Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers with high morbidity and mortality worldwide [1-3], which is characterized by poor prognosis and recurrent episodes [4]. Nowadays, the pathogenesis of CRC is not fully clarified, but it is widely accepted that inflammation or inflammatory bowel diseases (IBDs) are highly associated with CRC, namely colitis-associated CRC (CAC) [5]. IBDs, such as ulcerative colitis (UC) and Crohn's disease (CD), are chronic and relapsing-remitting inflammatory diseases [6]. Inflammatory cytokines are important factors for the formation of a tumor-supportive microenvironment in IBD [7]. Chronic intestinal inflammation has been shown to support tumor initiation through oxidative stress-induced mutations. A pro-inflammatory microenvironment that develops possibly as a result of defective intestinal barrier function and host-microbial interactions enables tumor promotion [8].

Gut microbiota plays a role in influencing the progression of CRC. Alterations in the structure of gut microbiota often lead to microecological imbalance, which is commonly considered as an important link in the pathogenesis of IBD and CRC [9]. Although the precise mechanisms remain elusive, increasing evidence suggest gut microbial abundance has a close relationship with CRC incidence. For example, Bifidobacterium, Lactobacillus, and Ruminococcus significantly decreased in the intestine of CRC patients [9].

The Wnt signaling pathway is widely acknowledged to play a leading role in the onset of CRC development [10]. β-

[ABSTRACT] Pai-Nong-San (PNS), a prescription of traditional Chinese medicine, has been used for years to treat abscessation-induced diseases including colitis and colorectal cancer. This study was aimed to investigate the preventive effects and possible protective mechanism of PNS on a colitis-associated colorectal cancer (CAC) mouse model induced by azoxymethane (AOM)/dextran sodium sulfate (DSS). The macroscopic and histopathologic examinations of colon injury and DAI score were observed. The inflammatory indicators of intestinal immunity were determined by immunohistochemistry and immunofluorescence. The high throughput 16S rRNA sequence of gut microbiota in the feces of mice was performed. Western blot was used to investigate the protein expression of the Wnt signaling pathway in colon tissues. PNS improved colon injury, as manifested by the alleviation of hematochezia, decreased DAI score, increased colon length, and reversal of pathological changes. PNS treatment protected against AOM/DSS-induced colon inflammation by regulating the expression of CD4+ and CD8+ T cells, inhibiting the production of HIF-α, IL-6, and TNF-α, and promoting the expression of IL-4 and IFN-γ in colon tissues. Meanwhile, PNS improved the components of gut microbiota, as measured by the adjusted levels of Firmicutes, Bacteroidetes, Proteobacteria, and Lactobacillus. PNS down-regulated the protein expression of p-GSK-3β, β-catenin, and c-Myc, while up-regulating the GSK-3β and p-β-catenin in colon tissues of CAC mice. In conclusion, our results suggested that PNS exhibits protective effect on AOM/DSS-induced colon injury and alleviates the development of CAC through suppressing inflammation, improving gut microbiota, and inhibiting the Wnt signaling pathway.

(KEY WORDS) Pai-Nong-San; CAC; AOM-DSS; Gut microbiota; Wnt signaling pathway

Catenin, a key component of Wnt signaling, is controlled by a destruction complex composed of axis inhibition protein (Axin), adenomatosis polyposis protein (APC), casein kinase (CK1) and glycogen synthase kinase-3β (GSK3β) [11]. In response to Wnt signaling activation, these complexes are disaggregated, and free β-catenin is stabilized and rapidly accumulates in the cytoplasm and nucleus and interacts with the transcription factor TCF4/LEF to activate the transcription of Wnt target genes such as CCND1 and c-Myc [12]. The network analysis for the proteomics study indicated that differentially expressed c-Myc protein might contribute to CAC progression [13]. Consequently, these results suggest that suppression of Wnt/β-catenin activation may be a viable strategy for treating CAC.

