Pien Tze Huang ameliorates DSS-induced colonic inflammation in a mouse colitis model through inhibition of the IL-6/STAT3 pathway

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Abstract. Interleukin-6 (IL-6)/signal transducer and activator of transcription 3 (STAT3) pathway plays essential roles in the development of inflammatory diseases including ulcerative colitis (UC). Therefore, suppression of IL-6/STAT3 signaling provides a promising therapeutic strategy in UC. Pien Tze Huang (PZH), a well-known traditional Chinese formula, has been used in China and Southeast Asia for centuries as a folk remedy for various inflammatory diseases. However, the molecular mechanisms of its anti-inflammatory effects remain to be elucidated. In the present study, we generated a mouse colitis model by using dextran sulfate sodium (DSS) and evaluated the therapeutic efficacy of PZH against UC by observing the clinical manifestations. We found that PZH obviously alleviated DSS-induced colitis symptoms, including body weight loss, rectal bleeding and stool consistency. In addition, administration of PZH profoundly prevented DSS-induced colon shortening, and ameliorated colonic histopathological changes such as mucosal ulceration, infiltration of inflammatory cells, crypt distortion and hyperplastic epithelium. Moreover, PZH markedly inhibited the serum level of the inflammatory biomarker serum amylase A (SAA) in UC mice. Furthermore, PZH treatment significantly inhibited DSS-induced expression of IL-6 in colon tissues. Finally, the increased phosphorylation level of STAT3, induced either by DSS in experimental mice or by IL-6 in the differentiated human colorectal carcinoma cells, was significantly suppressed by PZH. These results suggest that the inhibition of IL-6/STAT3 signaling is a potential mechanism by which PZH is used in the treatment of UC.

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Abbreviations: TCM, Traditional Chinese Medicine; PZH, Pien Tze Huang; IBD, inflammatory bowel disease; UC, ulcerative colitis; IL-6, interleukin-6; STAT3, signal transducer and activator of transcription 3

Key words: Pien Tze Huang, Traditional Chinese Medicine, ulcerative colitis, IL-6/STAT3 pathway

Introduction

Inflammatory bowel disease (IBD), typically referring to ulcerative colitis (UC) and Crohn's disease (CD), is a group of chronically inflammatory disorders of gastrointestinal tract without specific etiology (1-4). Although these inflammatory conditions have been treated with 5-aminosalicylic acid derivatives, corticosteroids and immunosuppressants (5), most of these currently used treatments are inadequate due to severe side effects such as systemic immunosuppression (6-8). Thus, there is a need to develop novel and safer therapeutic agents. Recently, natural product-based treatments, particularly Traditional Chinese Medicine (TCM), have received great interest as they have relatively few side effects and have been used as alternative remedies for a variety of diseases including IBD (9-12).

The pathogenic mechanisms underlying IBD development are complex and heterogeneous, with the involvement of multiple cellular transduction pathways including interleukin-6 (IL-6)/signal transducer and activator of transcription 3 (STAT3) signaling. IL-6 is an important pro-inflammatory cytokine that has been shown to play a potential role in the pathogenesis of IBD. Elevated IL-6 levels have been detected in serum, inflammed colonic mucosal tissues and cultivated lamina propria mononuclear cells in IBD patients (13-16). Moreover, IL-6 level is correlated with the inflammatory

severity and therefore is considered as a clinical IBD-relevant parameter (17-23). Previous findings indicated that IL-6 signal transduction in IBD is not mediated by the membrane-bound receptor for IL-6 (IL-6R), but the soluble form of the IL-6R (sIL-6R), a process known as IL-6 trans-signaling (24-26). The IL-6/sIL-6R complex in turn binds to a common signal transducing receptor gp130, promoting dimerization of gp130 and then resulting in activation of the associated Janus kinases (JAKs). Activated JAKs phosphorylate gp130, leading to the recruitment and activation of STAT3 (27). STAT3 is an important transcription factor that plays an essential role in cell survival and proliferation (28,29). After activation via phosphorylation at tyrosine 705 by JAKs, STAT3 proteins in the cytoplasm dimerize and translocate to the nucleus where they regulate the expression of various critical genes, eventually leading to the development of IBD. Constitutive activation of STAT3 has been found in intestinal T cells from IBD patients (21,30) and commonly suggests severe disease activity (31). Therefore, suppressing the IL-6/STAT3 pathway provides a promising therapeutic strategy in IBD.

