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Research Article
Gentianopsis paludosa Regulates TL1A/DR3 and ZNF281 to Affect Inflammatory Bowel Disease-Associated Intestinal Fibrosis

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Abstract

Background and Objective: Colonic fibrosis is a common and serious complication of Inflammatory Bowel Disease (IBD). The TL1A/DR3 has been shown to be aberrantly expressed in patients with intestinal fibrosis in IBD and may be one of the key regulatory signaling pathways in intestinal fibrosis. The transcription factor ZNF281 has been proposed as a novel player in intestinal inflammation and fibrosis. Previous studies have shown that *Gentianopsis paludosa* (GP) can alleviate the symptoms of colonic fibrosis in rats, but the mechanism is not completely known. This study focused on the treatment of colonic fibrosis by GP via TL1A/DR3 and ZNF281. **Materials and Methods:** Lincomycin hydrochloride and 2,4,6-Trinitrobenzene Sulfonic Acid (TNBS) was used to induce colonic fibrosis rat models. As 0.68, 0.34 and 0.17 mg kg^{-1} of GP and salazosulfapyridine (SASP) were used to treat colonic fibrosis rats. The HE staining and Masson staining experiments were performed to evaluate histopathological damage and fibrosis. The expression of fibrosis markers (TGF-β1, α-SMA and ZNF281) were detected by immunohistochemistry and the expression of inflammatory factors (IL-1 and IL-13) were detected by ELISA. Western blotting was applied to detect the expression of TL1A/DR3 signal pathway-related proteins. **Results:** Lincomycin hydrochloride and TNBS caused significant colonic fibrosis in rats. The SASP and GP significantly reduced colonic tissue damage and fibrosis symptoms. This therapeutic effect was positively correlated with inhibition of TL1A/DR3 and ZNF281 related proteins. **Conclusion:** The GP ameliorates fibrotic lesions in rats with IBD and is associated with inhibition of the TL1A/DR3 signaling pathway and ZNF281. The GP is expected to be a clinical therapeutic agent for intestinal fibrosis in IBD.

Key words: *Gentianopsis paludosa*, TL1A/DR3, ZNF281, inflammatory bowel disease, intestinal fibrosis

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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

Inflammatory Bowel Disease (IBD) is a chronic intestinal disorder with an increasingly high incidence in Asia, including ulcerative colitis (UC) and Crohn's disease (CD)[1,2]. The pathogenesis of IBD is associated with intestinal mucosal barrier damage and immune imbalance caused by environmental, genetic and gut microbiota factors[3]. Intestinal fibrosis is a common and severe complication of IBD[4], characterized by excessive deposition of collagen-rich extracellular matrix in the intestinal tissue and excessive growth of the intestinal muscle layer, ultimately leading to luminal narrowing and even bowel obstruction, posing a threat to patients' lives[5]. Despite some progress in the treatment of IBD, the occurrence rate of IBD-associated intestinal fibrosis has not decreased. Approximately 75% of CD patients require surgical treatment due to intestinal fibrosis and stenosis, with high recurrence rates[6]. Fibrosis induced colon stenosis exists in 3-11.2% of UC patients, while UC patients without colon stenosis also exhibit varying degrees of fibrosis in their colon tissues[7,8]. There are currently no effective measures or treatments available due to the complex pathogenesis involved in this disease.

Gentianopsis paludosa (Hook. f.) Ma (GP) is an annual dry whole grass of Gentianaceae, which has the effect of clearing heat, removing dampness and detoxifying[9]. In Tibetan medicine, GP is often used to treat hepatitis, cholecystitis, diarrhea, gastroenteritis and so on. Lu *et al.*[10] confirmed that GP can significantly reduce the fibrosis symptoms of UC model rats induced by 2,4,6-Trinitrobenzenesulfonic acid (TNBS), which is related to the significantly down-regulation of mRNA expression of I type collagen, III type collagen and α-SMA and up-regulation of mRNA expression of E-cadherin. However, the therapeutic mechanism of GP on intestinal fibrosis is still not fully understood.

Tumor Necrosis Factor-Like Ligand 1A (TL1A) is a newly identified member of the tumor necrosis factor family, which was highly expressed in both serum and colon tissues of IBD and intestinal fibrosis[11,12]. The TL1A, which binds to death receptor 3, promotes intestinal fibrosis by regulating collagen and IL-31R[13]. The TL1A can be released by immune cells to promote Th1, Th2 and Th17 responses[14]. The TL1A promotes intestinal fibrosis in mice by regulating the secretion of interferon-γ and Interleukin-17 (IL-17) by T helper cells[15]. These results suggest that TL1A/DR3 may become a new immune target for inflammatory bowel disease-related intestinal fibrosis. However, whether the therapeutic effect of GP on IBD associated fibrosis is related to TL1A/DR3 has not been verified.