Pai-Nong-San (PNS), a prescription of traditional Chinese medicine, was documented in Jingui Yaolu by ZHANG Zhong-Jing in the Later Han Dynasty [14]. This formula is composed of three kinds of Chinese herbal medicines: the dried immature fruit of Aurantii fructus immutatus (Citrus aurantium L. Sp. Pl, CA), the dried root of Paeoniae Radix Alba (Paeonia lactiflora Pall. Reise Russ. Reich, PL) and the dried root of Platycodonis Radix (Platycodon grandiflorus (Jacq.) A. DC. Monogr. Campan, PG). PNS was widely used for the treatment of diseases with abscessation, such as colitis [15]. It was reported to possess the effects of anti-inflammation [16], expelling pus, and drawing toxin [17]. Although PNS is an effective prescription with the above effects, the potential therapeutic effects on CAC remains unknown. Thus, the present study was designed to investigate the protective/preventive effects of PNS on mice with azoxymethane (AOM)/dextran sodium sulfate (DSS)-induced CAC and explore the possible mechanism.

Materials and Methods

Reagents

Azoxymethane (AOM) was purchased from Sigma Chemical Company (St. Louis, MO). Dextran sodium sulfate (DSS, MW: 36 000–50 000) was obtained from Meilun Biological Technology Co., Ltd. (Dalian, China). Aspirin tablets were purchased from Kangli Pharmaceutical Co., Ltd. (Shijiazhuang, China). CA (AUCM-19031501), PL (AUCM-19031502), and PG (AUCM-19031503) were from a local herbal medicine market (Heifei, Anhui) and identified by Professor LIU Shou-jin in Anhui University of Chinese Medicine. The specimens used in the study were stored in Anhui University of Chinese Medicine to permit future reference and verification. Primary antibodies specific to HIF-α, IL-4, and IFN-γ were obtained from Bioss Biosynthesis Biotechnology Co., Ltd. (Beijing, China). Primary antibodies specific to TNF-α and IL-6 were provided by Wanlei Biotechnology (Shenyang, China). Primary antibodies specific to CD4 and CD8 were purchased from Abbkine (California, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. Primary antibodies specific to GSK-3β, p-GSK-3β (Ser9), β-catenin, and p-β-catenin (Ser33/37/Thr41) were purchased from Cell Signaling Technology (Shanghai, China), and antibody specific to c-Myc was obtained from Abcam Technology (Shanghai, China). Horseradish peroxidase-conjugated secondary antibody was purchased from Abbkine (California, USA). The standards including paconiflorin (purity ≥ 98%), hesperidin (purity ≥ 98%), naringin (purity ≥ 98%), and neohesperidin (purity ≥ 98%) were obtained from Desite Biotechnology Co., Ltd. (Chengdu, China). All other chemicals and solvents were analytical grade and purchased from Zhongshi Chemical Engineering Company (Shanghai, China).

Animals

Male Balb/c mice (6–8 weeks old, 20 ± 2 g) supplied from the Experimental Animal Center of Anhui Medical University, were allowed to adapt in a temperature-controlled room on a 12/12 h light-dark cycle with free access to standard chow and pure water for one week. Then, 36 mice were randomly divided into six groups: a normal group, a model group, an aspirin group (positive medicine) and PNS groups (high-, medium- and low-dose). The animal experimental protocol was reviewed and approved by the Institutional Ethics Committee of Anhui University of Chinese Medicine. Experiments were carried out following the approved guidelines.

Preparation of PNS

All raw herbs of PNS were powdered and passed through a 40-mesh sieve. They were mixed at a ratio of 5:5:2 (CA to PL to PG), and suspended in pure water before administration.

UPLC analysis

Preparation of solutions

First, 1.67 g CA, 1.67 g PL and 0.67 g PG were ground into powders and mixed with 75% methanol, before ultrasonic dispersion for 30 min followed by centrifugation at 5400 r·min⁻¹ for 10 min. Then, the supernatant was collected and passed through 0.22 μm filters. Similarly, the standards were accurately weighed, dissolved with 75% methanol, and then mixed to prepare a mixed reference solution. Five different concentrations of mixed reference solutions were prepared to create a standard curve for calculation of content.

Analytical conditions

The chromatographic column was ACQUITY UPLC BEH C18 (2.1 mm × 100 mm, 1.7 μm). The elution profile of two solvents, methanol (A) and water (B) were as follows: 0–3.0 min, 20% A; 3.0–4.0 min, 20%–35% A; 4.0–9.0 min, 35% A; 9.0–13.0 min; 35%–50% A; 13.0–14.0 min, 50%–75% A; 14.0–17.5 min, 75% A, 17.5–22.0 min, column wash and stabilization. The flow rate was 0.2 mL·min⁻¹. The volume of injection was 2 μL. The column temperature was maintained at 35 °C. The detection wavelength was 254 nm.