Pien Tze Huang (PZH) is a well-known TCM formula that was first prescribed by a royal physician more than 450 years ago in the Ming Dynasty. The main ingredients of PZH include *Moschus*, *Calculus bovis*, *Snake gall* and *Radix notoginseng*. These products together confer PZH properties of heat-clearing and detoxification (32). Since in the TCM system the accumulation of toxic dampness and heat is one of the major causative factors for inflammation and inflammation-related cancer, PZH has been used in China and Southeast Asia for centuries to clinically treat a variety of inflammatory diseases and cancers (33-50). However, the precise mechanism of anti-inflammatory activity of PZH remains largely unclear. Using dextran sulfate sodium (DSS)-induced mouse colitis model, in the present study we evaluated the therapeutic efficacy of PZH against UC and elucidated the possible molecular mechanisms.

Materials and methods

Materials and reagents. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, Trypsin-EDTA were obtained from Life Technologies Corporation (Grand Island, NY, USA). A 24-well cell culture insert was purchased from BD Biosciences (Franklin Lakes, NJ, USA). DSS (molecular weight, 40,000 kDa) was purchased from MP Biomedicals (Solon, OH, USA). Antibodies for western blot analysis were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). The immunoblot detection system (ECL Plus) and bicinchoninic acid (BCA) protein assay reagent were obtained from Pierce Biotechnology, Inc. (Rockford, IL, USA). Mouse IL-6 or serum amyloid A (SAA) detection ELISA kit was purchased from BioLegend, Inc. (San Diego, CA, USA) or Immunology Consultants Laboratory, Inc. (Portland, OR, USA), respectively. All the other chemicals, unless otherwise stated, were obtained from Sigma Chemicals (St. Louis, MO, USA).

Preparation of PZH. PZH was obtained from and authenticated by the sole manufacturer Zhangzhou Pien Tze Huang Pharmaceutical Co., Ltd. (Zhangzhou, China; Chinese FDA approval no. Z35020242). The stock solution of PZH was prepared by dissolving PZH powder in saline to a concentration of 20 mg/ml, followed by sonication for 30 min. The working concentrations for cell-based experiments were made by diluting the stock solution in the cell culture medium.

Table I. Score of the disease activity index.

Score	Weight loss	Rectal bleeding	Stool consistency
0	0%	Normal	Normal
1	1-5%	-	-
2	6-10%	Positive hemoccult	Loose
3	11-20%	-	-
4	>21%	Gross bleeding	Diarrhea

Disease activity index was calculated as the sum of scores of each category.

Animals. Seven-week-old male BALB/c mice (weight, 20-22 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and were acclimatized for 1 week before the experiment. Animals were housed individually in a room maintained at 22˚C under a 12-h day/night cycle. Food and water were given *ad libitum* throughout the experiments. The animal experiments conducted in this study were approved by the Animal Care and Use Committee of Fujian University of Traditional Chinese Medicine.

In vivo mouse colitis study. Mouse colitis model was established as we described previously (51). Briefly, acute colitis was induced by administration with 3% DSS in the drinking water for 8 days. On the first day of model construction, the animals were randomly divided into three groups (n=5): The normal control group in which mice received neither DSS stimulation nor PZH treatment; the DSS-induced model or PZH-treated group in which mice received DSS stimulation and were given intra-gastric administration with saline or 234 mg/kg, respectively, daily for 12 days. The progression of colitis was monitored in a blinded manner, including measurement of body weight, evaluation of stool consistency, and presence of rectal bleeding tested by guaiac paper. Disease activity index (DAI) was represented as the sum of scores for weight loss, stool consistency and rectal bleeding (Table I).

