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Original Article

Astragaloside IV reduces intestinal fibrosis in rats with TNBS-induced colitis

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Abstract: To investigate the anti-fibrotic effects of Astragaloside IV in rats with experimental colitis, and to explore the possible mechanisms, Sprague-Dawley Rats with colitis were induced by 2,4,6-trinitrobenzene sulphonic acid (TNBS). Forty-eight rats were randomly divided into normal group, TNBS model group, SB203580 group and Astragaloside IV group. 150 mg/kg TNBS dissolved in 50% ethanol were gavaged into each rat in the three groups but not the normal group. Rat in the normal group was gavaged with an equal volume of 0.9% NaCl solution. Rats in the SB203580 group were intraperitoneally injected with 10 mg/kg of SB203580 daily for 4 days. Rats in the Astragaloside IV group were gavaged with 25, 50 or 100 mg/kg of Astragaloside IV daily for 21 days. At the end of the experiment, colon tissue samples were collected, and the injury and fibrosis of the colon were detected by HE staining and Masson collagen staining, respectively. Expression of transforming growth factor (TGF)-β1, connective tissue growth factor (CTGF), collagen I, collagen III, fibronectin (FN) and α-SMA was determined by western blotting. Compared with the TNBS model group, the histological scores and fibrosis were improved significantly in the SB203580 group as well as Astragaloside IV group. Expression of TGF-β1, CTGF, Collagen I, Collagen III, FN and α-SMA in colonic tissues in the SB203580 group and Astragaloside IV group was significantly decreased compared with that in the TNBS model group. In conclusion, Astragaloside IV reduces intestinal fibrosis in rats with TNBS-induced colitis.

Keywords: Astragaloside IV, TNBS, intestinal fibrosis, TGF-β1, p38

Introduction

Inflammatory bowel disease (IBD) comprises ulcerative colitis (UC) and Crohn's disease (CD), which is characterized by distinct clinical, histopathological, endoscopic, and radiological features and chronically relapsing inflammation of the bowel of unknown origin [1, 2]. Due to the complex etiology of human IBD genetic heterogeneity, many of our current understanding of the pathogenesis of IBD has been studied from a variety of animal models [3, 4]. Chemically induced murine models of intestinal inflammation are the most commonly used and best described because they have been shown to be similar to human IBD in multiple aspects. The hapten 2,4,6-trinitrobenzene sulfonic acid (TNBS), administered as anenema, originally was used in rats to induce chronic intestinal inflammation [5] and in BALB/c mice that reiterates the progression to fibrosis seen in CD [6]. Melgar *et al*. demonstrated that single exposure to dextran sulfate sodium (DSS) makes acute colitis progress to chronic colitis with involvement of adaptive immunity in B6 but not in BALB/c mice [7]. Although the histopathological colitis lesions in murine after chemicals administration showed chronicity, the colitis might reflect prolonged repair of acute colitis and it is not defined simply as chronic colitis. Therefore, the pathophysiology of chronic colitis remains enigmatic and should be investigated further.

Intestinal fibrosis is usually considered as a common consequence of IBD and occurs in CD [8], which invariably recurs after surgical intestinal resection [9], but may develop severe complications leading to intestinal obstruction and surgery. The lack of a better understanding of pathophysiology of intestinal fibrosis is striking because of our current inability to diagnose intestinal fibrosis early and accurately, treat it properly, and take measures to prevent it.

Animal models for intestinal fibrosis have recently been described. Chronic TNBS administration triggers intestinal fibrosis [10], and after stopping TNBS, the expression of genes related to inflammation, acute phase response, and cell proliferation declines, but the fibrosis-related proteins remains elevated [11]. Adenovirus-induced expression of TGF-β leads to the differentiation of fibroblasts into smooth muscle cells, thickening of the intestinal wall, and massive ECM deposition with obstruction as well as colonic inflammation [12]. The balance between the onset of an inflammatory process and the potential for tissue repair and fibrosis may depend on the synthesis and degradation of extracellular matrix (ECM) [13, 14]. Of the several molecules (cytokines, growth factors) regulating the development, proliferation, differentiation and activation of myofibroblasts, TGF-β appears to play a pivotal role [15, 16]. TGF-β intracellular effects are mediated mainly by Smad proteins, including activation of myofibroblasts, stimulation of α-SMA, collagens, CTGF, TIMPs and PAI-1, and inhibition of MMPs [8, 17]. Important other Smad-independent intracellular signal transduction pathway involving profibrotic effects of TGF-β has not yet been defined.