Establishment of a CAC model and drug administration

Briefly, CAC mice were intraperitoneally injected (i.p.) with 10 mg kg⁻¹ AOM. After seven days, the mice were given three regular cycles of water (2 weeks) 2.5% DSS (1 week) by gavage. At the beginning of DSS stimulation (day
22), the mice were intragastrically administered with a low-(0.8 g kg⁻¹), medium- (1.6 g kg⁻¹), high-dose (3.2 g kg⁻¹) of PNS or aspirin (0.013 g·kg⁻¹) (Scheme 1).

**Sample collection**

During DSS stimulation, the inflammation of CAC mice was scored as previously described [10]. Colon tissues were removed on day 78, and then rinsed with ice-cold 0.9% NaCl for histopathology examination and immunohistochemistry staining. Tissues were removed from the colon for Western blot analysis.

**Histopathological examination**

Colon tissues were fixed in 4% paraformaldehyde and dehydrated with increasing concentrations of alcohol and xylene, before embedded in paraffin blocks. The embedded tissues were cut into 5 µm thick sections which were then stained with hematoxylin and eosin (HE) for microscopic examination.

**Immunohistochemistry**

The colon tissue sections were transferred into 10 mmol L⁻¹ citrate buffer solution. Endogenous peroxidase activity was blocked with 0.03% H₂O₂ for 10 min. The sections were washed by PBS thrice for 5 min, incubated in goat serum at room temperature for 1 h, and then incubated with primary antibodies to IL-4, IL-6, TNF-α, or IFN-γ at 4 ℃ overnight. After washed by TBST, the sections were incubated in kits of the polymer detection system (PV-6000, ZSGB-BIO, China), and visualized by diaminobenzidine (DAB). Then, 30 fields of immunohistochemistry sections were randomly selected and the integrated optical density (IOD) was calculated by Image-pro Plus 6.0.

**Immunofluorescence**

Pretreatment for the colon tissue sections was the same as immunohistochemistry assay. Mixed primary antibodies to CD4⁺ and CD8⁺ T cells were used to immerse the sections at 4 ℃ overnight. After washed, the sections were incubated in appropriate mixed secondary antibodies for 20 min and counterstained by DAPI. Then, 30 fields of immunohistochemistry sections were randomly selected and the integrated optical density (IOD) was calculated by Image-pro Plus 6.0.

**16S rRNA gene sequence analysis**

Before euthanasia, the feces of mice in each group were collected and stored at −80 ℃. The obtained feces were sent to Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) for DNA extraction, PCR amplification, and microbiota diversity analysis. Sequencing analysis included sequencing data processing, OUT clustering and species annotation, sample complexity analysis, and multi-sample comparison analysis.

**Western blot**

Total protein was extracted with RIPA lysis buffer from the colon tissue samples followed by homogenization on ice. Protein concentration was determined by the BCA method. Samples were separated on SDS-PAGE and transferred to PVDF membranes on ice. After blocking with 5% skim milk, the membranes were incubated with primary antibodies at 4 ℃ overnight, followed by horseradish peroxidase-conjugated secondary antibodies and visualized using the enhanced chemiluminescence kit (Millipore, Billerica, MA). Semi-quantitative analysis was performed using densitometry.

**Statistical analysis**

All results were obtained from at least three repeated experiments. Statistical comparison was performed by the one-way ANOVA followed by Turkey’s test. A value of P of less than 0.05 was significantly different.

**Results**

**The chemical component of PNS**

Under the above UPLC conditions, a total of nine peaks were detected in the PNS samples, where paeoniflorin, naringin, hesperidin, and neohesperidin were the main components (Fig. 1, Table 1). According to the linear equations established, the contents of the four components were 0.397 ± 0.198, 1.631 ± 0.407, 0.273 ± 0.083, and 2.425 ± 0.629 mg·g⁻¹, respectively.

**Preventive effect of PNS on AOM/DSS-induced CAC in mice**

As shown in Fig. 2B, the weight of the mice in the model group significantly decreased compared with the normal group. However, the weight loss was reversed by PNS ad-
ministration in a dose-dependent manner.

