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Original Article

Shenling Baizhu Powder (参苓白术散) Ameliorates Pi (Spleen)-Deficiency-Induced Functional Diarrhea in Rats*

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ABSTRACT **Objective:** To explore the mechanism of Pi (Spleen)-deficiency-induced functional diarrhea (FD) model rats treated by Shenling Baizhu Powder (参苓白术散, SBP). **Methods:** Thirty male Sprague-Dawley rats were randomly divided into 5 groups including control, model, low-, medium-, and high-dose SBP groups (SBPLDG, SBPMDG, SBPHDG), 6 rats in each group, respectively. Pi-deficiency-induced FD rats model was developed through *Radix et Rhizoma* Rhei gavage for 7 days. After modeling, the rats were treated with 3 doses of SBP [0.93, 1.86, and 3.72 g/(kg·d)], and the rats in the control and model groups were given pure water for 7 days. The diarrhea index was calculated. On the 7th and 14th days, the traveled distance of rat was measured by the open field test. Serum D-xylose content was determined by the phloroglucinol method and interleukin (IL)-10 and IL-17 levels were measured using an enzyme-linked immunosorbent assay kit. The content of Treg cells was determined by flow cytometry. **Results:** Compared with the control group, the diarrhea index and IL-17 level in the model group were significantly higher and the total exercise distance and D-xylose content significantly decreased (P<0.05). The expression of IL-10 in the SBPHDG group was significantly up-regulated, and serum D-xylose level and Treg cells increased significantly compared with the model group (P<0.05). **Conclusion:** High-dose SBP exhibited ameliorating effects against Pi-deficiency induced FD, which might be attributed to its modulations on intestinal absorption function as well as adaptive immunity in mesenteric lymph nodes of rat.

KEYWORDS functional diarrhea, Pi (Spleen)-deficiency, Shenling Baizhu Powder, T helper type 17 cells, Treg cells, Chinese medicine

Functional diarrhea (FD), which is differentiated from inflammatory bowel disease, microscopic colitis, and chronic infection, is one kind of functional gastrointestinal diseases.[1] It is characterized by continuous or repeated loose (paste) or watery stools, and more than 75% of bowel movements are not accompanied by abdominal pain.[2,3]

In the Chinese medicine (CM) theory, FD belongs to the category of "diarrhea", and the main pathogenic organ is located in Pi (Spleen) and Wei (Stomach). Shenling Baizhu Powder (参苓白术散, SBP), is a classical prescription for the treatment of Pi-deficiency diarrhea in the clinic. It has been extensively used for treating FD in China.[4,5] Singh, et al[6] found that treatment of FD significantly relieved patients' anxiety. Another study proved that SBP could repair gastrointestinal mucosal and protect the gastrointestinal mucosal barrier in Pi-deficiency induced diarrhea mice.[7] However, the underlying treatment mechanism for FD is unclear.

Accumulating evidence suggests that the immune system may play an essential role in the pathogenesis of diarrhea. T helper type 17 (Th17) and Treg cells share a typical precursor cell (the naive CD4 T cell). Th17 cells cause autoimmunity and inflammation, whereas Treg cells inhibit the inflammation and maintain immune homeostasis. Th17 cells produce interleukin (IL)-17, IL-22, and IL-23, and recruit neutrophils and promote inflammation at the site of infection. By contrast, Treg cells produce anti-inflammatory cytokines such as IL-10 and transforming growth factor-β (TGF-β), suppress the activity of a variety of immune cells, thereby inhibiting the immune responses.[8,9] A study has shown that the immune dysfunction in diarrhea patients correlates with

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Pi-deficiency.[10] We conducted this study to evaluate the treatment effect and mechanism of SBP on Pi-deficiency-induced FD by testing cytokine levels of IL-17 and IL-10 in rats.

METHODS

Animals

Thirty healthy male Sprague-Dawley rats aged 2-month old [200 ± 20 g, license No. SCXK (Lu) 20140007] were generated and group-housed (6 per cage) under specific pathogen-free conditions at a constant room temperature of 22–25 ℃ with a 12-h light/dark cycle (lights on 8 AM, off 8 PM). Animal experiment was approved by the Committee on Animal Care and Use of Institute of Laboratory Animal Science, Jinan University, China, and performed according to the recommended guidelines.

