

Periplaneta Americana Extract may Attenuate 2,4,6-Trinitrobenzenesulfonic Acid-Induced Intestinal Fibrosis by Inhibiting TGF- β /Smad Signaling Pathway

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Research

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Abstract

Purpose

Intestinal fibrosis is an incurable digestive disease accompanied by stricture formation, and it has an increasing incidence in recent years. Periplaneta americana is one of the medicinal insects with a long history. There are few reports on the effect of intestinal fibrosis. This study aims to evaluate the inhibitory effect of PA treatment on intestinal fibrosis.

Methods

TNBS was used to establish intestinal fibrosis model by enema in BALB/c mice. The mice were treated with PA (50, 100, 200 mg/kg body weight) and 5-aminosalicylic acid (5-ASA) (40mg/kg) by gavage once a day for 6 weeks. At the end of the last week, the mice were sacrificed. Colon samples were collected for H&E and Masson staining. The mRNA and protein expression of α -smooth muscle actin (α -SMA), collagen I and the transforming growth factor- β (TGF- β) / Smad signaling pathway were conducted by real-time PCR and western blot analysis. In vitro, TGF- β 1 was used to induce intestinal fibrosis at human colon fibroblasts (CCD-18Co). And using real-time PCR and western blot methods to detect the expression of α -SMA and collagen I.

Results

PA inhibited the expression of α -SMA and collagen I in vivo and in vitro. But the difference was that PA inhibited the TGF- β /Smad signaling pathway in vivo, and the same results had not been obtained in vitro. Conclusion: PA may attenuate intestinal fibrosis by inhibiting TGF- β /Smad signaling pathway, but more experiments were needed to prove it in vitro.

Conclusions

PA has potential pharmacological effects in inhibiting intestinal fibrosis, and the TGF- β /Smad signaling pathway seemed promising.

Introduction

Intestinal fibrosis is currently a common clinical unsolved disease, and is more common in complications of IBDs [1,2]. Fibrosis occurs in more than one-third of CD patients. About 50% of patients with CD have fibrotic stenosis, and 75% of them will eventually receive surgery [3]. With the extension of the onset time, intestinal fibrosis will also have other complications, such as stenosis [4], fistula, and even colon cancer [5]. The progress of intestinal lesions varies greatly and may last for a long time, and fibrosis more easily occurs in deep ulcers or transmural fissures [6]. Despite the significant progress in the inflammation treatment of IBDs, the incidence of intestinal stenosis and fibrosis has not been significantly reduced [7]. Normally, excessive accumulation of scar tissue in the intestinal wall is a manifestation of intestinal

fibrosis, accompanied by the distribution of inflammation, this accumulation may run through the thickness of the entire intestinal wall and may cause stenosis [8].

Fibrosis is the result of long-term local chronic inflammation, mainly manifested in ECM protein deposition, which is produced by activated fibroblasts [9]. TGF- β 1 isoform is not only closely related to fibrosis of various organs (including intestinal fibrosis), but also has a significant relationship with ECM synthesis. Moreover, the TGF- β /Smad signal pathway is generally considered to be related to fibrosis, Smad protein is a typical TGF- β signal pathway [10]. TGF- β 1 is the central pathway of organ fibrosis in most if not in all organs, including gastrointestinal tract and heart. Despite the important clinical impact, disease pathogenesis is not fully understood, and no targeted therapies able to revert fibrosis are currently available.

TCM is widely used in the treatment of various clinical diseases in Asia and has a long history. In addition, many TCM exhibit special pharmacological effects in the treatment of human chronic diseases [11]. The extract of PA contains many ingredients that can kill bacteria, fungi, viruses, protozoa and even cancer cells. PA has been studied as an alternative therapy for many diseases, such as heart disease, burn wounds [12-13], cancer [14], etc. Studies have also shown that PA could resist liver fibrosis [15], renal fibrosis [16], and may reduce the accumulation of ECM components by inhibiting the expression of TGF- β 1 and α -smooth muscle actin (α -SMA). Even, PA can improve pulmonary fibrosis through TGF- β /Smad signaling pathway [17]. Although the research on PA has made great progress in recent years, the role of the drug in intestinal diseases is rarely reported, especially in intestinal fibrosis. In view of these findings, this study aims to evaluate whether PA could inhibit the activation of fibroblasts and the progression of intestinal fibrosis by blocking TGF- β /Smad signaling pathway. TNBS-induced mouse intestinal fibrosis model was used in this study, which focused on the colonic protective effect of PA in intestinal fibrosis.