Sample preparation. At the end of the experiment, the animals were anaesthetized with Avertin. Blood was collected via right heart ventricle puncture in lightly heparinized syringes and kept on ice. Sera were separated by 5 min centrifugation at 5,000 x g and stored at -80˚C prior to the analysis. The colons were excised and length was measured. One portion of each distal colon was cut and fixed in 10% formalin for histological examination. The remainder of each distal colon was snap-frozen in liquid nitrogen and stored at -80˚C for further analysis of the tissue IL-6 level and STAT3 phosphorylation. Proteins in frozen colons were extracted using T-PER Tissue Protein Extraction Reagent kit according to the manufacturer's

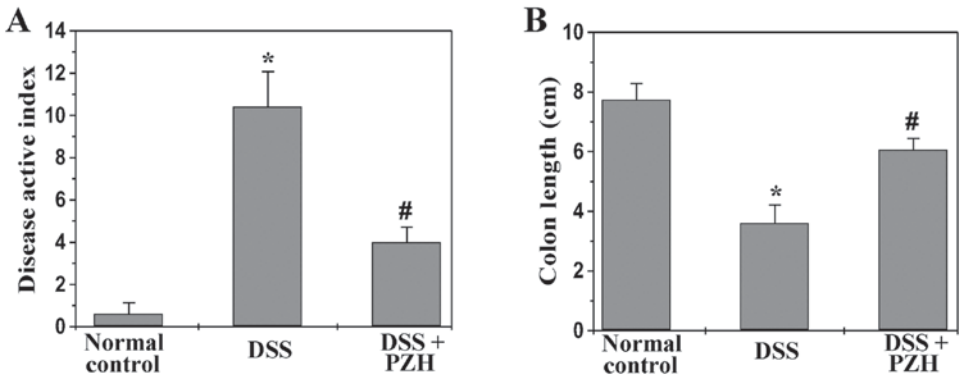

Figure 1. Effect of PZH on the development of DSS-induced ulcerative colitis in mice. Colitis was induced by DSS. On the first day of model construction, mice received an intragastric administration of saline or PZH, daily for 12 days. (A) The progression of DSS-induced colitis was monitored daily in a blinded manner, including the observation of body weight loss, stool consistency and rectal bleeding. The disease activity index (DAI) was calculated as described in Table I. (B) At the end of the experiments, the animals were anesthetized. The colons were excised and the length from the cecum to the anus was measured. Data shown are the means ± SD from 5 individual mice in each group. *P<0.05, vs. normal controls; #P<0.05, vs. mice stimulated with DSS but not treated with PZH. PZH, Pien Tze Huang; DSS, dextran sulfate sodium.

protocol. Protein concentrations were determined by BCA protein assay kit.

Histopathological evaluation. The formalin fixed section of distal colons were processed and stained with hematoxylin and eosin (H&E) and evaluated under light microscopy in a blinded manner by an experienced pathologist.

Measurement of serum SAA level or expression of IL-6 in colon by ELISA. The level of SAA in the sera and the expression of IL-6 in colonic tissues were measured using ELISA kits according to the manufacturer's protocol. Absorbance was read at 450 nm using a microplate reader (model ELX800; BioTek Instruments, Inc., Winooski, VT, USA). All the samples were analyzed in triplicate.

Western blot analysis. Equivalent amounts of protein were resolved in 12% Novex Bis-Tris gel electrophoresis (NuPAGE; Life Technologies Corporation). Proteins were then transferred into nitrocellulose membranes in an iBlot Western Blotting system (Invitrogen, Grand Island, NY, USA). The membranes were blocked for 1 h at room temperature with super SuperBlock® Blocking Buffers (Pierce Biotechnology, Inc.). They were then incubated at 4˚C overnight with primary antibodies against p-STAT3 (rabbit, polyclonal, 1:1,000, CST, cat. no. 9131), regular STAT3 (rabbit, polyclonal, 1:1,000, CST, cat. no. 9132)and β-actin (rabbit, polyclonal, 1:2,000, CST, cat. no. 4967) in blocking buffer. After the membranes were washed, they were further incubated with appropriate horseradish anti-rabbit IgG, HRP-linked antibody (1:5,000, CST, cat. no. 7074) for 1 h at room temperature. The membranes were analyzed using enhanced chemiluminescence Plus reagents and scanned with the Storm Scanner (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA).