Disease activity index (DAI), a vital index reflecting the effect of PNS on the CAC model, was recorded during CAC modeling. It was calculated on the basis of body weight and feces, using the following equation: DAI score = (weight loss score + stool trait score + hematochezia score)/3 (Table 2). In addition to continuous weight loss, mice in the model group showed severe diarrhea, hematochezia, and eventually anal prolapse. In Fig. 2C, the DAI value was the highest in the model group, and the increasing trend in CAC mice was dose-dependently inhibited by PNS administration.

Colorectal length is commonly considered as one of the inflammatory signs of colitis. As shown in Figs. 2D and 2E, the colon length of mice in the model group was significantly shorter than that of the normal group. But PNS treatment remarkably increased the colorectal length of CAC mice in a dose-dependent manner. The number of colon tumors are shown in Figs. 2D and 2F. Compared with the model group, there are multiple visible tumors in the colon of mice in the

### Table 1  Identification of chromatographic peaks

<table>
<thead>
<tr>
<th>Peak number</th>
<th>Name</th>
<th>t&lt;sub&gt;R&lt;/sub&gt;/min</th>
<th>Component source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>paeoniflorin</td>
<td>6.43</td>
<td>Paeonia lactiflora</td>
</tr>
<tr>
<td>4</td>
<td>naringin</td>
<td>11.21</td>
<td>Citrus aurantium</td>
</tr>
<tr>
<td>5</td>
<td>hesperidin</td>
<td>11.85</td>
<td>Citrus aurantium</td>
</tr>
<tr>
<td>6</td>
<td>neohesperidin</td>
<td>12.32</td>
<td>Citrus aurantium</td>
</tr>
</tbody>
</table>

score + stool trait score + hematochezia score)/3 (Table 2). In addition to continuous weight loss, mice in the model group showed severe diarrhea, hematochezia, and eventually anal prolapse. In Fig. 2C, the DAI value was the highest in the model group, and the increasing trend in CAC mice was dose-dependently inhibited by PNS administration.

Colorectal length is commonly considered as one of the inflammatory signs of colitis. As shown in Figs. 2D and 2E, the colon length of mice in the model group was significantly shorter than that of the normal group. But PNS treatment remarkably increased the colorectal length of CAC mice in a dose-dependent manner. The number of colon tumors are shown in Figs. 2D and 2F. Compared with the model group, there are multiple visible tumors in the colon of mice in the

### Table 1  Identification of chromatographic peaks

<table>
<thead>
<tr>
<th>Peak number</th>
<th>Name</th>
<th>t&lt;sub&gt;R&lt;/sub&gt;/min</th>
<th>Component source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>paeoniflorin</td>
<td>6.43</td>
<td>Paeonia lactiflora</td>
</tr>
<tr>
<td>4</td>
<td>naringin</td>
<td>11.21</td>
<td>Citrus aurantium</td>
</tr>
<tr>
<td>5</td>
<td>hesperidin</td>
<td>11.85</td>
<td>Citrus aurantium</td>
</tr>
<tr>
<td>6</td>
<td>neohesperidin</td>
<td>12.32</td>
<td>Citrus aurantium</td>
</tr>
</tbody>
</table>

score + stool trait score + hematochezia score)/3 (Table 2). In addition to continuous weight loss, mice in the model group showed severe diarrhea, hematochezia, and eventually anal prolapse. In Fig. 2C, the DAI value was the highest in the model group, and the increasing trend in CAC mice was dose-dependently inhibited by PNS administration.

Colorectal length is commonly considered as one of the inflammatory signs of colitis. As shown in Figs. 2D and 2E, the colon length of mice in the model group was significantly shorter than that of the normal group. But PNS treatment remarkably increased the colorectal length of CAC mice in a dose-dependent manner. The number of colon tumors are shown in Figs. 2D and 2F. Compared with the model group, there are multiple visible tumors in the colon of mice in the