Materials And Methods

PA: Sichuan GoodDoctor Pharmaceutical Group. TNBS: Dalian Meilun Biotechnology Co., LTD. BALB/c mice: JiNan Pengyue Experimental Animal Breeding Co., Ltd. 5-ASA: Beijing Solarbio Science & Technology Co., Ltd. The gene sequences were synthesized by Sangon Biotech (ShangHai, China). Primary antibodies (anti- β -actin (43kDa), anti-collagen I (139kDa), anti-TGF-beta1 (12.5kDa), anti-Smad2 (58kDa), anti-phospho-Smad2 (58kDa), anti-Smad3 (48kDa), anti-phospho-Smad3 (48kDa) and anti-Smad7 (46kDa), anti- α -SMA (48kDa)): Abcam (Cambridge, UK). Peroxidase-conjugated goat-antimouse IgG (H+L): Zsgb-Bio (Beijing, China). Peroxidase-conjugated goat antirabbit IgG (H+L): Zsgb-Bio (Beijing, China). TRITC-conjugated goat antirabbit IgG): Zsgb-Bio (Beijing, China).

Experimental protocols in Mice

Female BALB/c mice (8-10 weeks old. body weight 18-22 g.) were kept under the Animal Center of Anhui Medical University (Hefei, China). All mice were housed under standard environmental conditions at controlled temperature ($22 \pm 2^\circ\text{C}$), humidity ($50 \pm 10\%$), and light (12 h light/dark cycle), they were free access to standard diet and water. A total of 110 mice were randomized into six groups, listed as follow:

Control group, TNBS group, PA groups (50mg/kg, 100mg/kg, 200mg/kg), 5-ASA group (40 mg/kg). The dosage of PA was selected according to the references [18]. The amount of TNBS per week was: 1.0, 1.0, 1.5, 1.5, 2.0, and 2.0 mg, prepared with 45% ethanol. The mice were fasted overnight and gently anesthetized with ether. A 3.5-F catheter was connected to a 1 ml syringe, the animals were anesthetized, 100 µl/20g of TNBS was aspirated with an enema. Slowly insert the catheter 4 cm from the anus. The key step: proceed very slowly, so as not to damage or destroy the colon wall. If any resistance you feel during catheter insertion, remove the catheter and try to reinsert it gently. Slowly inject 100 µl / 20g of 45% ethanol + TNBS solution into the colon cavity. Slowly remove the catheter from the intestine and place the mouse head down for 1 min. TNBS was given to mice once a week, physiological saline, PA and 5-ASA were given to mice by gavage every day. The whole experiment lasted for 6 weeks. The mice were executed at the seventh week, and blood was taken from the eyeballs before execution.

Hematoxylin and Eosin (H&E) Staining

All mice colon samples were washed and immersed in a 4% formaldehyde solution and then embedded in paraffin at 4% paraformaldehyde. Then the tissue was dehydrated in an ascending series of ethanol, embedded in paraffin. Serial sections (3.5mm) were stained with hematoxylin and eosin (H&E). The stained sections were observed under a Nikon Eclipse E800 (Nikon Corporation, Tokyo, Japan).

Table1: The histological activity index (HAI)

Masson staining

The aforementioned paraffin embedded slices (5 µm) were stained with Weigert solution (Sigma-Aldrich, Merck KGaA) for 5-10 min. After being fully washed, sections were treated with Ponceau fuchsin acid solution for 5-10 min, immersed in 2% acetic acid aqueous solution for 1min, then differentiated in 1% phosphomolybdic acid aqueous solution for 3-5 min. Without washing with water, the sections were treated with aniline blue for 5 min then immersed in 0.2% acetic acid aqueous solution for 1 min. Slices were permeabilized with xylene and mounted with neutral resin.

Cell Culture and Treatments

CCD-18Co cells were cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin at 37°C in a humidified 5% CO₂ atmosphere. Cells were seeded in 96-well plates at 1×10⁴ cells per well, and incubated overnight. Cells were given 5.0 ng/ml TGF-β1 for 12h in serum-free DMEM medium. The PA groups were pretreated with the indicated concentrations: 1.0, 2.0, 4.0, 8.0, 16.0, 32.0 mg/ml, 5-ASA positive groups concentrations: 5, 10.0, 20.0 µmol/l. The cells were further cultured for 24 hours, and then they were monitored.

MTT assay

After the previous step of cell treatment, remove the supernatant and rinse gently with PBS twice, and finally 20 µl MTT was added to each well in the dark, then incubated for 4 h at 37°C. Subsequently,

remove the supernatant was, 150 μ l DMSO was added to each well. 96-well plates were shaken for 15min. Benchmark microplate reader (Thermo, Massachusetts, USA) was used to detect the optical density values (OD) at 490 nm.

Real-Time PCR analysis for mRNA expression

Total RNA was extracted from colon samples using Trizol reagent (Invitrogen). 5X PrimeScript RT Master Mix was used to perform the reverse transcription. These steps must be performed at low temperatures to prevent RNA damage. ABI 7500 Real Time System (Applied Biosystems, Foster City, CA, USA) was used to perform real-time PCR by using the Platinum SYBR Green PCR SuperMix-UDG kit. The amplification conditions: 95°C for 30 s, 40cycles at 95°C for 5 s, 60°C for 34 s. The gene sequences (5'-3') were synthesized as table 2. The relative expression levels of genes were calculated using control β -actin mRNA and the $2^{-\Delta\Delta C_t}$ method. RQ (Relative Quantification) values were used for statistical analysis.