Cell culture. Human colon cancer Caco-2 cells were purchased from the American Type Culture Collection (Rockville, MD, USA). Cells (passages 20-40) were grown in DMEM supplemented with 20% (v/v) FBS, 1,000 mg/l of glucose, 50 U/ml penicillin and 50 μg/ml streptomycin in a 37˚C humidified incubator with 5% CO_2. Caco-2 cells usually reached confluence 3 days after seeding, and differentiated into enterocyte-like cells 18-20 days post-confluence. To test the effect of PZH on IL-6-induced phosphoralytion of STAT3, we seeded these fully differentiated cells in 24-well biocameral inserts (area, 0.33 μm^2; pore size, 0.4 μm insert). On the day of the experiment, the medium was removed and supplemented with 0.5% FBS medium. Differentiated Caco-2 cells (20 days post-confluence) in 24-well biocameral inserts were pre-incubated with 0.5 mg/ml of PZH for 2 h followed by stimulation with 10 ng/ml of IL-6 for 30 min.

Measurement of cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Differentiated Caco-2 cells (20 days post-confluence) in 96-well plates were treated with various concentrations of PZH for 24 h. The cytotoxic effect of PZH on Caco-2 cells was examined by the MTT colorimetric assay. Briefly, MTT (100 μl) (0.5 mg/ml in PBS) was added to each well, and the samples were incubated for an additional 4 h at 37˚C. The purple-blue MTT formazan precipitate was dissolved in 100 μl DMSO. The absorbance was measured at 570 nm using a spectrophotometer reader (Model ELX800; BioTek Instruments, Inc., Winooski, VT, USA).

Statistical analysis. Data were presented as mean ± SD for the indicated number of independently performed experiments. The data were analyzed using the SPSS package for Windows (version 17.0; SPSS, Inc., Chicago, IL, USA). Statistical analysis was carried out using Student's t-test and one-way ANOVA, followed by LSD's test or Dunnett's test. Differences with P<0.05 were considered to be statistically significant.

Results

PZH potentially inhibits the development of DSS-induced UC in mice. To determine whether PZH could inhibit the development of UC, we evaluated the clinical manifestations in experimental mice. The DAI was calculated based on the observation of body weight, stool consistency and rectal bleeding.

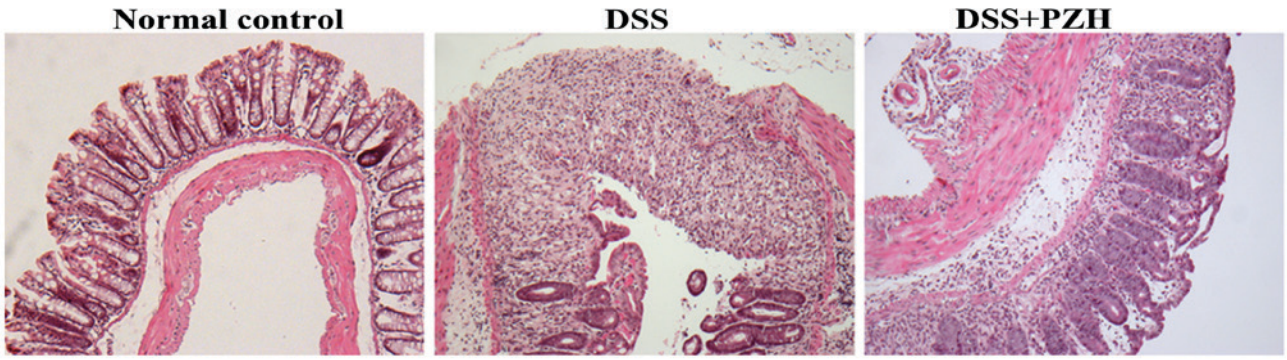

Figure 2. Effect of PZH on colon histological damage in mice with DSS-induced ulcerative colitis. Colon tissues were processed with H&E staining. Histopathological changes were observed under the microscope. Images were captured at a magnification of x100. PZH, Pien Tze Huang; DSS, dextran sulfate sodium; H&E, hematoxylin and eosin.