Several antifibrotic drugs (chemical and biological) have been tested in experimental models of tissue fibrosis being able to inhibit, mitigate or even reverse the fibrogenesis/fibrosis process [18]. Astragaloside IV is the main compound of Astragalus saponins, in which more than 40 constituents have been identified from the astragalus root. Astragaloside IV can improve the cardiac function of the ischemic myocardium and myocardial infarction in rats [19]. Astragaloside IV can promote the proliferation of human umbilical vein endothelial cells and angiogenesis and keep blood vessels from reducing *in vivo* [20]. Astragaloside IV has protective effects on ultraviolet A-induced photoaging in human fibroblasts [21] and ischemia-reperfusion injury after liver transplantation in rats [22]. However, there is little information about the therapeutic effect of Astragaloside IV on animal models of intestinal fibrosis in TNBS-induced colitis.

Basing ourselves upon these data there is no doubt that Astragaloside IV may play a central role in anti-fibrosis in the organ. Nevertheless, there are many interesting questions regarding the molecular mechanisms for action and signaling pathways in colitis. On the other hand, the novelty of this study is that the effects of the Astragaloside IV in colonic mucosa under TNBS-induced colitis in rats were tested for the first time as well as the involvement of the signaling pathways implicated in experimental intestinal fibrosis development. The TNBS-induced colonic injury was measured by histological and biochemical analysis. TGF-β1, CTGF, Collagen I, Collagen III, FN and α-SMA expression were analyzed in order to gain a better insight into the action mechanism(s) of the observed protective effects of Astragaloside IV.

Materials and methods

Experimental animals

Male Wistar rats supplied by Super-B&K laboratory animal Co. Ltd. (Shanghai, China) and weighing 200 ± 15 g, were placed in a controlled room (temperature 24-25°C, humidity 70-75%, lighting regimen of 12L/12D) and fed with a normal laboratory diet. Rats were deprived of food for 24 h prior to the induction of colitis, but allowed free access to tap water throughout. 8 animals were randomly assigned to each group. Experiments followed a protocol approved by the local animal Ethics Committee and Shanghai Jiao Tong University Affiliated Sixth People's Hospital.

Induction of colitis

Colitis was induced according to the procedure described by Morris *et al*. Briefly, rats were slightly anaesthetized with ether following a 24 h fast, and then a medical-grade polyurethane canal for enteral feeding (external diameter 2 mm) was inserted into the anus and the tip was advanced to 8 cm proximal to the anus verge. 1 ml TNBS (150 mg/kg, Sigma-Aldrich Company Ltd., Spain) dissolved in 50% ethanol was instilled into the colon through the cannula. Following the instillation of the TNBS/ethanol, the animals were maintained in a head-down position for a few minutes to prevent leakage of the intracolonic instillation. Rats in the control group received an equal volume of 0.9% physiological saline instead of the TNBS solution. Rats in the SB203580 group were intraperitoneally injected with 10 mg/kg of SB203580 daily for 4 days after day 4 induc-

tion of colitis. Rats in the Astragaloside IV group were gavaged with 25, 50 or 100 mg/kg of Astragaloside IV daily for 21 days after day 4 induction of colitis. The animals were sacrificed, using an overdose of anaesthetic, 22 days after induction of colitis.

Histological studies

Specimens were dehydrated and embedded in paraffin and 4 μm tissue sections were cut using a Leica Biosystem Rotary Microtome (Leica Microsystem Nussloch GmbH, Wetzlar, Germany). Next, sections of the tissues were placed on slides, deparaffinized and sequentially stained with hematoxylin and eosin (Richard-Allan Scientific, Kalamazoo, MI, USA) or Massonn trichrome. Under an identical light microscope, the stained tissue sections on slides were analyzed at magnification ×200.