Table 2:Primer sequences used for RT-PCR analysis

Western blot analysis

Proteins were extracted from colon samples using RIPA lysis buffer, PMSF and protease inhibitors, and they were separated on 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to PVDF membranes. Membranes were blocked with 5% non-fat dried milk for 1h at room temperature, and then incubated with primary antibodies overnight at 4°C, including anti- β -actin, anti-collagen I, anti-TGF-beta1, anti-Smad2, anti-phospho-Smad2, anti-Smad3, anti-phospho-Smad3 and anti-Smad7, anti- α -SMA. Subsequently, the membrane was incubated with secondary antibodies for 1 h and washed them three times with PBS. The blots were visualized using Super Signal West Pico (Bridgen, Beijing, China).

Statistical Analysis

Statistical significance was determined by analysis of variance (ANOVA) using GraphPad Prism version 8.0 (GraphPad Prism, CA, USA). Data were expressed as mean \pm standard deviation (SD). A value of $P < 0.05$ was considered statistically significant.

Results

PA Attenuates Intestinal Fibrosis in Mice

Severity of colonic inflammation was evaluated by HE staining assay. As shown in Figure 1, colonic tissues from the control group was almost normal with little inflammatory infiltration necrosis and edema, lamina propria and mucosal muscle layers were well arranged, there is almost no loss of crypts and cups cell. In TNBS group, colonic crypts and cups cell loss was more, and a large number of inflammatory cells infiltrated in all layers of the colon. With the increase dose of PA, the protective effect

on the colon structural integrity became more obvious, especially in the 200mg/kg PA group, and the 5-ASA group also showed a protective effect on the colon.

PA reduces collagen expression in the colon of mice with intestinal fibrosis

As shown in figure 2, the collagen expression in control group was less, but it significantly increased in TNBS group. In the PA groups, the decreased collagen expression could be observed obviously. The most significant inhibitory effect of collagen expression appeared in 5-ASA group. It was not difficult to find that the 100mg/kg and 200mg/kg PA had almost the same degree of inhibition as the 5-ASA group.

PA Suppresses α -SMA and Collagen I Expression in Mice with Intestinal Fibrosis

Real time-PCR and western blot assays were performed to evaluate the expression levels of fibrosis-related mRNA and proteins in intestinal tissue. As shown in Figure 3, compared with control group, mRNA and protein levels of collagen I and α -SMA were obviously up-regulated in TNBS group, while they were significantly down-regulated in 100mg/kg, 200mg/kg PA groups and 5-ASA group.

PA Attenuates CCD-18Co Cells Fibrosis and Inhibits the Expression of α -SMA and collagen I

MTT assay was used to determine the safe concentration of PA's effect on CCD-18Co cells. In figure 4A, compared with control group, TGF- β 1 group was not observed proliferative effect on CCD-18Co cells. TGF- β 1 stimulated and induced CCD-18Co cells to produce ECM, but the proliferation did not be promoted. It could be observed that 8-32mg/ml PA inhibited cell proliferation obviously, which might be a toxic concentration. The concentration of 1, 2, 4 mg/ml PA was selected in this study. As shown in Figure 4B-E, mRNA expression of α -SMA and collagen I in the TGF- β 1 group were higher than control group. Compared with the TGF- β 1 group, both PA groups and the 5-ASA group were able to reduce the mRNA expression of α -SMA and collagen I. The protein expression of α -SMA was significantly higher in TGF- β 1 group, and was lower in PA and 5-ASA groups. Compared with control group, the protein expression of collagen I showed a significant increase in TGF- β 1 group, and it was reduced in PA groups.

PA Protects against Intestinal Fibrosis by Inhibiting TGF- β /Smad signaling pathway in Mice

In order to detect the expression of TGF- β /Smad signaling pathway, RT-PCR and Western blot were used to detect the expression of TGF- β 1, Smad2, p-Smad2, Smad3, p-Smad3, and Smad7. As shown in figure 5, in the gene expression results (figure A-D), the expression of Smad2, Smad3 and TGF- β 1 were all up-regulated in TNBS group, and down-regulated after 100mg/kg and 200mg/kg PA treatment. Although 5-ASA group could also inhibit their expression, but the effect was not as good as PA. In addition, the expression of Smad7 showed the opposite result. In the protein expression results (figure E-J), the expression of p-Smad2, Smad2 and p-Smad3, Smad3 were up-regulated in TNBS group, and down-regulated in PA groups and 5-ASA group. The expression result of Smad7 and TGF- β 1 was basically consistent with gene expression.

