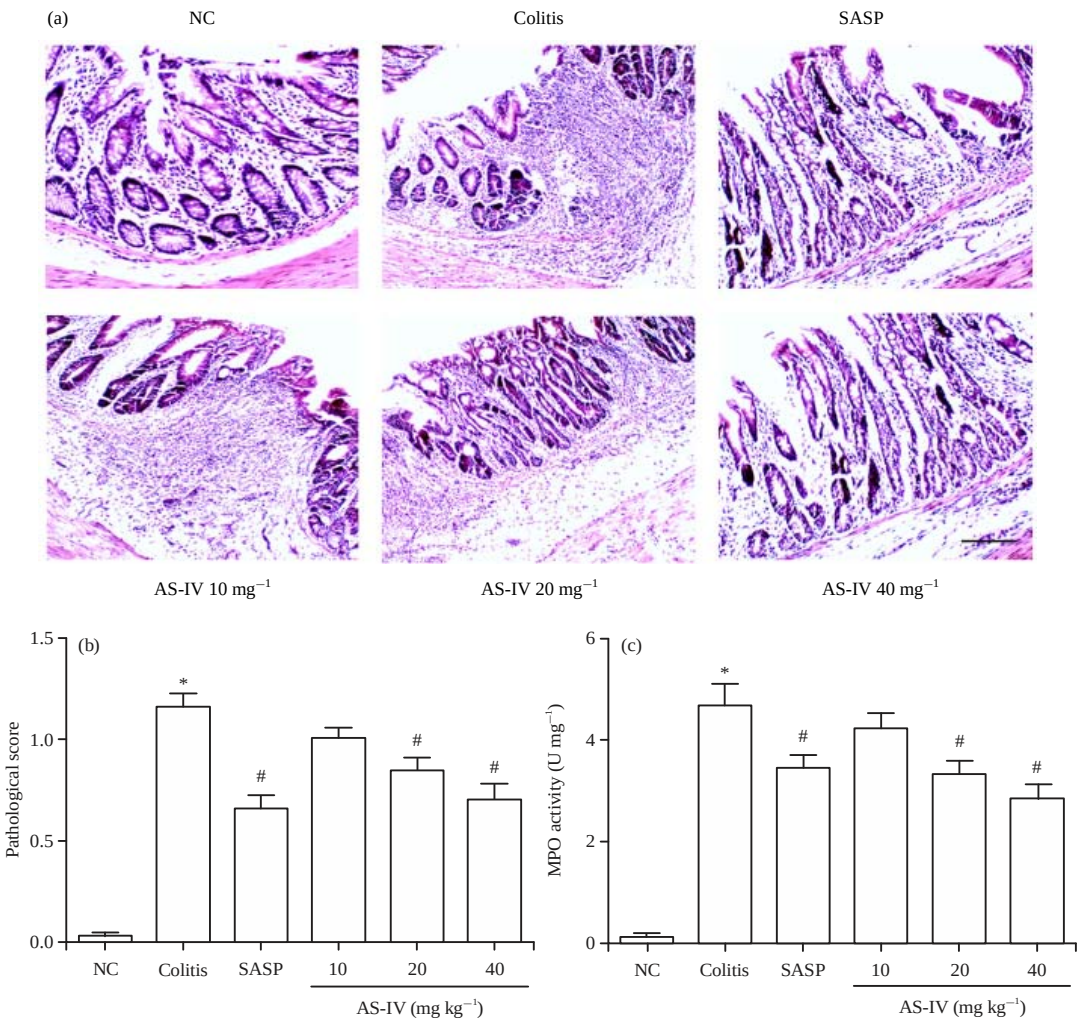

Fig. 1(a-c): Effects of Astragaloside IV (AS-IV) on colonic inflammation in colitis rats. Panel A depicts the representative histological changes of the colon tissue (H and E staining, Scale bar, 100 μm). Statistical analysis of the pathological score and myeloperoxidase (MPO) activity are shown in panel B and panel C, respectively

Data are presented as Mean ± SEM, n = 8 per group. *p<0.05 vs. normal control (NC), #p<0.05 vs. colitis group. SASP: Salicylazosulfapyridine treated group

activity (p<0.01) and AS-IV treatment dose-dependently decreased pathological score and MPO activity in colitis rats, significant differences were found both in high (40 mg kg^{-1}) and median (20 mg kg^{-1}) dose of AS-IV-treated rats (p<0.05).