Zinc Finger Protein 281 (ZNF281) is overexpressed in various cancers, including cervical, liver, pancreatic and colorectal cancers[16-19]. Additionally, it has also been identified in intestinal inflammation and fibrosis. The ZNF281 acts as an inducible transcription factor for epithelial-mesenchymal transition (EMT) and is involved in the regulation of pluripotency, stem cells and cancer[20]. The EMT is also one of the important mechanisms for the formation and development of fibrosis[21]. This suggests that ZNF281 may be one of the crucial regulators of intestinal fibrosis formation and progression. However, whether GP can regulate EMT through ZNF281 and affect the progression of intestinal fibrosis has not been reported.

To verify the possible mechanism of GP on IBD-associated intestinal fibrosis, a series of experiments were conducted on the colonic fibrosis model induced by hydrochloric acid lincomycin and TNBS in rats. This study provides valuable insights into the treatment of intestinal fibrosis with GP.

MATERIALS AND METHODS

Study area: This study was conducted in the Life Science Research Center of Hebei North University from February, 2021 to December, 2022.

Drugs: The herbs were collected from Zhangjiakou, Hebei, China and identified as *Gentianopsis paludosa* (Hook. f.) Ma by Zhao Hengcheng of Hebei North University. As 250 g GP was soaked in 8 times 75% ethanol for 0.5 hr and extracted for 1.5 hrs. The residue was refluxed with 5 times of 75% ethanol for 1.5 hrs and the filtrate was collected twice. The ethanol was recovered by rotary evaporation instrument (Shanghai Yarong Biochemical Instrument Factory, Shanghai, China) and dried in evaporation dishes. The dry powder was dissolved in normal saline before use.

Colonic fibrosis rat models and grouping: Thirty-six SD male rats (SPF grade, weight 180-200 g) were purchased from Beijing Sbeifu Biotechnology Co. Ltd. All animal experiments were performed in accordance with the national guide for the care and use of laboratory animals. The experimental design was approved by the Institutional Ethics Committee of Hebei North University. The rats were randomly divided into normal control group, model group, GP high-(0.68 mg kg^{-1}), medium-(0.34 mg kg^{-1}) and low-(0.17 mg kg^{-1}) dose groups and sulfasalazine positive control group (SASP, 342 mg kg^{-1}), with 6 rats in each group. According to the reference report by Qi *et al.*[22] and Latella *et al.*[23], except for the normal group of rats, which were given an equivalent volume of physiological

saline (Shijiazhuang Fourth Pharmaceutical Group, Shijiazhuang, Hebei, China), all other groups of rats were orally administered with 85 mg kg^{-1} lincomycin hydrochloride (Chengdu Tongde Pharmaceutical Co. Ltd., Chengdu, Sichuan, China) once a day for five consecutive days. Except for the normal control group, all other groups of rats were induced with TNBS (Sigma, America) to establish a rat model of intestinal fibrosis. As 75% ethanol solution (Tianjin Kemiou Chemical Reagent Co. Ltd., Tianjin, China) of TNBS (125 mg kg^{-1}, 1 mL/rat) was injected into the colon of ether (Tianjin Kemiou Chemical Reagent Co. Ltd., Tianjin, China) anesthetized rats. Normal control group rats were injected with an equal amount of physiological saline. After injection, the rat's tail was lifted and held for 5 min. Drug intervention was administered for 7 days. Colonic tissue and serum samples were collected for subsequent experiments 2 hrs after the last dose.

General condition of rats: The weight, diet, activity, reaction, hair color, feces and other general conditions of rats in each group were observed during modeling and administration.

Pathological examination of colon tissue: The colonic tissues of rats were fixed in 4% paraformaldehyde fixing solution (Beijing Solarbio Technology Co. Ltd., Beijing, China) and the colonic tissues were embedded in conventional paraffin. After the sections were dewaxed, HE staining (Beijing Solarbio Technology Co. Ltd., Beijing, China) and Masson staining (Beijing Solarbio Technology Co. Ltd., Beijing, China) were performed to observe the pathological changes and degree of fibrosis of colon tissues.

Protein expressions of TGF-β1, α-SMA and ZNF281 in colon tissue were detected by immunohistochemistry: The colonic tissues of rats were fixed in 4% paraformaldehyde fixing solution and the colonic tissues were embedded in conventional paraffin. After the sections were dewaxed, the protein expression levels of ZNF281, α-SMA and TGF-β1 (Wuhan Boster Bioengineering Co., Ltd., Wuhan, Hubei, China) in colon tissues were detected by immunohistochemistry.