Discussion

Studies have shown that PA has many pharmacological activities, and even found that PA has potential effect on inhibiting liver fibrosis [18] and renal fibrosis [16]. However, the effect of PA on intestinal fibrosis is unknown. In this experiment, a model of intestinal fibrosis was constructed firstly. Rectal administration of TNBS and ethanol to cause colon inflammation in susceptible mouse strains is a rapid method [19]. When TNBS-induced chronic colitis model of BALB/c mice was used, it showed the characteristics of fibrosis. Its histological characteristics are mainly manifested in: transmural inflammation, inflammatory cell infiltration (macrophages, neutrophils and lymphocytes) [20]. In the TNBS group, intestinal section staining showed a large number of inflammatory cells, indicating significant inflammation of the colon. However, inflammatory cells were relatively reduced in the PA groups, which may be the result of anti-inflammatory effects of PA. This might have a positive effect on inhibiting intestinal fibrosis.

In addition, we had mentioned earlier that another manifestation of intestinal fibrosis was the deposition of ECM [21], the deposition of ECM was manifested in a large amount of α -SMA and collagen (typically type I collagen) expression. ECM is produced by activated fibroblasts and is regulated by pro-/anti-fibrotic factors [22]. Cells associated with intestinal fibrosis, typically colonic subepithelial myofibroblasts, have been shown to be the major producers of ECM [23]. When colon tissue is damaged, myofibroblasts are activated and contract by shrinking the expression of α -SMA. Then, ECM multiply and migrate to the wound site and secrete large amounts of ECM components such as collagen and fibronectin. Therefore, in our experiments, the genes and proteins expression of α -SMA and collagen I showed similar results in vivo and in vitro. Wherefore, PA might be able to inhibit intestinal fibrosis by reducing ECM. In our experiments, the gene and protein expression of α -SMA and collagen I increased in the TNBS group, which might be the cause of intestinal fibrosis. Under the treatment of PA, the expression of α -SMA and collagen I were decreased, and the 100mg/kg and 200mg/kg of PA showed significant inhibitory effects, suggested that PA could reduce intestinal fibrosis at this concentration.

According to statistics, the most important profibrotic mediators include TGF- β , insulin-like growth factor (IGF)-1 and -2, activins, connective tissue growth factor (CTGF), various cytokines, such as Interleukin (IL)-1, -4, -6, -13, -17), angiogenic factors and so on [9, 24]. TGF- β is expressed in a large number of cell types and organs in mammals and is linked to ECM. The TGF- β 1 isoform can promote ECM synthesis and fibroblast contraction [25]. In this experiment, TGF- β 1 was used to stimulate human colonic fibroblasts (CCD-18Co), which caused ECM secreted. The results showed that the up-regulation of α -SMA and collagen I expression in the TGF- β 1 group, it was the evidence of intestinal fibrosis. After PA treatment, their expression were down-regulated, further indicated that PA could also attenuate intestinal fibrosis in vitro.

TGF- β is a multifunctional cytokine involved in the fibrosis of almost all organs and tissues (including the gastrointestinal tract). One of the most typical signaling pathways is the Smad pathway. Activated TGF- β receptor mediate Smad protein expression and regulate the expression of type I collagen [26-28].

Overexpression of TGF- β in mice can cause intestinal fibrosis and obstruction, preventing intestinal TGF- β /Smad signaling mice from surviving colonic fibrosis. Additionally, Smad7 can inhibit TGF- β signaling. Disrupting the TGF- β /Smad pathway or increasing the expression of Smad7 can effectively reduce the occurrence of intestinal fibrosis. The increase in p-Smad2/3 or the decrease in Smad7 is consistent with the profibrotic effect of the TGF- β /Smad pathway [29]. Despite the risks, TGF- β is still a very important target in the progress of intestinal fibrosis. Consequently, TGF- β signaling is a potential strategy for the treatment and alleviation of intestinal fibrosis [10]. In vivo results, PA treatment caused a decrease in the expression of Smad2, p-Smad2, Smad3, p-Smad3, and TGF- β 1 protein molecules in the TGF- β /Smad signaling pathway, and an increase expression of Smad7. Therefore, PA could inhibit intestinal fibrosis through the TGF- β /Smad signaling pathway in vivo. However, PA was not a monomer, and the results in vitro experiments had not been consistent with the results in vivo of TGF- β /Smad signaling pathway inhibition. In this experiment, we also observed that some protein expression results were inconsistent with mRNA expression. This may be due to the posttranscriptional processing, transcriptional products undergoes degradation, translation, and posttranslational modification [30].

Conclusions

This result indicated that PA might have a potential role in inhibiting intestinal fibrosis by regulating the TGF- β /Smad signaling pathway. In the next experiment, we will deeply study and consider exploring other factors related to fibrosis effects of signal pathways. We hope that our experiment will contribute to the treatment of intestinal fibrosis.

Abbreviations

2,4,6-trinitrobenzenesulfonic acid: TNBS, *Periplaneta americana*: PA, Inflammatory bowel diseases:IBDs, 5-aminosalicylic acid: 5-ASA, Transforming growth factor- β 1: TGF- β 1, Human colon fibroblasts: CCD-18Co, α -smooth muscle actin: α -SMA, Traditional Chinese Medicine: TCM, Excessive extracellular matrix: ECM

Declarations

Ethics approval and consent to participate

The animal study was approved by the Committee for Experimental Animal Use and Care of Anhui Medical University.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Author Contributions

Jing Liu: conception and design of study, acquisition of data. Pin Lv: conception and design of study, acquisition of data. Xiang Rao: revising the manuscript critically for important intellectual content. Jiajia Wang: Financial support. All authors read and approved the final manuscript.