Biochemical measurements

Colon tissues were hydrolyzed and then centrifuged at 1,000× g for 10 min, and the supernatant was collected in order to determine the content of hydroxyproline using Hydroxyproline assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Real-time PCR

Total RNA was extracted from colon specimens with Trizol (Invitrogen) according to the standard protocol then reverse transcribed. Thereafter, cDNA was amplified using the ABI7700 sequence-detector system (Applied Biosystems, Foster City, CA, USA). The primers of genes were as follow: TGF-β1, F: 5'-AAGGACCTGGGTTGGAAGTG-3', R: 5'-TGGTTGTAGAGGGCAAGGAC-3'; CTGF, F: 5'-CGTAGACGGTAAAGCAATGG-3', R: 5'-AGCAGCAAACACTTCCTC-3'; α-SMA, F: 5'-AACACGGCATCATCACCAAC-3', R: 5'-CACAGCCTGAATAGCCACATAC-3'; FN, F: 5'-GCCGTGGTCCTAACAAATCTC-3', R: 5'-GGCGGTGACATCAGAAGAATC-3'; Collagen I, F: 5'-TCAAGATGTGCCACTCTG-3', R: 5'-ACCTTCGCTTCCATACTC-3'; Collagen III, F: 5'-GTCCACAGCCTTCTACAC-3', R: 5'-TCCGACTCCAGACTTGAC-3'; GAPDH, F: 5'-GTCGGTGTGAACGGATTTG-3', R: 5'-TCCCATTCTCAGCCTTGAC-3'. Relative quantification of the gene expression was performed by normalization of the signals of different genes with the GAPDH signal. The ΔΔCt method for relative quantification of gene expression was used to determine mRNA expression levels.

Protein extraction and western blotting

Western blotting was performed as previously described [23]. Colon tissues were harvested and lysed on ice for 30 min in radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China) and separated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Amersham Inc., Arlington Heights, IL) by electroblotting. In the next step, the membranes were incubated with specific primary antibodies for TGF-β1, CTGF, α-SMA, FN, Collagen I, Collagen III, p-p38, p38 and GAPDH. Each filter was washed three times for 15 min and incubated with the secondary horseradish peroxidase-linked anti-goat, anti-rabbit or anti-donkey IgG antibodies. The antibody-antigen binding was visualized via the Super-Signal West Pico ECL Substrates (Pierce). Band intensity (total gray) was quantified via the Image J software.

Statistical analysis

The statistical differences were determined using One-way ANOVA and Student's t test in GraphPad Prism software, version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Differences among groups were analyzed using Student's two-tailed. All values are expressed as a mean ± S.D. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Protective effects of Astragaloside IV in TNBS-induced colitis in rats

On histological examination of the colon from control rats, the histological features were typical of a normal structure (**Figure 1A**). The histopathological features included transmural necrosis, edema and diffuse inflammatory cells (polymorphonuclear leukocytes, lymphocytes, and eosinophils) infiltration in the mucosa. Mucosal injury was produced after TNBS administration, characterized by necrosis of epithelium, focal ulceration of the mucosa and diffuse infiltration of inflammatory cells in the mucosa and submucosa (**Figure 1A**). Treatment with Astragaloside IV or p38 inhibitor SB203-580 reduced the morphological alterations associated with TNBS administration protecting the mucosal architecture (**Figure 1A**). The