Enzyme-Linked Immunosorbent Assay (ELISA): Rat IL-1 ELISA kit (Shenzhen New Bioscience, Shenzhen, China) and rat IL-13 ELISA kit (Shenzhen New Bioscience, Shenzhen, China) were used to detect the protein levels of IL-1 and IL-13 in rat serum according to the manufacturer's instructions. Different concentrations of IL-1 and IL-13 standard solution were prepared to make the standard curve. The OD values were measured at 560 nm using an enzymatic marker (Bio-Rad Laboratories, Inc., America).

Western blot: Colon tissue samples were collected and processed into small pieces. Tissue pieces were incubated in High Performance RIPA Lysis Buffer (Beyotime, Shanghai, China) containing PMSF for protein extraction. The total protein concentration was determined using the BCA Protein Concentration Assay kit (Beyotime, Shanghai, China). The samples were separated using Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE, Biosharp, Guangzhou, Guangdong Province, China) according to the different molecular weights of the proteins. Equal amounts of proteins were loaded onto PVDF membranes (Beijing Solarbio Technology Co. Ltd., Beijing, China) and incubated with specific primary antibodies TL1A (1:5000, Abcam, England), DR3 (1:500, Proteintech, America), Caspase-8 (1:500, Proteintech, America), Claudin-2 (1:500, Proteintech, America) and GAPDH (1:5000, Proteintech, America). The PVDF membranes were then further incubated using the appropriate secondary antibody. Membranes were imaged and analyzed using a scanner (SinSage Technology Co. Ltd., Beijing, China)[24].

Statistical analysis: The SPSS 22.0 software was used for statistical analysis. Differences between groups were compared using independent samples t-test. Data were expressed as Mean±Standard Deviation (SD). The $p<0.05$ was considered statistically significant.

RESULTS

General condition of rats: During the experiment, the general behavior of rats in the normal control group was normal and the weight of rats decreased at 24 hrs of fasting and increased at other times. After 4-5 days of lincomycin hydrochloride, rats showed slow weight growth, lassitude, sloth, withered and colorless hair, adhesions of feces and loose stools. After colonic administration of 2,4,6-trinitrobenzenesulfonic acid, the rats in the model group showed depression, dry and yellow fur, reduced activity, unresponsiveness, dilute blood stools and weight loss ($p<0.05$ compared to rat body weight in normal group). Compared with the model group, the rats in the SASP, GP-H and GP-M groups showed significant improvement in mental status, activity and responsiveness, white and lustrous fur and increased body weight ($p<0.05$ compared to rat body weight in the model group), especially in the GP-H and GP-M groups. The rats in the GP-L group did not recover significantly ($p>0.05$ compared to rat body weight in the model group) (Fig. 1).

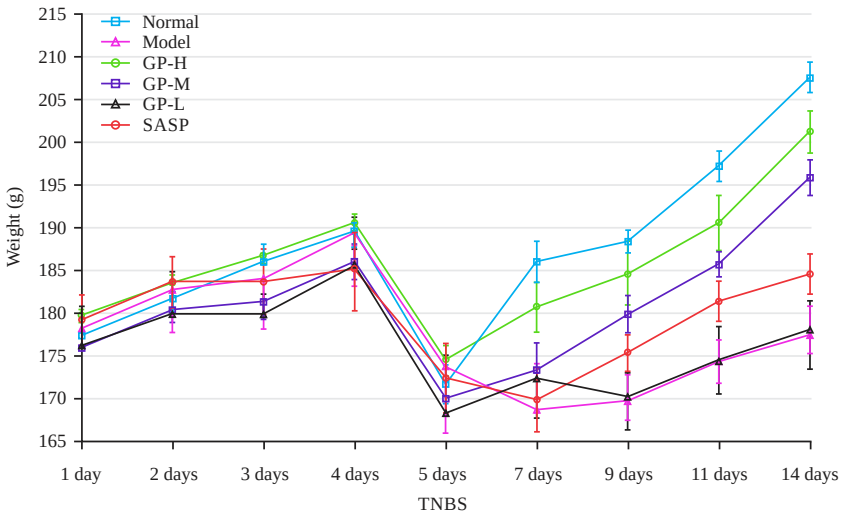

Fig. 1: Mean weight change of rats in each group

Normal: Normal control group, Model: Model group, GP-H: GP high-dose groups, GP-M: GP medium-dose group, GP-L: GP low-dose group and SASP: Sulfasalazine positive control group