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Tables

Table 1: The Disease Activity Index points

Points	Weight loss(%)	Stool consistency/diarrhea	Blood
0	None	Normal	Negative hemocult
1	1%-5%	Soft but still formed	Negative hemocult
2	6%-10%	Soft	Positive hemocult
3	11%-18%	Very soft; wet	Blood traces in stool visible
4	>18%	Watery diarrhea	Gross rectal bleeding

Table 2:Primer sequences used for RT-PCR analysis

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
β -actin	TCCTCCTGAGCGCAAGTACTCT	GCTCAGTAACAGTCCGCCTAGAA
α -SMA	TGGCCACTGCTGCTTCCTCTTCTT	GGGGCCAGCTTCGTCATACTCCT
Collagen I	TCTGGAGAGGCTGGTACTGC	GAGCACCAAGAAGACCCTGA
Smad2	ACTAACTTCCCAGCAGGAAT	GTTGGTCACTTGTTTCTCCA
Smad3	GGAGCCGAGTACAGGAGACA	AAAGGTCCATTCAGGTGCAG
Smad7	TTTGTGTATTTATTTCTTTCTCTC	CACTCTCGTCTTCTCCTC
TGF- β 1	CCCTGGACACCAACTATTGC	TGCGGAAGTCAATGTACAGC

Figures

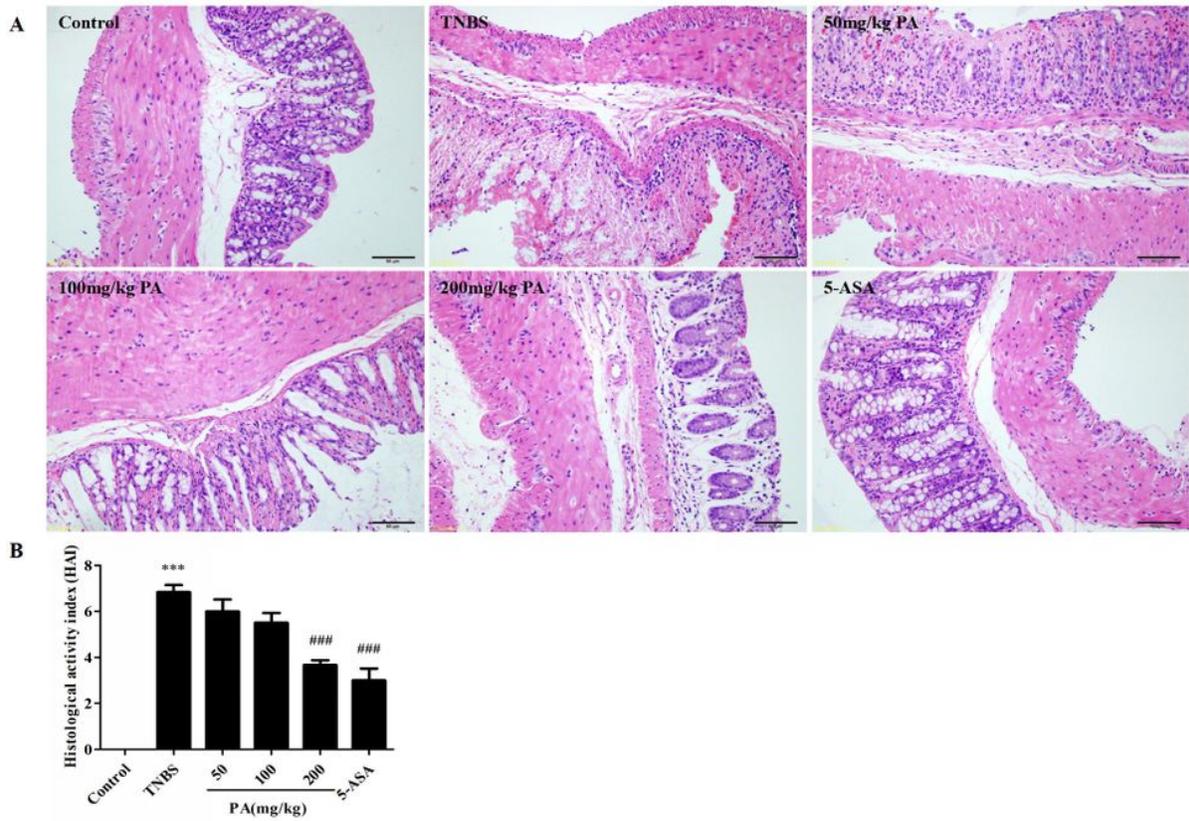


Figure 1

H&E staining on mice colon tissue (A) H&E staining observation of intestinal pathological changes in mice(100×). (B) HAI scores. *** $p < 0.001$ vs control group. ### $p < 0.001$ vs TNBS group.