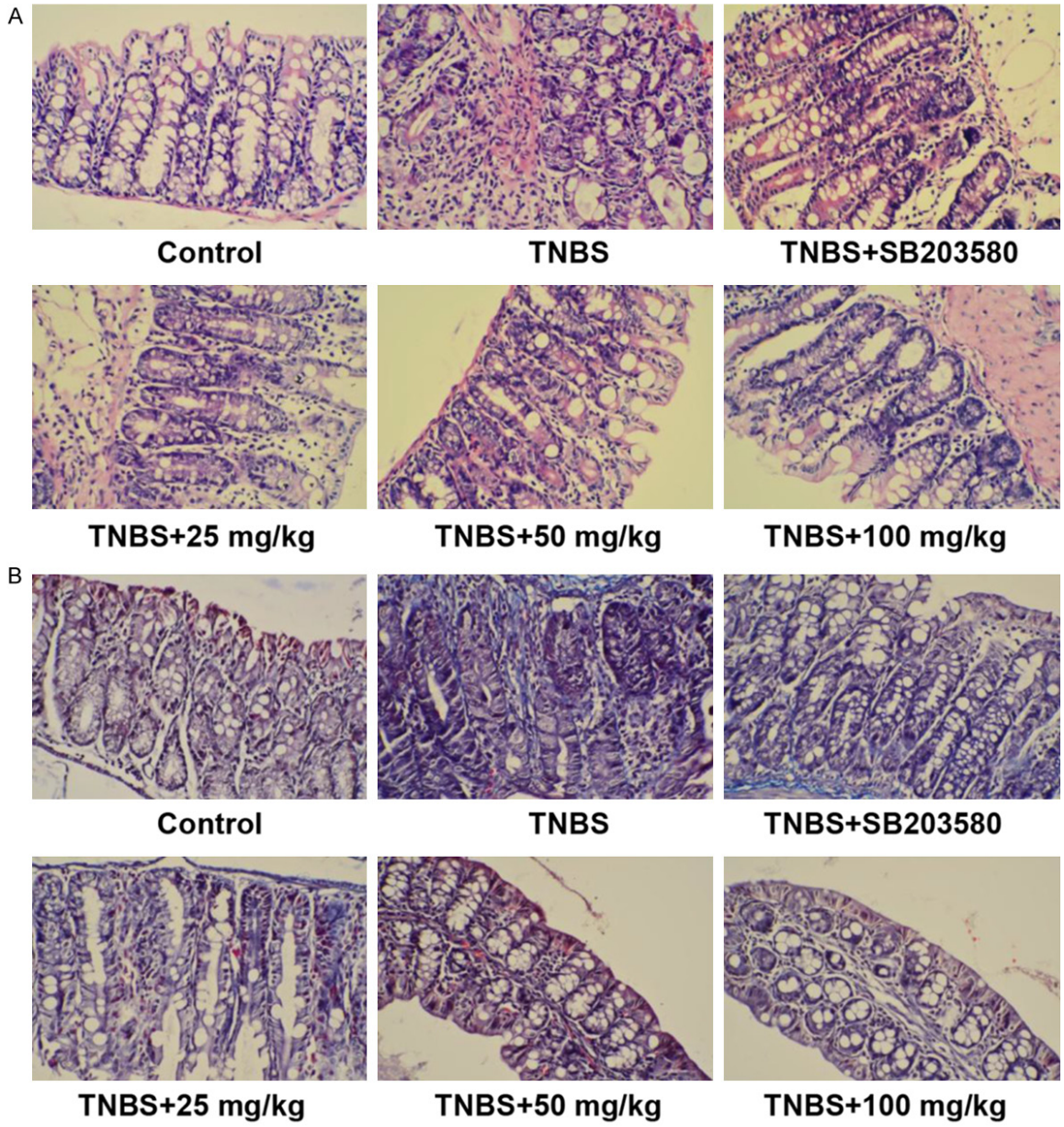

Figure 1. Effect of Astragaloside IV on colon injury in TNBS-induced colitis model. A. Histological appearance of rat colonic mucosa after haematoxylin andeosin stain (HE). B. Masson trichromic staining of rat colonic mucosa.

expression of collagen fibers in the mucosa, submucosa and muscularis was enhanced by Masson staining in TNBS-induced rats (**Figure 1B**). In TNBS rats, the affected colonic wall area consisted of granulomatous tissue in which fibroblasts and fibrosis were evident both in the submucosa and serosa together with regenerative changes in the overlying epithelium. Due to collagen deposition, in this group of rats a severe disarrangement of colonic architecture was often present. The colon of TNBS/SB203580 or TNBS/Astragaloside IV-treated rats showed nearly normal mucosal architecture and mild fibrosis. Compared to TNBS rats, colonic fibrosis was slightly reduced in TNBS rats treated with SB203580 or Astragaloside IV. The destruction of the glands and the proliferation of fibrous tissue were significantly reduced than those in the TNBS model group (**Figure 1B**). The expression of collagen fibers in the mucosa, submucosa and muscularis was significant decreased in Astragaloside IV- or SB203580-treated rats compared with that in TNBS-induced rats.