Effect of AS-IV on the permeability of intestinal epithelial barrier: As shown in Fig. 2, serum level of D-LA and DAO in colitis rats were markedly increased when compared to that of the normal rats (p<0.05), SASP treatment significantly reduced the level of D-LA and DAO in colitis (p<0.05), AS-IV treatment dose-dependently decreased the serum level of D-LA and DAO, significant differences were found both in high (40 mg kg^{-1}) and median (20 mg kg^{-1}) dose of AS-IV-treated groups (p<0.05).

Effect of AS-IV on goblet cells and the expression of Muc-2 and Muc-3: As shown in Fig. 3a, compared to the normal control, the mRNA expression of Muc-2 and Muc-3 in the colon of colitis rats were significantly decreased (p<0.01), while high and media dose of AS-IV treatment markedly increased the mRNA expression of Muc-2 and Muc-3 in the colon (p<0.01). As shown in Fig. 3b, the number of goblet cells in the colon of colitis rats was significantly increased, while AS-IV treatment dose-dependently decreased the number of goblet cells, significant difference was found in high-dose of AS-IV treatment group (p<0.01). The representative goblet cells in Fig. 3c showed that, the size of goblet cells in colitis group were smaller than that of the normal rats, even though

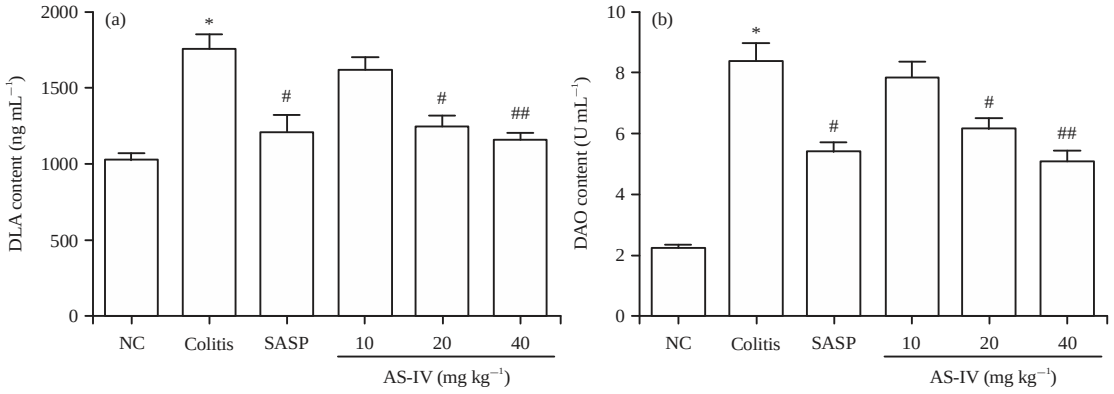

Fig. 2(a-b): Effects of Astragaloside IV (AS-IV) on serum level of D-lactate (DLA) and diamine oxidase (DAO) in colitis rats. Data are presented as Mean±SEM, n = 8 per group. *p<0.05 vs. normal control (NC), #p<0.05 vs. colitis group. SASP: Salicylazosulfapyridine treated group