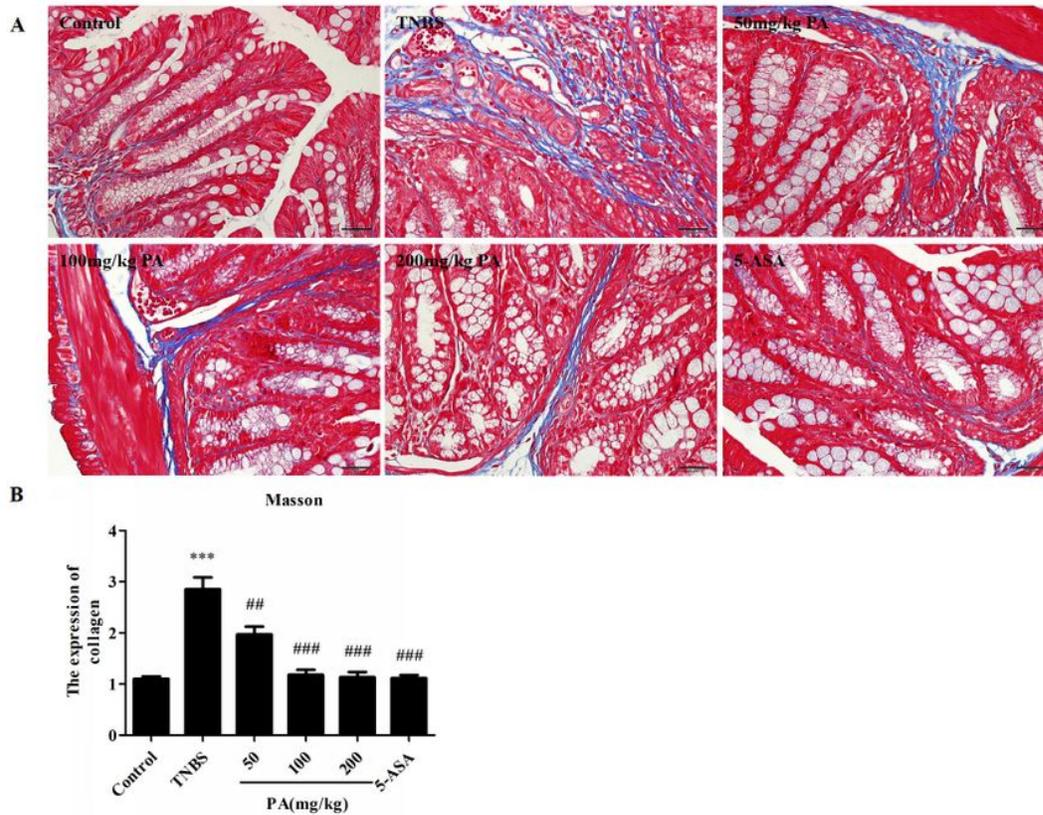


Figure 2

Masson staining on mice colon tissue (A) Masson staining image in mice colon (400×). (B) Statistical analysis of collagen expression in Masson staining images. *** $p < 0.001$ vs control group. ## $p < 0.01$, ### $p < 0.001$ vs TNBS group.