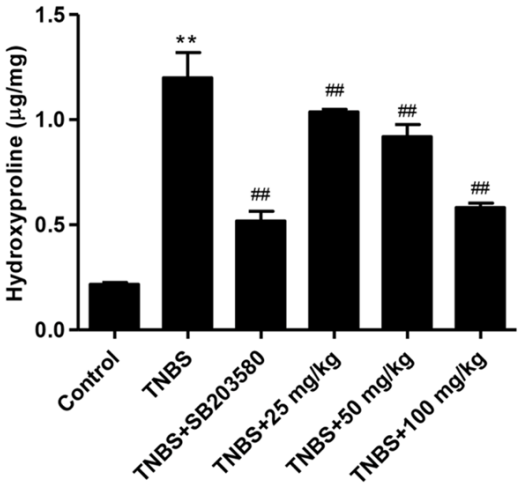

Figure 2. Effects of Astragaloside IV on hydroxyproline levels in TNBS-induced colitis model. Control groups, TNBS and SB203580 or Astragaloside IV-treated animals after 22 days. $^{**}P<0.01$ compared with control; $^{\#\#}P<0.01$ compared with TNBS.

Hydroxyproline level is significantly decreased after Astragaloside IV administration

Our data also showed that hydroxyproline content increased significantly in colonic mucosa of TNBS group compared with control rats. In addition, under our experimental conditions, the tested doses of Astragaloside IV or SB203580 significantly reduced the rise in the hydroxyproline generation compared with TNBS group (**Figure 2**).

Effect of Astragaloside IV on protein expression and p38 activity

The expression levels of TGF-β1, CTGF, α-SMA, FN, Collagen I and Collagen III were measured by Real-time PCR and western blotting of colonic mucosa. As shown in **Figure 3A** and **3D**, the expression of TGF-β1 and CTGF was significantly increased in TNBS-induced rats at both mRNA and protein levels. Moreover, Astragaloside IV or SB203580 treatment significant decreased mRNA and protein expression of TGF-β1. While, only SB203580 or high dose of Astragaloside IV can decrease CTGF expression (**Figure 3A**, **3D**, **3G**). mRNA and protein expression of α-SMA and FN mRNA expression were significantly increased in response to TNBS, but decreased in Astragaloside IV or SB203580-treated rats. However, only high dose of Astragaloside IV can correct TNBS-induced FN expression in rats (**Figure 3B**, **3E**, **3G**). Collagen I and Collagen III mRNA and protein expression was increased by TNBS treatment and decreased in SB203580 or high dose of Astragaloside IV-treated rats (**Figure 3C**, **3F**, **3G**). Finally, we also determined the activity of p38 in TNBS-induced rats. As shown in **Figure 3G** and **3H**, p-p38/p-38 fold change was significantly increased in TNBS-induced rats, while SB203580 or Astragaloside IV can decrease p-38 activation induced by TNBS.

Discussion

Disruption of any one of a number of specific immune defense mechanisms may lead to the development of chronic intestinal inflammation such as inflammatory bowel disease (IBD), which includes ulcerative colitis (UC) and Crohn's disease (CD). Transmural inflammation of Crohn's disease (CD) is associated with the phenotypic transition of the mesenchymal cells, which results in cell proliferation and collagen deposition, and then leads to the distortion of tissue architecture, fibrosis, stenosis and obstruction [24, 25]. Patients with CD often showed excessive deposition of ECM, thickening of all layers of the gut wall, overgrowth of muscular layers of the intestine, proliferation of profibrogenic mesenchymal cells in the colon and overexpression of profibrogenic cytokines and growth factors, which are the key factors for intestinal fibrosis and prerequisites for ideal animal models of intestinal fibrosis [26, 27]. In the present study, we have demonstrated for the first time that Astragaloside IV attenuated intestinal fibrosis in rats with TNBS-induced colitis.

In fibrotic intestinal diseases, marked thickening of all layers of the intestinal wall is observed. In this report, we showed morphological signs of cell damage, the colon wall of rats with TNBS-induced colitis showed thickening. SB203580 or Astragaloside IV administration with anti-fibrotic effects significantly improved the course and macroscopic finding of TNBS-induced colonic colitis, as well as the histological severity of the fibrosis of the colonic wall, compared to the rats treated with TNBS. These observations suggest that SB203580 or Astragaloside IV with anti-fibrotic properties could be an effective therapeutic strategy for the cure and the prevention of intestinal fibrosis. In the TNBS model, SB203580 or Astragaloside IV caused a reduction in the damage