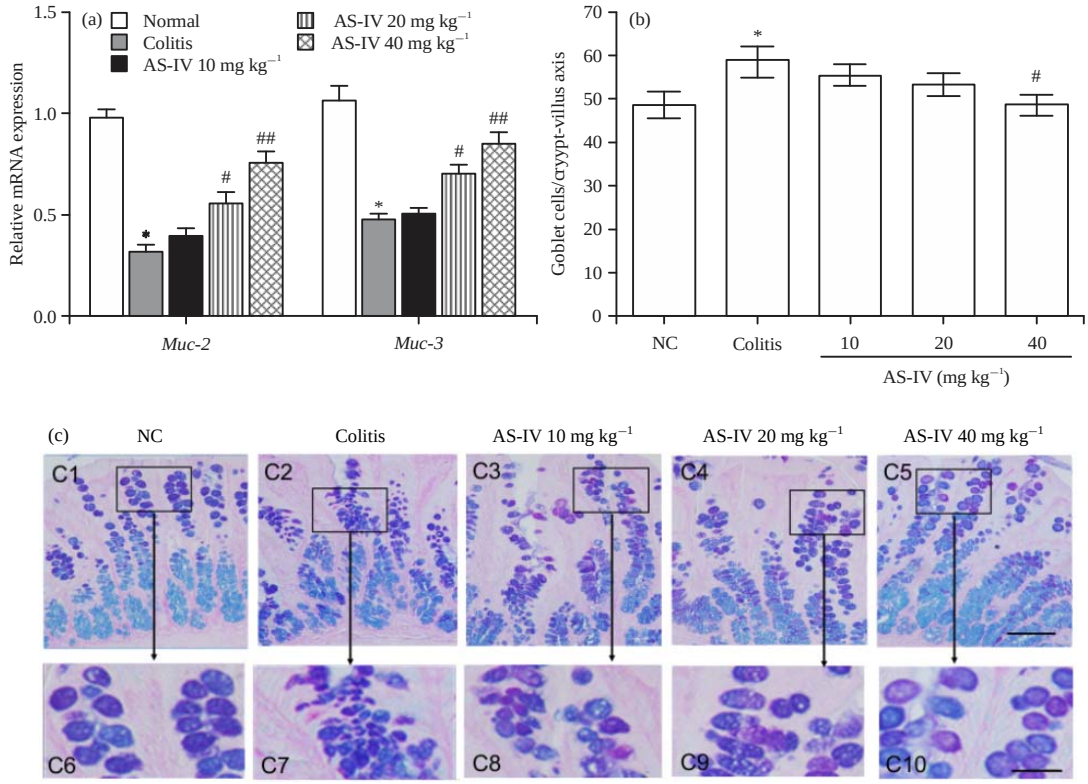

Fig. 3(a-c): Effects of Astragaloside IV (AS-IV) on goblet cells and mucins expression in the colon of colitis rats. Statistical analysis of the relative mRNA expression of Muc-2 and Muc-3 and goblet cells number are shown in panel A and panel B, respectively. Panel C depicts the representative histological change of goblet cells in the colon tissue (PAS staining, C1-C5, scale bar = 100 μm C6-C10, scale bar = 50 μm)
Data are presented as Mean±SEM, n = 8 per group. *p<0.05 vs. normal control (NC), #p<0.05, ##p<0.01 vs. colitis group

the cell number was increased, SASP and AS-IV treatment reversed the alteration of goblet cells both in cell number and cell size.

Effect of AS-IV on the proliferation of colonic cells and wnt signaling: As shown in Fig. 4a and b, the protein expression of PCNA, a marker of colonic epithelial cells proliferation, in colitis