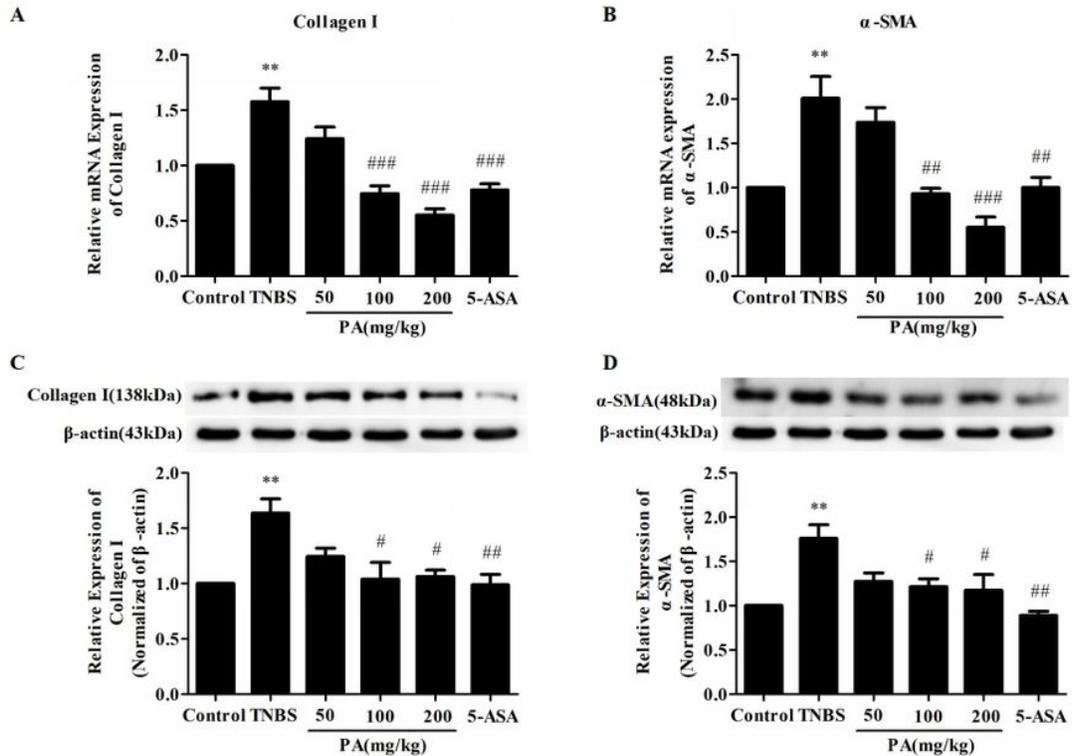


Figure 3

The mRNA and protein expression of collagen I and α -SMA in mice (A) Relative mRNA expression of collagen I. (B) Relative mRNA expression of α -SMA. (C) Immunoblot and protein expression analysis of collagen I. (D) Immunoblot and protein expression analysis of α -SMA. ** $p < 0.01$ vs control group. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs TNBS group.