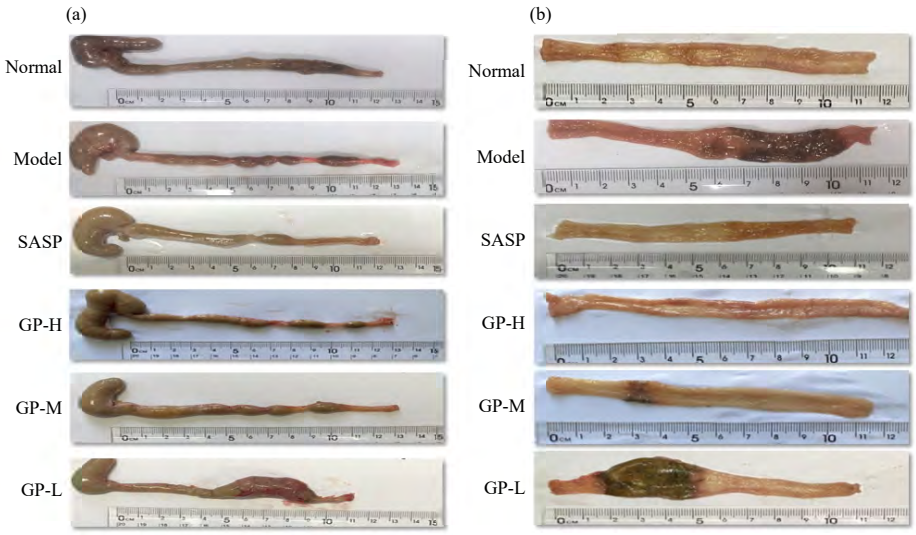

Fig. 2: Morphology of colonic and lining damage in each group of rats

Normal: Normal control group, Model: Model group, SASP: Sulfasalazine positive control group, GP-H: GP high-dose groups, GP-M: GP medium-dose group and GP-L: GP low-dose group

Colonic morphology: The length of colon was not statistically different between groups ($p>0.05$), but the length of intestinal wall injury showed significant differences. The length of intestinal wall injury in the model group was significantly larger than that in the normal control group and the injury showed black lesions ($p<0.05$). No significant lesions were observed macroscopically in the rats of GP-H and SASP groups ($p<0.05$). Minor lesions were observed in the GP-M group ($p<0.05$). However, rats in the GP-L group were similar to the model group with large intestinal wall damage and obvious lesions ($p>0.05$). The above results suggested that GP can dose-dependently reduce the pathological damage of colonic tissue (Fig. 2).

HE detects histopathological injury of the colon: In the normal control group, the intestinal wall structure of rats was intact. The mucosal layer, submucosal layer, muscle layer and outer membrane structures were clearly distinguished. The mucosal layer crypt and cup cells were of the same size and shape and arranged in parallel. The muscle layer tissue was closely arranged. Compared with the normal control group, the colon structure of rats in the model group was significantly

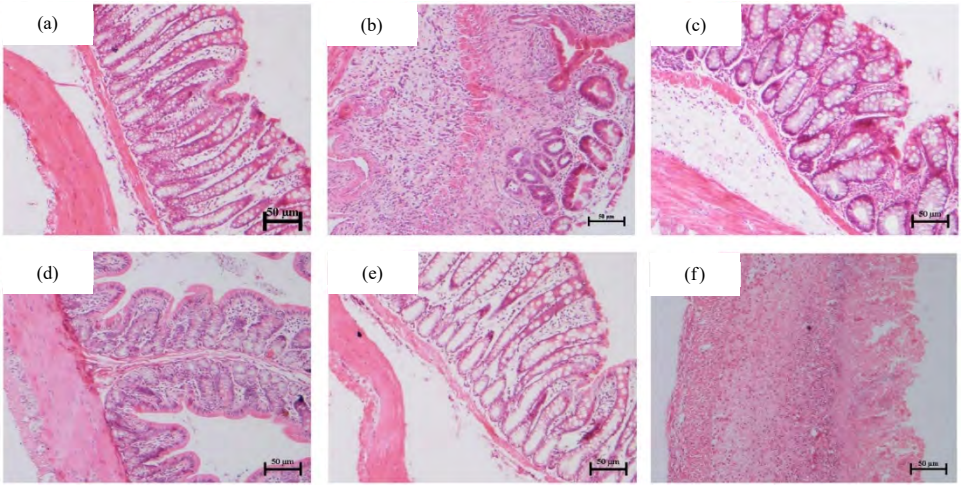

Fig. 3(a-f): HE staining of colonic tissue of rats in each group, (a) Normal, (b) Model, (c) SASP, (d) GP-H, (e) GP-M and (f) GP-L
Normal: Normal control group, Model: Model group, SASP: Sulfasalazine positive control group, GP-H: GP high-dose groups, GP-M: GP medium-dose group and GP-L: GP low-dose group