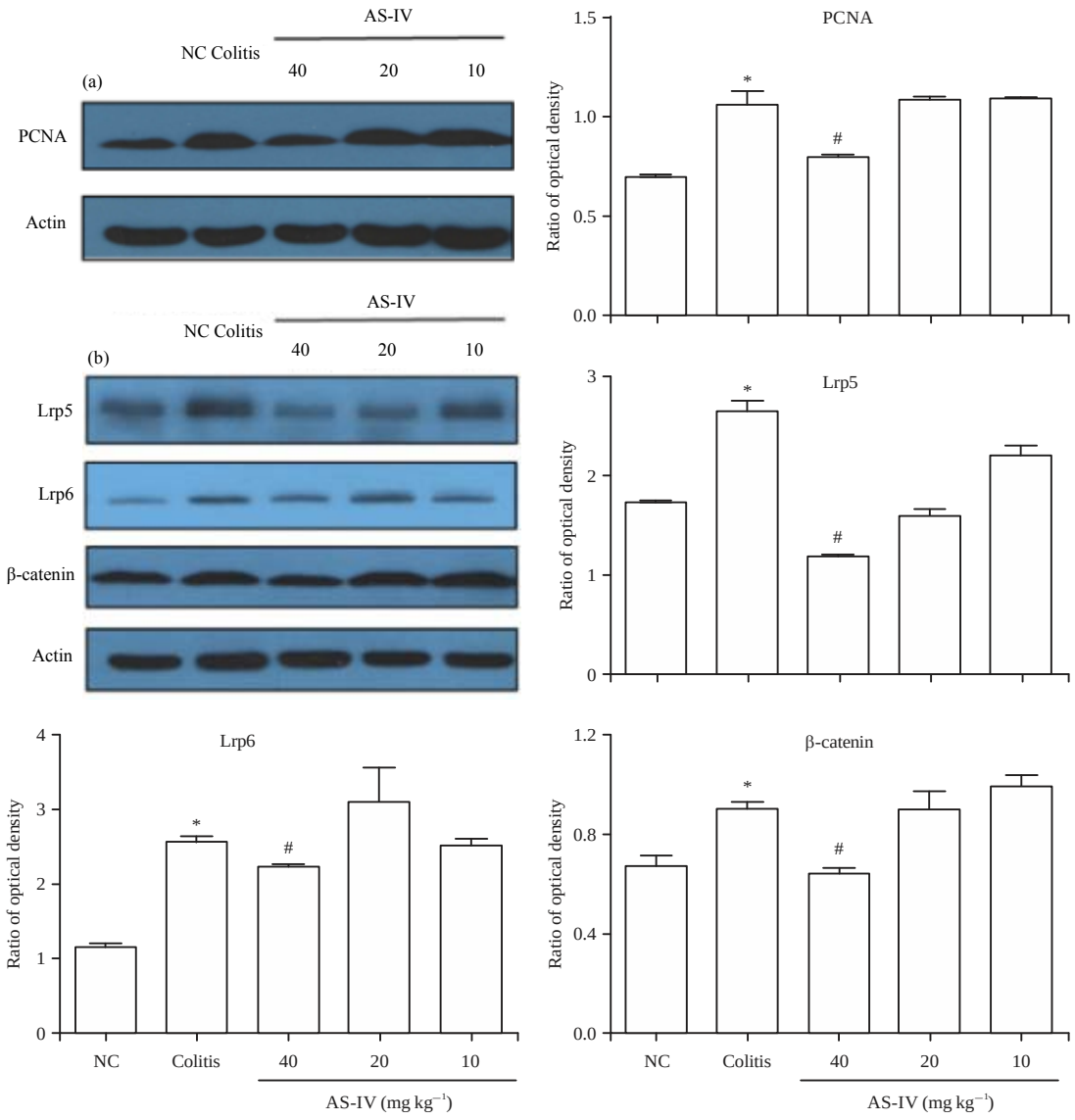

Fig. 4(a-b): Effects of Astragaloside IV (AS-IV) on protein expression of Wnt signaling pathway in the colon of colitis rats. Representative Western immunoblots of PCNA, Lrp5, Lrp6 and β-catenin as well as the statistical analysis of the protein expression level were shown in Panel A and Panel B, respectively

Data are presented as Mean±SEM, n = 8 per group. *p<0.05 vs normal control (NC), #p<0.05 vs colitis group

group was markedly increased when compared to that of the normal control (p<0.01), AS-IV administration significantly decreased the expression of PCNA (p<0.01). The expression of Wnt signaling protein, i.e., Lrp5, Lrp6 and β-catenin were all significantly increased in the colon tissues of colitis rats (p<0.05), while high dose of AS-IV administration markedly restored the expression of Lrp5, Lrp6 and β-catenin to the normal level (p<0.05).

Effect of AS-IV on the differentiation of colonic goblet cells and notch signaling: To investigate the effect and mechanism of AS-IV on goblet cells differentiation under colonic inflammation, the mRNA expression Rath1, Gfi1 and Klf4 in Notch signaling were further examined. As shown in Fig. 5, the mRNA expression of Rath1, Gfi1 and Klf4 in the colon of colitis rats were all significantly reduced when compared to the normal group (p<0.05), while high-dose of AS-IV treatment markedly elevated the expression of Rath1, Gfi1 and Klf4 of colitis (p<0.05), indicating that AS-IV treatment could promote the differentiation of goblet cells during inflammation.

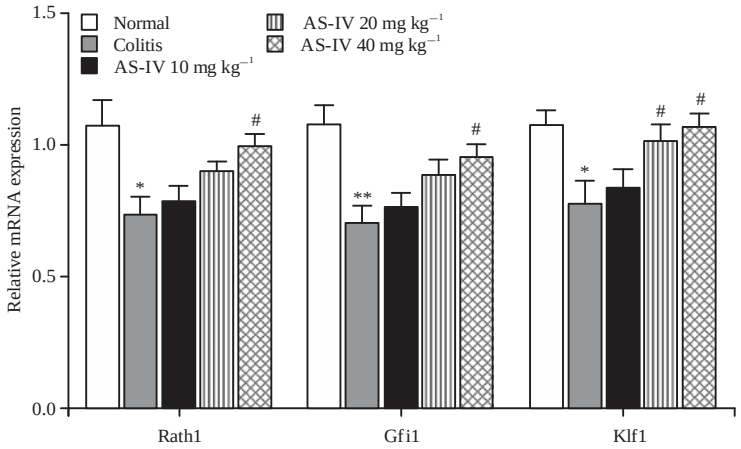

Fig. 5: Effects of Astragaloside IV (AS-IV) on the mRNA expression of Rath1, Gfi1 and Klf4 in Notch signaling in the colon of colitis rats.

Data are presented as Mean±SEM, n = 8 per group. *p<0.05, **p<0.01 vs normal control (NC), #p<0.05 vs colitis group

DISCUSSION

In this present study, it was investigated that the therapeutic effect of AS-IV on mucosal injury in the colon of colitis rats and also its effect on mucins production and goblet cells in the colon.