### Table 1  Identification of chromatographic peaks

<table>
<thead>
<tr>
<th>Peak number</th>
<th>Name</th>
<th>t&lt;sub&gt;R&lt;/sub&gt;/min</th>
<th>Component source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>paeoniflorin</td>
<td>6.43</td>
<td>Paeonia lactiflora</td>
</tr>
<tr>
<td>4</td>
<td>naringin</td>
<td>11.21</td>
<td>Citrus aurantium</td>
</tr>
<tr>
<td>5</td>
<td>hesperidin</td>
<td>11.85</td>
<td>Citrus aurantium</td>
</tr>
<tr>
<td>6</td>
<td>neohesperidin</td>
<td>12.32</td>
<td>Citrus aurantium</td>
</tr>
</tbody>
</table>

score + stool trait score + hematochezia score)/3 (Table 2). In addition to continuous weight loss, mice in the model group showed severe diarrhea, hematochezia, and eventually anal prolapse. In Fig. 2C, the DAI value was the highest in the model group, and the increasing trend in CAC mice was dose-dependently inhibited by PNS administration.

Colorectal length is commonly considered as one of the inflammatory signs of colitis. As shown in Figs. 2D and 2E, the colon length of mice in the model group was significantly shorter than that of the normal group. But PNS treatment remarkably increased the colorectal length of CAC mice in a dose-dependent manner. The number of colon tumors are shown in Figs. 2D and 2F. Compared with the model group, there are multiple visible tumors in the colon of mice in the

### Table 1  Identification of chromatographic peaks

<table>
<thead>
<tr>
<th>Peak number</th>
<th>Name</th>
<th>t&lt;sub&gt;R&lt;/sub&gt;/min</th>
<th>Component source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>paeoniflorin</td>
<td>6.43</td>
<td>Paeonia lactiflora</td>
</tr>
<tr>
<td>4</td>
<td>naringin</td>
<td>11.21</td>
<td>Citrus aurantium</td>
</tr>
<tr>
<td>5</td>
<td>hesperidin</td>
<td>11.85</td>
<td>Citrus aurantium</td>
</tr>
<tr>
<td>6</td>
<td>neohesperidin</td>
<td>12.32</td>
<td>Citrus aurantium</td>
</tr>
</tbody>
</table>
model group and the tumor incidence was 100%. The number of tumors in the PNS groups significantly decreased compared with that of the model group. Notably, in the high-dose PNS group, few tumors were observed in the colon of CAC mice.

As shown in Fig. 2G, the histopathology of colon sections in the normal group showed that the crypts were compactly arranged and the mucosal and muscular layers were intact. In the model group, the crypts were branched, extremely irregular, and tree-shaped, with back to back gland formation. Compared with the model group, the crypt cavities of colon tissues in the medium- and high-dose PNS groups expanded. The above results indicated that PNS treatment significantly improved colon cancer in mice.

The anti-inflammatory effect of PNS on AOM/DSS-induced CAC in mice

As shown in Fig. 3, a large number of CD4+ and CD8+ T cells aggregated in the colon tissues of CAC mice. Compared with the model group, PNS and the positive medicine significantly reduced the number of CD4+ and CD8+ T lymphocytes.

Fig. 4 shows the immunohistochemical staining and IOD analysis of inflammatory factors in the colon tissues of each group. For anti-inflammatory mediators (Figs. 4A and 4B), compared with the normal group, the secretion levels of IL-4 and IFN-γ in the CAC group significantly decreased ($P < 0.001$). Moreover, compared with the normal group, the secretion levels of IL-6 and TNF-α in the CAC group significantly increased ($P < 0.001$) (Figs. 4C and 4D). In contrast, PNS recovered the levels of these inflammatory mediators in a dose-dependent manner. Besides, PNS also significantly decreased the expression of HIF-α in the colon tissues of CAC mice (Fig. 4E).