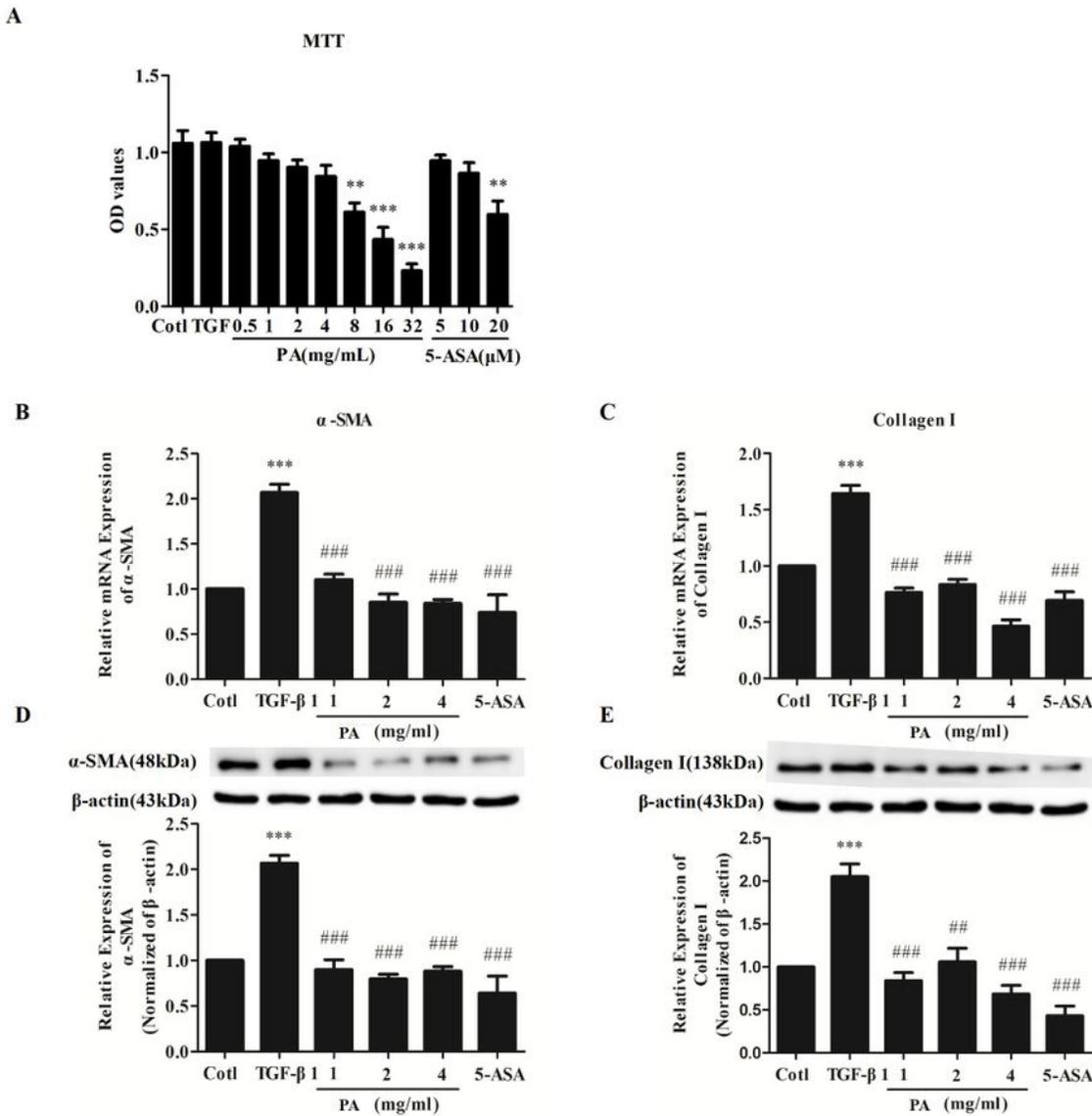


Figure 4

MTT assay and the mRNA and protein expression of α-SMA and collagen I in CCD-18Co cells (A) MTT assay result. (B) Relative mRNA expression of α-SMA. (B) Relative mRNA expression of collagen I. (C) Immunoblot and protein expression analysis of α-SMA. (D) Immunoblot and protein expression analysis of collagen I. ***p<0.001 vs control group. ##p<0.01, ###p<0.001 vs TGF-β1 group.

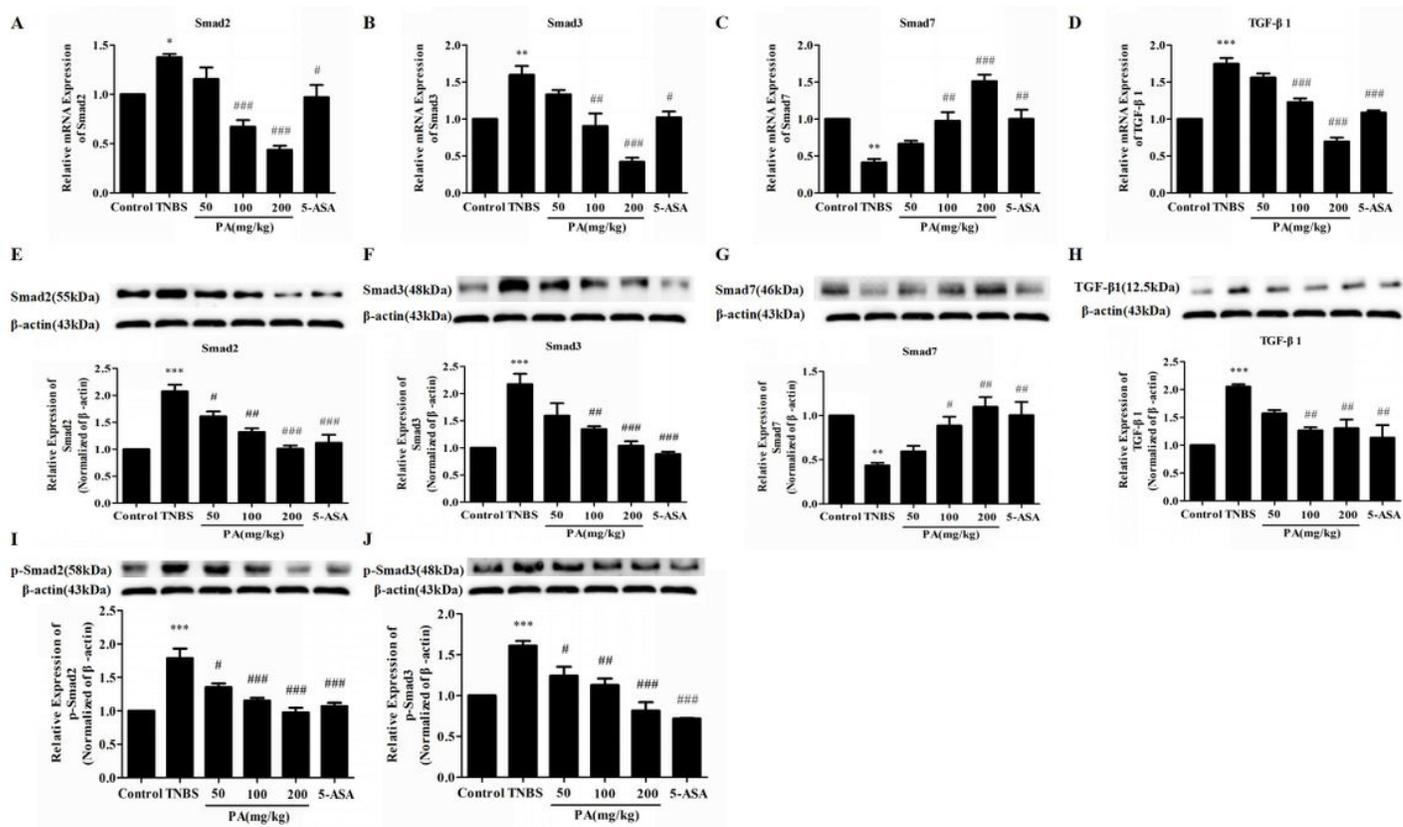


Figure 5

The mRNA and protein expression of TGF-β/Smad signaling pathway in mice (A-D) Relative mRNA expression of Smad2, Smad3, Smad7 and TGF-β1. (E-J) Immunoblot protein expression analysis of Smad2, Smad3, Smad7, TGF-β1, p-Smad2 and p-Smad3. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control group. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs TNBS group.