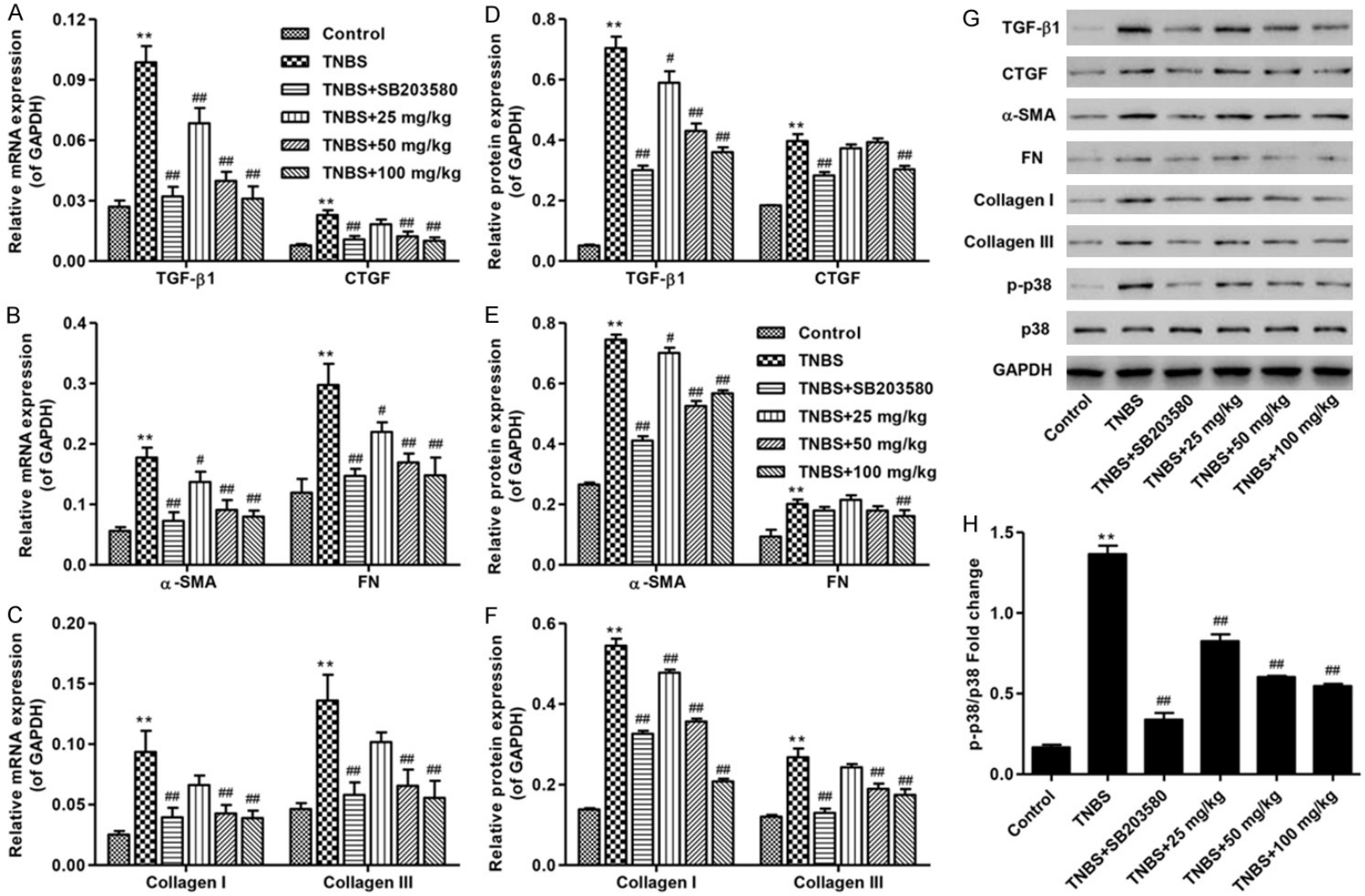

Figure 3. Effects of Astragaloside IV on TFG-β1 and p38 expression in TNBS-induced colitis model. The expression of TGF-β1, CTGF, α-SMA, FN, Collagen I and Collagen III was measured by Real-time PCR (A-C) and western blot assay (D-G). (G, H) The expression of p38 and p-p38 was measured by western blot assay. $^{**}P<0.01$ compared with control; $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with TNBS.

score also accompanied by attenuation in hydroxyproline production, an indicator of fibrosis. Hydroxyproline is a major component of the protein collagen and plays key roles for collagen stability, this therefore may be of special interest because fibrosis is a major complication of IBD, which is mediated by intestinal fibroblasts [28, 29]. In line with our finding, in an experimental acute and chronic model of TNBS-induced colitis, the hydroxyproline determination, used as indicator of collagen production and fibrosis, showed a marker increase [30]. Moreover, Liu et al. reported that Astragaloside IV delayed the formation of liver fibrosis and decreased the serum levels of hydroxyproline content in liver [31].