Effect of PNS on the dysregulation of gut microbiota in CAC mouse model

Figs. 5A and 5B show the change of gut microbiota diversity in each group. In the CAC group, the alpha diversity of the gut microbiota decreased, and the composition ratio also changed. The Sorbs index and Shannon index were used to assess alpha diversity. The diversity index of the PNS group was closer to the value of the normal group compared with the positive group. Figs. 5C and 5D are the results of the percent of community abundance on different levels. On the phylum level, *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* were the dominant phyla that constituted the gut microbiota. Compared with the normal group, the abundance of *Bacteroidetes* and *Proteobacteria* in the CAC group decreased, but the abundance of *Firmicutes* significantly increased. After treatment, the PNS group adjusted all three phyla to a normal level, while the positive group improved *Bacteroidetes* alone (Fig. 5C). On the genus level, *Lactobacilus*, *Muribaculaceae*, and *Bacteroides* accounted for a large proportion. Compared with the normal group, the abundance of *Lactobacillus* in the CAC group significantly decreased, and the abundance of *Muribaculaceae* was similar in all groups. However, the level of *Lactobacillus* was close to the normal group after treatment (Fig. 5D). The results indicated PNS can regulate the structure of the microbiota. Figs. 5E and 5F show the results of the beta diversity of the gut microbiota. The hierarchical clustering tree (Fig. 5E) and Non-Metric Multi-Dimensional Scaling (NMDS) (Fig. 5F) were used to explore the similarities and differences in gut microbiota composition between

### Table 2 DAI scoring standards

<table>
<thead>
<tr>
<th>Score</th>
<th>Weight loss (%)</th>
<th>Stool trait</th>
<th>Hematochezia</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>1</td>
<td>1−5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>6−10</td>
<td>Loose</td>
<td>Little</td>
</tr>
<tr>
<td>3</td>
<td>11−15</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>16−20</td>
<td>Thin</td>
<td>Obvious</td>
</tr>
</tbody>
</table>

Fig. 3 Effect of PNS on the expression of CD4 and CD8 T cells in the colon of CAC mice assessed by immunofluorescence. NOR: the normal group; CAC: the model group; L: the low-dose PNS group; M: the medium-dose PNS group; H: the high-dose PNS group. Data are means ± SD ($n = 6$). *$P < 0.05$, **$P < 0.001$ vs the NOR group; ***$P < 0.01$, ****$P < 0.001$ vs the CAC group, tested by one-way ANOVA.
different groups. The results showed that the PNS group was similar to the normal group, while the CAC group was separated from the normal group.

**Effect of PNS on the Wnt signaling pathway activation in CAC mouse model**

As shown in Fig. 6, compared with normal mice, CAC mice presented significantly reduced expression of GSK-3β and β-catenin protein in tumor tissues, while the expression of p-GSK-3β and β-catenin protein significantly increased. PNS treatment dose-dependently increased the expression of GSK-3β and β-catenin compared with the CAC group. Compared with the normal group, the expression of c-Myc in the tumor of the model group significantly increased (P < 0.001) (Fig. 6D). After PNS treatment, the expression of c-Myc in the tumor tissues of CAC mice was significantly reduced, especially in the PNS high-dose group (P < 0.001). These results demonstrated that PNS inhibited the over-activation of the Wnt signaling pathway in CAC mice.

**Discussion**

CRC is the fourth most frequent cause of cancer deaths worldwide [19], while the etiological factor and mechanism of CRC remain not fully understood. An increasing number of researches suggest that IBD may be associated with an increased risk for CRC development [20-23]. The initiating mutation in colitis is characterized by the infiltration of immune cells, and their functions in antigen presentation. In this process, this activated inflammatory response can in turn cause colon destruction such as fibrosis, abscess, fistula, and cancer. An experimental study has reported that the suppression of inflammatory responses has an immense significance in the prevention of CRC [23]. Accordingly, the early prevention of IBD and finding an effective therapeutic strategy play an important role in the treatment of CRC.

In this study, we aimed to investigate the potential beneficial effects of PNS on AOM/DSS-induced CAC and attempted to elucidate the underlying molecular mechanisms. Pharmacodynamic studies showed that mice in the PNS group exhibited less weight loss than those in the CAC group, and the changes of DAI score were dose-dependently attenuated by PNS administration. As an indirect index to reflect the colorectal inflammation, colon length in mice of the PNS group was recovered. Meanwhile, the pathological changes of intestinal tissues were dramatically attenuated, which was manifested by the relative complete crypts and less uncontrolled cell proliferation and arrangement. It is noteworthy that the effects on colon length, weight loss, tu-
Consistent with this observation, our results showed that the number of CD4⁺ and CD8⁺ T cells in the colon tissues of AOM/DSS-induced mice was significantly higher than that of the normal group. However, PNS treatment dose-dependently decreased their numbers, DAI score, and pathological changes were improved in the high-dose PNS group compared with the aspirin group. Thus, these results supported the protective effects of PNS on AOM/DSS-induced colon injury.