AS-IV displayed significant therapeutic effect against TNBS-induced mucosal depletion and colonic inflammation by reducing histological damage score and MPO activity. This finding is consistent well with previous report showing therapeutic effect of AS-IV on mucosal healing in colitis rats[14]. It is well known that DAO is an ideal indicator of intestinal mucosal structure and function, the elevated serum DAO level reflects the destruction of the intestinal mucosal barrier[16]. Function of intestinal mucosa barrier can also be assessed by detecting serum DLA, for serum accumulation of DLA reflects increased intestinal permeability and impaired mucosa barrier[17]. This study found that AS-IV treatment markedly reduced the serum level of DLA and DAO of colitis rats, this finding was in accordance with its effect on mucosal depletion and inflammation. As previous report showed that AS-IV could promote the healing of cutaneous wounds and ulcers[13,18], hypothesis that the novel role of AS-IV in mucosa repair may contribute to its therapeutic effect on colitis.

Mucosal epithelia is essential for the maintenance of mucosal homeostasis and health, which is in part accomplished by goblet cells[19]. Mucus layer is formed by several mucins secreted by goblet cells, which acts as a barrier between the luminal contents and the epithelial surface[20]. It is reported that loss or dysfunction of intestinal goblet cells enabled bacteria to contact the epithelium, which contributed to the pathogenesis of IBD[21]. The present study found that mRNA expression of muc-2 and muc-3 was decreased in the colon of colitis rats, while AS-IV administration markedly increased the expression of muc-2 and muc-3, indicating that the mucosa healing effect of AS-IV in colitis may be associated with the recovery of mucus layer. Interestingly, our data also showed that colonic goblet cells in colitis rats were increased, but the cell size became much smaller, AS-IV treatment markedly reversed the alteration of goblet cells both in number and size. It is reported that the proliferative capacity of intestinal cells could be enhanced under inflammation[22], it is possible that the proliferation of goblet cells might also be promoted during colitis in order to repair the damage. However, the changes in goblet cells number seems conflict with the decreased expression of muc-2 and muc-3, this study speculated that the changed size of goblet cells might contribute to the decreased expression of mucins.

Balanced rate of proliferation and differentiation of intestinal cells is essential for maintaining epithelial homeostasis. Wnt/β-catenin as well as Notch signaling both play crucial roles in regulating the proliferation and of intestinal epithelial cells and intestinal cells fate decision[23-25]. The protein expression in Wnt/β-catenin pathways, i.e., Lrp5, Lrp6 and β-catenin, in the colon of colitis rats was significantly elevated, while the genes that required for the differentiation of goblet cells in Notch signaling, i.e., Rath1, Gfi1 and Klf4, were markedly decreased, indicating the imbalanced proliferation and differentiation of goblet cells during inflammation. It was also found that the alteration of Wnt and Notch signaling in colitis rats could be markedly reversed by AS-IV administration, indicating that AS-IV might facilitate the shift from cells proliferation to goblet cells differentiation in colitis condition. Knowing that inflammatory cytokines could

stimulate intestinal epithelial cell hyper proliferation[26,27], it was believed that the regulatory effect of AS-IV on the proliferation and differentiation of goblet cells might contribute to its effect on mucosa repair. Based on the data of this study, it still did not know the exact mechanism about AS-IV's effect on goblet cells fate decision. Concerning the evidence that intestinal cells hyperplasia is cytokine predominance, speculate that the regulatory effect of AS-IV on goblet cells may result from its effect on cytokine production, for AS-IV has been reported to down-regulate the production of inflammatory cytokines[28], further study are needed to clarify this issue.

CONCLUSION

This study discovers that AS-IV supplementation can attenuate colonic inflammation via improving mucosal barrier function, the effect of AS-IV on regulating the proliferation and differentiation of goblet cells may be responsible for the restored mucins production and mucus layer recovery. Results from this present study, for the first time, provide experimental evidence for the novel effect of AS-IV on intestinal epithelial barrier function, which is beneficial for the development of new medications for IBD therapy. This study will help the researcher to uncover the critical role of unbalanced proliferation and differentiation of goblet cells in the disruption of intestinal mucus barrier.

SIGNIFICANCE STATEMENT

Results from this present study, for the first time, provide experimental evidence for the novel effect of AS-IV on intestinal epithelial barrier function, which is beneficial for the development of new medications for IBD therapy. This study will help the researcher to uncover the critical role of unbalanced proliferation and differentiation of goblet cells in the disruption of intestinal mucus barrier, which do provide significance statement about this study.

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