Astragaloside IV is one of the main active ingredients in *Astragalus membranaceus* and exhibits various activities, including vasodilating effect, preventing endothelial dysfunction, improving cardiac cell energy metabolism, anti-inflammatory effect, and antioxidant effects [32, 33]. Similar to our results for the first time that Astragaloside IV inhibited TNBS-induced intestinal fibrosis in rats was observed, previous studies showed that Astragaloside IV protects against the progression of renal fibrosis by inhibiting inflammation via the TLR4/NF-κB signaling pathway [34] and suppresses bleomycin-induced pulmonary fibrosis in rats via attenuation of oxidative Stress and inflammation [35]. The effects of Astragaloside IV on the cellular and molecular mechanisms underlying intestinal fibrosis have never been evaluated.

Intestinal fibrosis is related to abnormal accumulation of ECM proteins produced by activated intestinal mesenchymal cells [36]. Several evidence suggest that different molecules control of intestinal fibrosis, including stem cell factor (SCF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin-like growth factor-1 and -2 (IGF-1 and -2), connective tissue growth factor (CTGF), interleukin-1 and -13 (IL-1 and -13), endothelins (ET-1, ET-2, ET-3), angiotensin II, transforming growth factor-α (TGF-α), basic fibroblast growth factor (bFGF), peroxisome proliferator-activated receptor-γ (PPAR-γ), and TGF-β superfamily members [8, 37]. In the present study, we found that the expression of TGF-β1, CTGF, α-SMA, FN, Collagen I and Collagen III was significantly increased in response to TNBS treatment, while SB203580 or Astragaloside IV inhibited the expression of these protein. Thus, the model showed enhanced abnormal collagen deposition which mimics the fibrosis of the intestine of CD, and could become a beneficial tool for the analysis of the pathophysiology of intestinal fibrosis. Consistently, the increased colonic expression of Collagen I, Collagen III, α-SMA, CTGF and TGF-β1 was also found in TNBS-induced chronic colitis in rats [37]. Meng *et al.* reported that Astragaloside IV inhibits renal tubulointerstitial fibrosis in rats and also inhibit expression of α-SMA and TGF-1 in NRK-49F and HK-2 cells *in vitro* [38]. Therefore, intestinal fibrosis in chronic colitis could be explained at least in part via TGF-β-dependent transition of mesenchymal progenitor fibroblasts into profibrogenic fibroblasts and myofibroblasts with increased production of collagens [39]. The precise mechanism including the role of CTGF, IGF-I as well as TGF-β1 should be further investigated.

We also examined the expression and activation of the p38 MAPK by western blot analysis. Intracolonic administration of TNBS resulted in a significant increase in the phosphorylation of p38 MAPK protein, indicating that the p38 MAPK protein activation could be caused by TNBS. Interestingly, administration of SB203580 or Astragaloside IV was able to diminish the activation of p38 MAPK. p38 MAPK is a key modulator of several target genes that ultimately control infiltration of monocytic cells, acute intestinal inflammation and intestinal electrolyte and water secretion. Our results are in agreement with a previous study by Camacho-Barquero et al. [40] in which it has been shown that p38 MAPK was activation in acute experimental colitis. Abrogation of p38 MAPK by SB203580 evidently eliminates the inhibitory effects of Astragaloside IV on genes relevant to the activation of hepatic stellate cells (HSC), the key effectors in hepatic fibrogenesis, suggesting that Astragaloside IV inhibits fibrogenesis by inhibiting p38 MAPK activation [41].

In conclusion, the results of this study demonstrated that the association of Astragaloside IV prevents the development of colonic fibrosis in an experimental model of TNBS-induced colitis in rats. The antifibrotic properties were in part related to their effects on the modulation of the p38 MAPK signaling pathway. The results of

this study provide new insights into reversing colonic fibrosis through the inactivated p38 effects of Astragaloside IV, and further studies are needed to clarify the underlying mechanism and enhance the performance of Astragaloside IV in colitis.

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Disclosure of conflict of interest

None.

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