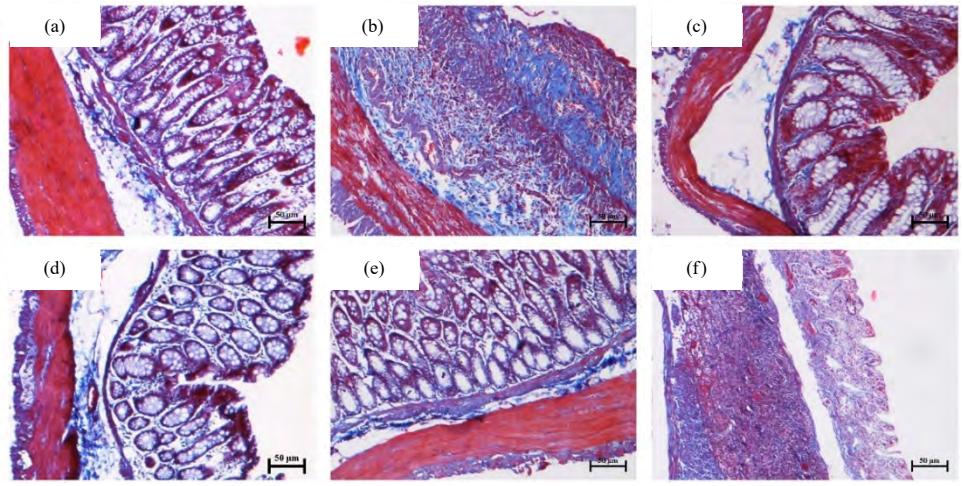

Fig. 4(a-f): Masson staining of colonic tissue of rats in each group, (a) Normal, (b) Model, (c) SASP, (d) GP-H, (e) GP-M and (f) GP-L
Normal: Normal control group, Model: Model group, SASP: Sulfasalazine positive control group, GP-H: GP high-dose groups, GP-M: GP medium-dose group and GP-L: GP low-dose group

changed, which showed that the intestinal wall was thickened, the crypt structure and cup-shaped cell structure were disordered and changed from columnar to elliptical and the inflammatory infiltration was obvious and the boundary between mucosa and submucosa was not clear. The intestinal wall structure of the rats in GP-H, GP-M and SASP groups was similar to that of the normal groups and the histomorphology was relatively normal, especially the mucosal layer crypt and cup cells in GP-H and GP-M groups were uniform in size and shape and arranged in parallel and the muscle layer tissue was closely arranged. In contrast, the colon structure of GP-L group was disordered and inflammatory infiltration was obvious. The above results suggested that GP could improve the histopathological damage of colon and showed a dose-dependent effect (Fig. 3).

Masson detects colonic tissue fibrosis: In the normal control group, there was a small amount of blue-stained fibrous tissue in the submucosa layer. Compared with the normal control group, the blue-stained fibrous tissue in the mucosal layer and submucosa of the model group was significantly increased. This indicated that the degree of fibrosis was severe and the model was successful. The blue-stained collagen fibers in the GP-H group, GP-M group and SASP group were significantly reduced and the degree of fibrosis was not reduced in the GP-L group (Fig. 4).