As shown in Fig. 1, upon DSS stimulation mice exhibited apparent manifestations of colitis which was characterized by body weight loss, diarrhea and rectal bleeding. Administration of PZH significantly ameliorated DSS-induced colitis symptoms. The DAI score of normal control, DSS-stimulated model or PZH-treated group was 0.6±0.54, 10.4±1.67 or 4.0±0.71, respectively (P<0.05; Fig. 1A). Moreover, colons were harvested from mice in each group after sacrifice; and length from cecum to anus was measured. We found that PZH treatment profoundly prevented DSS-induced colon shortening. The average colonic length in normal control, model or PZH-treated group was 7.74±0.34, 3.6±0.41 or 6.06±0.38 cm, respectively (P<0.05; Fig. 1B). Taken together, PZH is potent in suppressing the development of UC *in vivo*.

PZH alleviates colon histological damages in mice with DSS-induced UC. We next evaluated the histopathological changes of colon tissues through H&E staining. As shown in Fig. 2, the normal control mice displayed normal colonic histology with an intact epithelium, well-defined gland lengths and no leukocyte infiltration in the mucosa, whereas DSS induced severe colonic histological damages such as mucosal ulceration, inflammatory cell infiltration, crypt distortion and hyperplastic epithelium. However, PZH treatment significantly alleviated DSS-induced histopathological changes in colonic mucosa.

PZH reduces SAA level in mice with DSS-induced UC. It has been shown that the level of the inflammatory marker SAA is increased in UC patients. We therefore performed ELISA assay to examine SAA level in experimental mice. As shown in Fig. 3, PZH treatment significantly inhibited DSS-induced increase of serum level of SAA in UC mice. The SAA level in mice of normal control, model or PZH-treated group was 304.9±62.8, 1,667.9±202.9 or 558.2±92.5 μg/ml, respectively (P<0.05).

PZH suppresses IL-6/STAT3 signaling pathway both in mice with DSS-induced UC and in inflammatory intestinal epithelial cells. To investigate the mechanism of the anti-inflammatory activity of PZH, we evaluated the activation of IL-6/STAT3 pathway in colon tissues of experimental mice. The protein expression of IL-6 in colon tissues was determined by ELISA

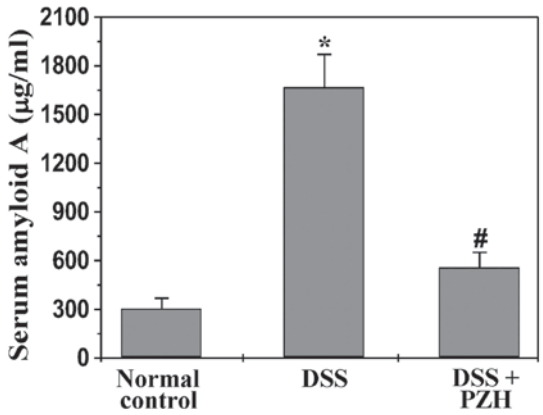

Figure 3. Effect of PZH on the level of SAA in mice with DSS-induced ulcerative colitis. The levels of SAA in sera were determined by ELISA. Data shown were the means ± SD from 5 individual mice in each group. *P<0.05, vs. normal controls; #P<0.05, vs. mice stimulated with DSS but not treated with PZH. PZH, Pien Tze Huang; SAA, serum amyloid A; DSS, dextran sulfate sodium.

assay and STAT3 activation was examined by western blot analysis using antibody that recognizes STAT3 phosphorylation at Tyr705. As shown in Fig. 4, the expression of IL-6 and the phosphorylation level of STAT3 in DSS-induced UC mice was significantly increased, as compared to that in normal control mice; which, however, was significantly inhibited by PZH treatment.