We performed chemical composition analysis on the PNS methanol extract and preliminarily identified peaks 1, 4, 5 and 6 as paeoniflorin, naringin, hesperidin and neohesperidin by combining with the retention time of the standard substance. The other five peaks were not matched due to lack of standard products. Apart from these four components, literatures have shown that other main components of these herbs including oxgpaeonigflorin, benzoylpaeoniforin, albiforin, hesperetin, naringenin, platycoside A, platycoside B, and platycoside D, et al. [23-25]. The components of PNS were diverse, but due to limited UPLC conditions, all the components were not shown here. Therefore, in order to clarify the effective material basis of PNS in more detail, the following experiments can be combined with UPLC-Q/TOF-MS and UNIFI informatics platform to identify the chemical components of PNS.

Then, we detected the number of CD4⁺ and CD8⁺ T cells and the expression of inflammatory indicators to investigate the immunological mechanism of PNS-relieved colon injury. As the lymphocytes with immunomodulatory function, CD4⁺ and CD8⁺ T cells play vital roles in maintaining immune homeostasis [26]. A previous study showed that increased infiltration of CD8⁺ T cells may mediate colon tissues damage caused by high expression of perforin [27]. Consistent with this observation, our results showed that the number of CD4⁺ and CD8⁺ T cells in the colon tissues of AOM/DSS-induced mice was significantly higher than that of the normal group. However, PNS treatment dose-dependently decreased their

**Fig. 5** Effect of PNS on the dysregulation of gut microbiota in CAC mouse model. Alpha diversity analysis diagram of Sobs index diagram (A) and Shannon index (B) The gut microbiota composition analysis at phylum level (C) and genus level (D) The hierarchical clustering tree (E) and Non-Metric Multi-Dimensional Scaling (NMDS) (F) NOR: the normal group; CAC: the model group; POS: the aspirin group; PNS: the PNS group. Data are means ± SD (n = 3). *P < 0.05 vs the NOR group, #P < 0.05 vs the CAC group, tested by one-way ANOVA.
To further elucidate the molecular mechanism by which PNS regulated inflammatory processes in CAC mouse model, key signaling molecules along Wnt pathway were examined by Western blot. In our study, colon tissues from normal mice demonstrated low levels of c-Myc, β-catenin and p-GSK-3β of inflamed colon tissues. In line with its suppression of pro-inflammatory response, the aberrant over-activation of the Wnt/β-catenin signaling pathway in CAC mouse model was greatly abolished by PNS as well. Wnt/β-catenin activation is implicated in chronic inflammation, organ fibrosis, and a variety of human cancers. Accumulation of β-catenin in the nucleus is related to poor prognosis in CRC patients. Furthermore, nuclear β-catenin cooperates with TCF/LEF to activate the expression of Wnt/β-catenin signaling target genes. For example, the c-Myc oncogene plays a central role in the development of carcinogenesis and it is overexpressed in CRC. Thus, we postulate that its modulation of inflammatory processes might be associated with inhibiting the Wnt/β-catenin signaling pathway.

Gut microbiota is closely related to the occurrence of intestinal inflammation. In general, abnormal changes in the composition of gut microbiota can lead to the destruction of the intestinal barrier, thus activating inflammatory response and aggravating the damage in intestinal mucosa. In CAC mice, the proportion of Bacteroidetes in the colonic microbes increased, while the proportion of Firmicutes decreased. However, PNS improved gut microbiota composition of CAC mouse model such as Firmicutes, Bacteroidetes, and Proteobacteria. In addition, it significantly increased the proportion of Lactobacillus, leading to a higher proportion of beneficial genera. These results indicated that PNS possesses the ability to regulate the disturbance of gut microbiota.

In conclusion, the present study demonstrated that PNS exhibits protective effects on AOM/DSS-induced colon injury in mice and alleviates the development of colitis-associated CRC through suppressing inflammation, improving gut microbiota, and inhibiting the Wnt signaling pathway.

References
[4] Lasry A, Zinger A, Ben-Neriah Y. Inflammatory networks un-


[35] Baldus SE, Monig SP, Huxel S, et al. MUC1 and nuclear β-catenin are coexpressed at the invasion front of colorectal carcinomas and are both correlated with tumor prognosis [J]. Clin Cancer Res, 2004, 10(8): 2790-2796.