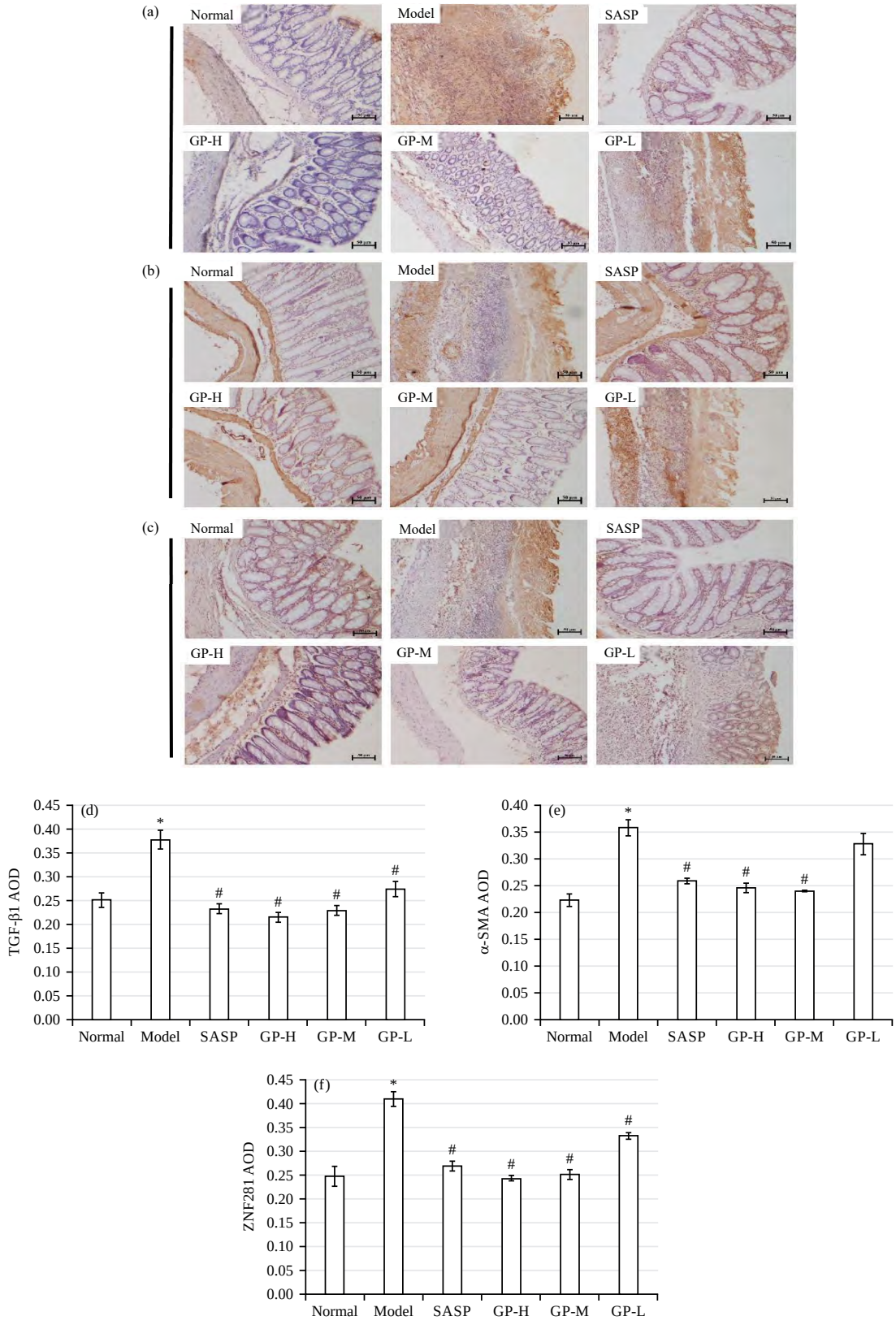

Fig. 5(a-f): GP inhibits the overexpression of ZNF281, α-SMA and ZNF281 proteins in intestinal fibrosis rats

Compared with the normal group, *p<0.05 indicates a statistically significant difference. Compared with the model group and #p<0.05 indicates a statistically significant difference