By stimulating the differentiated human colorectal carcinoma Caco-2 cells with IL-6, we generated an inflammatory cell model of human intestinal epithelium to examine the *in vitro* effect of PZH on IL-6/STAT3 pathway. As shown in Fig. 5, IL-6 stimulation resulted in a significant increase of pSTAT3 level in Caco-2 cells. However, PZH treatment profoundly inhibited IL6-induced STAT3 phosphorylation. The levels of non-phosphorylated STAT3 remained unchanged after the treatment with IL-6 and/or PZH.

PZH did not display cytotoxicity in intestinal epithelial cells. To exclude the possibility that the *in vitro* suppressive activity of PZH on STAT3 pathway was due to cytoxicity, we determined its effect on the viability of differentiated Caco-2 cells

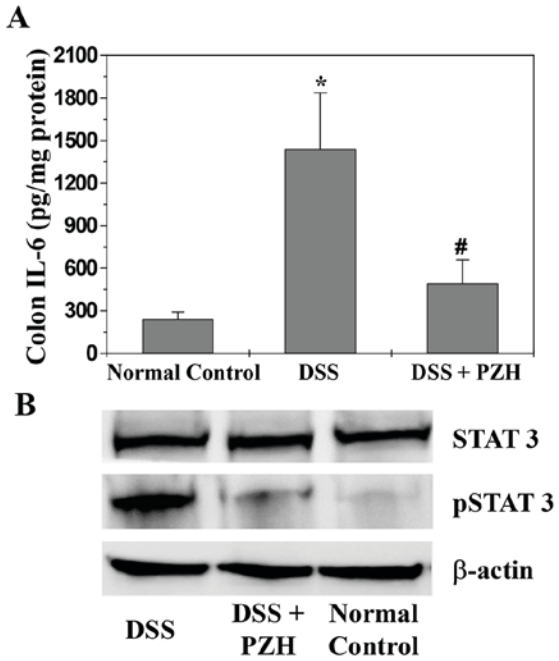

Figure 4. Effect of PZH on the expression of IL-6 and STAT3 phosphoralytion in mice with DSS-induced ulcerative colitis. (A) The levels of IL-6 in colon tissues were determined by ELISA. Data shown are the means ± SD from 5 individual mice in each group. *P<0.05, vs. normal controls; #P<0.05, vs. mice stimulated with DSS but not treated with PZH. (B) STAT3 phosphorylation in colon tissues was determined by western blot analysis. β-actin was used as the internal control. Images were representatives of colon tissues randomly selected from 6 individual mice in each group. PZH, Pien Tze Huang; IL-6, interleukin-6; STAT3, signal transducer and activator of transcription 3; DSS, dextran sulfate sodium.

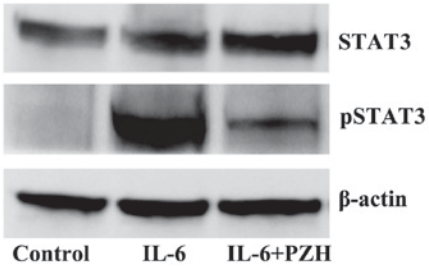

Figure 5. Effect of PZH on IL-6-mediated STAT3 phosphorylation in Caco-2 cells. Differentiated Caco-2 cells (20 days post-confluence) were pre-treated with PZH for 2 h followed by stimulation with 10 ng/ml of IL-6 for 30 min. STAT3 phosphorylation was determined by western blot analysis using an antibody that recognizes phosphorylated STAT3 at Tyr^{705}. β-actin was used as the internal control. Images were representative of three independent experiments. PZH, Pien Tze Huang; IL-6, interleukin-6; STAT3, signal transducer and activator of transcription 3.

using MTT assay. As shown in Fig. 6, Caco-2 cell viability was not affected by treatment with PZH, suggesting that the inhibitory effect of PZH on IL-6-induced STAT3 activation in intestinal epithelial cells did not result from its cytotoxic action.