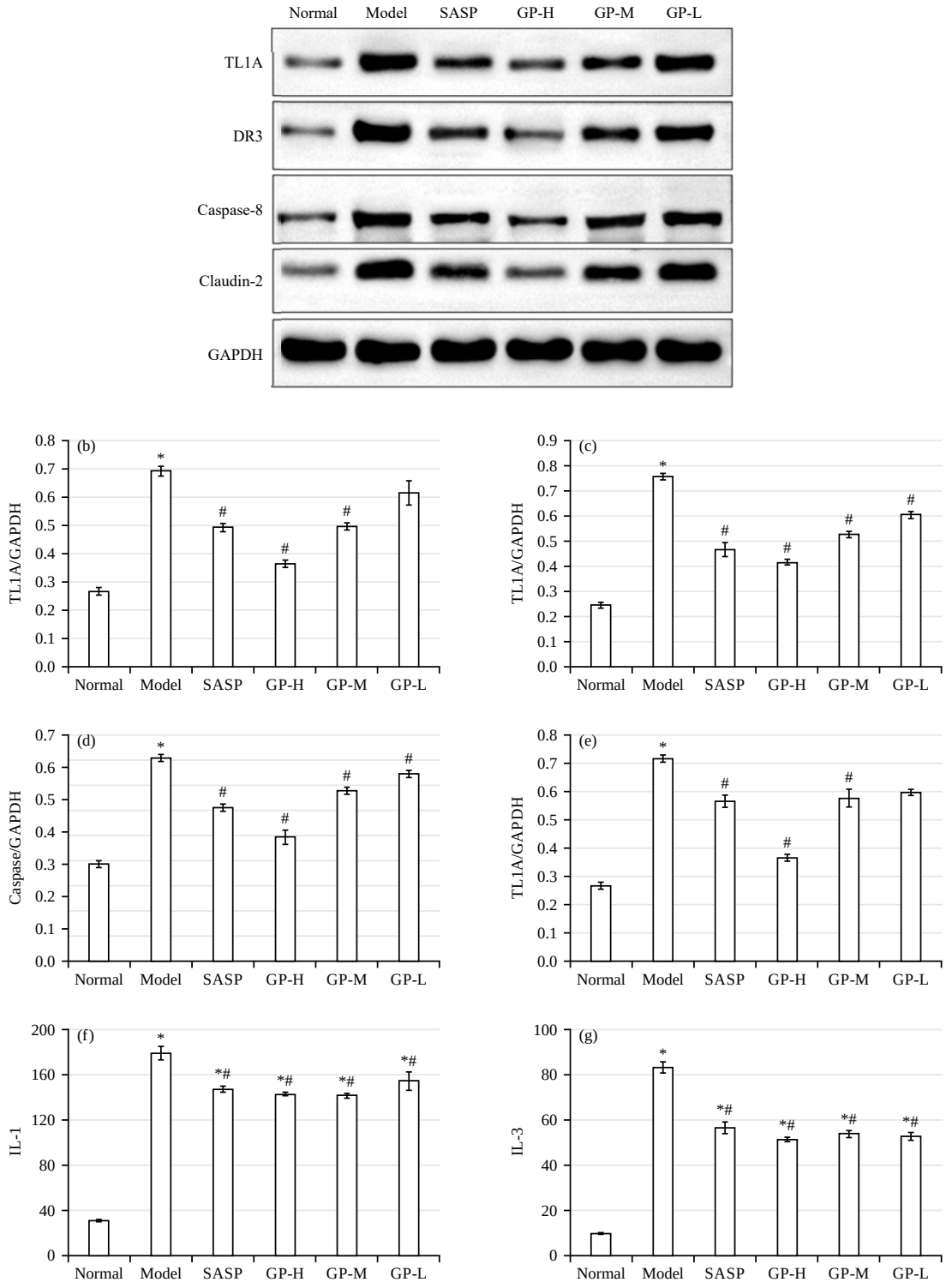

Fig. 6(a-g): GP inhibits the overexpression of TL1A/DR3 signaling pathway in intestinal fibrosis rats, (a-e)Western blotting to detect the expression of TL1A, DR3, Caspase-8 and Claudin-2 protein in colon tissue and (f-g) ELISA assay to detect IL-1 and IL-13 proteins in colonic tissues

Compared with the normal group, *p<0.05 indicates a statistically significant difference, Compared with the model group, #p<0.05 indicates a statistically significant difference

GP inhibited the expression of TGF-β1, α-SMA and ZNF281 in intestinal fibrosis rats: The results of immunohistochemistry showed that the expression of TGF-β1, α-SMA and ZNF281 were significantly higher in the model group compared with the normal control group ($p<0.05$). Compared with the model group, TGF-β1, α-SMA and ZNF281 were significantly decreased in the SASP, GP-H and GP-M groups ($p<0.05$). The GP-L group only significantly down-regulated TGF-β1 and ZNF281 ($p<0.05$) (Fig. 5a-f).

GP inhibits the overexpression of TL1A/DR3 signaling pathway in intestinal fibrosis rats: Western blotting was used to detect TL1A/DR3 signaling pathway proteins. The expression levels of TL1A, DR3 and Caspase-8 were much higher in the model group than in the normal group ($p<0.05$). The SASP significantly down-regulated three proteins ($p<0.05$) and GP dose-dependently down-regulated three proteins ($p<0.05$), especially in the GP-H group (Fig. 6a-d). The IL-1 protein was detected by ELISA. The linear regression equation of the standard curve was calculated with the concentration of the standard as the horizontal coordinate and the OD value as the vertical coordinate. The regression equation for IL-1 was $Y = 0.149+0.014X$ ($R^2 = 0.996$). The result showed that the expression of IL-1 protein in the model group was significantly higher than that in the normal group ($p<0.05$). After treatment with SASP or GP, IL-1 expression were significantly reduced ($p<0.05$) (Fig. 6e-f). Meanwhile, IL-13 protein expression was also detected by ELISA. The regression equation for IL-13 was $Y = 0.170+0.046X$ ($R^2 = 0.996$). The result showed that the expression of IL-13 protein in the model group was significantly higher than that in the normal group ($p<0.05$). After treatment with SASP or GP, IL-13 expression were significantly reduced ($p<0.05$) (Fig. 6g). Western blotting was used to detect claudin-2 protein. The result showed that the expression of claudin-2 protein in the model group was significantly higher than that in the normal group ($p<0.05$). After treatment with SASP or GP, claudin-2 expression were significantly reduced ($p<0.05$) (Fig. 6a and f).

DISCUSSION

The TNBS is a common induction agent for animal models of IBD and intestinal fibrosis. In recent years, studies on TNBS-induced acute intestinal fibrosis animal models have been increasing[25]. In the pre-experiment, acute intestinal fibrosis rat models can be rapidly established through enema using a 1:1 volume ratio of 5% TNBS and 75% ethanol solution. This modeling method is straightforward, effective and has a low mortality rate. Lincomycin hydrochloride is a commonly used inducer of intestinal flora dysbiosis, which has the characteristics of short modeling time and long maintenance time[26]. Literature research has confirmed that the flora dysbiosis is correlated with the occurrence and development of liver fibrosis and pulmonary fibrosis[27,28]. Similar results were also obtained in our preliminary experiments, which showed that the flora dysbiosis induced by lincomycin hydrochloride could aggravate the TNBS-induced intestinal fibrosis lesions in rats. Therefore, this study utilized hydrochloric acid lincomycin and TNBS to induce intestinal fibrosis rat models.

Intestinal luminal stenosis and intestinal obstruction caused by intestinal fibrosis are common complications of IBD. The EMT is an important mechanism in the development of intestinal fibrosis. The ZNF281 is a zinc-finger transcriptional regulator that is believed to function as an EMT-induced transcription factor. It activates the expression of TGF-β1 in colonic fibrotic tissues[29] which is an important pro-fibrotic factor promoting excessive extracellular matrix deposition such as collagen and leading to fibrosis development[30]. The α-SMA is one of the downstream signaling molecules of TGF-β1/Smad3. Activation of TGF-β1 increases the number of myofibroblasts, up-regulates α-SMA expression and increases extracellular matrix synthesis[31]. This study showed that ZNF281, TGF-β1 and α-SMA protein expression were elevated in colonic fibrosis rats, while the GP group significantly reduced the expression of all three proteins. It indicated that GP inhibited the occurrence of EMT by regulating the expressions of ZNF281, TGF-β1 and α-SMA proteins.

In recent years, an increasing number of studies have found that abnormal expression of TL1A and its membrane-bound death receptor 3 (DR3) was associated with the development and progression of IBD. It has been demonstrated that TL1A/DR3 can regulate the body's immune response to intervene in the IBD disease process. Overexpressed TL1A/DR3 can activate Caspase-8, induce IL-1 cytokine secretion and enhance the intrinsic immune response[32]. The findings of this experiment were similar to those reported in the literature. Elevated TL1A/DR3 protein expression in intestinal fibrosis model rats activated Caspase-8 and induced IL-1 cytokine secretion. The GP group significantly inhibited TL1A/DR3 overactivation and down-regulated Caspase-8 and IL-1 proteins. In addition, it was found that TL1A/DR3 activation promoted IL-13 secretion and up-regulated Claudin-2 protein expression. The GP treatment significantly down-regulated the expression of IL-13 and Claudin-2 proteins.

The GP ethanol extract demonstrates potential for treating IBD-related intestinal fibrosis. However, GP ethanol extract contains numerous compounds, which have varying therapeutic effects on IBD-related intestinal fibrosis. Moreover, this study has not yet been initiated. It will be the main focus of attention to study the purification, identification and pharmacological effects of the anti-fibrotic active components in GP.

CONCLUSION

This study investigated the protective effects of GP ethanol extract on IBD-related intestinal fibrosis and its potential mechanisms. The research results indicated that GP inhibited EMT by down-regulating the expression of ZNF281, TGF-β1 and α-SMA, demonstrating a significant anti-intestinal fibrosis effect. The GP also regulated the innate immune response by down-regulating the expression of TL1A/DR3, IL-1 and Caspase-8. The GP enhanced intestinal barrier function and protected the integrity of intestinal epithelial mucosa by inhibiting the expression of IL-13 and Claudin-2. Overall, as a traditional Chinese medicine, GP can alleviate intestinal IBD-related fibrosis and is expected to become a potential candidate for treating IBD and its fibrosis.

SIGNIFICANCE STATEMENT

Intestinal fibrosis is a common late-stage complication of IBD. Unclear pathogenesis, as well as unsatisfactory efficacy of current drugs, are important challenges in the treatment of intestinal fibrosis. Traditional Chinese medicine has advantages such as wide sources, multiple treatment targets, minimal side effects and significant clinical efficacy. Therefore, this study aims to evaluate the potential of GP in the treatment of IBD-related fibrosis. The results showed that GP can significantly alleviate the symptoms of intestinal fibrosis and its therapeutic mechanism is related to the inhibition of ZNF281 and TL1A/DR3 overexpression. This study not only reveals the potential pathogenesis of intestinal fibrosis but also confirms the possibility of GP as a potential therapeutic agent for IBD and its intestinal fibrosis.

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