Discussion

Despite recent advances in the drug treatment of IBD, many currently used pharmacotherapies contain intrinsic side effects

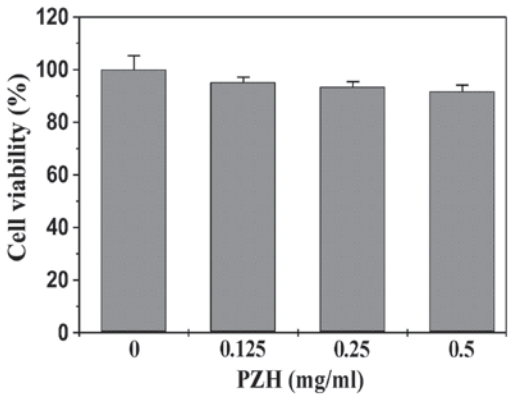

Figure 6. Effect of PZH on Caco-2 cell viability. Differentiated Caco-2 cells (20 days post-confluence) were treated with various concentrations of PZH for 24 h. Cell viability was determined by the MTT assay. The data were normalized to the viability of control cells. Data shown were the means ± SD from at least three independent experiments. PZH, Pien Tze Huang; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

such as systemic immunosuppression, which limits their long-term use (5-8). Natural products, including TCM, have received great interest since they have long been used to clinically treat inflammatory diseases and are relatively toxicity-free (9-12). PZH is a well-known TCM formula which has been used in China and Southeast Asia for centuries to clinically treat a variety of inflammatory diseases (33-50). However, the precise mechanism of anti-inflammatory activity of PZH remains largely unknown.

Using a DSS-induced mouse colitis model, in the present study, we evaluated the therapeutic efficacy of PZH against UC. We found that PZH significantly ameliorated DSS-induced colitis symptoms, including body weight, stool consistency and rectal bleeding. In addition, the administration of PZH profoundly prevented DSS-induced colon shortening, and alleviated colonic histopathological changes such as mucosal ulceration, infiltration of inflammatory cells, crypt distortion and hyperplastic epithelium. Moreover, PZH markedly inhibited the DSS-induced increase in serum levels of SAA, one of the inflammatory biomarkers which is commonly elevated in patients with UC. Thus, PZH is potent in suppressing the development of UC.

The IL-6/STAT3 pathway is an important signaling pathway that mediates inflammatory response. As a critical pro-inflammatory cytokine, IL-6 plays essential roles in the development of inflammatory diseases including IBD. Elevated IL-6 levels have been detected in the serum, inflammed colonic mucosal tissues and cultivated lamina propria mononuclear cells in IBD patients and in murine model with acute inflammation (13-16). In addition, serum IL-6 level is positively correlated with severity of intestinal histopathology (17-23). Generally, IL-6 exerts its bioactivities via binding to the sIL-6R, leading to the activation of JAKs and the downstream effectors such as STAT3 (24-27). Activated STAT3 proteins in the cytoplasm form homodimers and translocate to the nucleus to regulate the expression of various critical genes mediating antiapoptotic activities (28,29). Constitutive activation of STAT3 has been commonly found in lamina propria mononuclear cells from IBD patients, resulting in the resistance against apoptosis in

these cells and eventually the progression of IBD (21,30,31). Thus, the IL-6/STAT3 pathway has become a major target in the treatment of IBD. Using ELISA assay we found that PZH treatment significantly inhibited the DSS-induced expression of IL-6 in UC mice. Moreover, the increased phosphorylation of STAT3, induced by DSS in experimental mice or by IL-6 in the differentiated human colorectal carcinoma cells, was profoundly suppressed by PZH.

In conclusion, in the present study we report, to the best of our knowledge, for the first time that PZH attenuates intestinal inflammation in murine colitis model probably through inhibition of the IL-6/STAT3 pathway, demonstrating its potential clinical value in the treatment of IBD.

Acknowledgements

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Funding

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Availability of data and materials

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

Authors' contributions

JP and YC conceived and designed the experiments. LL, AS, JC and XK conducted the animal experiments. LL and TJS performed western blot and data analysis. AS and SS conducted ELISA and analysis. LL, JP and YC wrote and revised the manuscript.

Ethics approval and consent to participate

The animal experiments conducted in this study were approved by the Animal Care and Use Committee of Fujian University of Traditional Chinese Medicine.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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