Drugs and Reagents

SBP is composed of 11 Chinese medicinal herbs, including *Radix Ginseng* 15 g, *Poria* 15 g, *Semen Coicis* 9 g, *Rhizoma Atractylodis Macrocephlae* 15 g, *Radix Platycodi* 6 g, *Semen Nelumbinis* 9 g, *Fructus Amomi* 6 g, *Pericarpium Citri Tangerinae* 9 g, *Radix Glycyrrhizae* 9 g, *Dolicho Lablabl* 12 g, and *Rhizoma Dioscoreae* 15 g. Both *Radix et Rhizoma* Rhei and SBP Granules were purchased from Guangdong Yifang Pharmaceutical Co., Ltd. IL-17A monoclonal antibody (eBio17B7), FOXP3 monoclonal antibody (FJK-16s), CD25 monoclonal antibody (OX39), and CD4 monoclonal antibody (OX35) were purchased from the eBioscience company. Rat IL-17 enzyme linked immunosorbent assay (ELISA) kits (No.19010709R) and IL-10 ELISA kits (No.19010710R) were provided by Jiangsu Meimian Industrial Co., Ltd. (China). D-xylose assay kits (No. 20190410) were obtained from Nanjing Jiancheng Biotechnology Co., Ltd. (China).

Development of Pi-Deficiency FD Model and Drug Administration

The rats underwent adaptation to the environment for 10 days. Then, 30 rats were randomized divided into 5 groups by random number table (6 rats in each group), including control, model, low-, medium-, high-dose SBP groups (SBPLDG, SBPMDG, SBPHDG), respectively. The experimental process is shown in Figure 1.

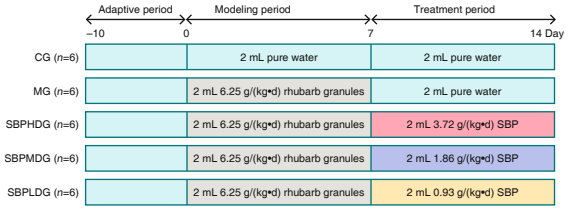

Figure 1. Animal Experiment Process

According to our previous experimental program,[11] during the modeling process, each rat was intragastrically administered with 2 mL of 6.25 g/(kg•d) *Radix et Rhizoma* Rhei Granules [15 g/(kg•d)], while rats in the control group were given pure water for 7 days. SBP was converted into the equivalent dose of rats according to the dosage of the adult and used as the drug dosage of the rats, and the dose of SBP given to rats were 0.93, 1.86, 3.72 g/(d•kg), respectively.[11] The rats in the control and model groups were given an equal dose of pure water for 7 days.

General Morphology Observation

All the rats were observed daily for fur color, food intake, behavioral activity, fecal texture, diarrhea, and body weight. Diarrhea index was calculated by observing the number of loose stools, the total number of stools, and the area of stains.[15] Diarrhea index=loose stool rate × loose stool level; loose stool rate=the number of loose stools per animal/total number of stools, and the loose stool is based on the presence or absence of stain on the filter paper. The loose stool level is classified into 4 grades according to the stain diameter of the stain on the filter paper:[12] grade 1≤1 cm; 1 cm<garde 2≤3 cm; 3 cm<grade 3≤5 cm; grade 4>5 cm.

Open Field Test

On the 7th and 14th day, the traveled distance of each rat was measured by the open field test (OFT).[13] The open-field arena, surrounded by a 35 cm high wall, 100 cm × 100 cm in size, which was divided into 9 square grid, and the central grid was defined as the center zone. The camera is placed directly above the center zone, which used to record the movements, total distance and total speed of each rat. The rat was placed in the center zone, and each rat was photographed for 5 min. The box surface was cleaned with a 70% alcohol solution after each rat testing.

Tissue Samples Collection

Intraperitoneal anesthesia was administered using 5% chloral hydrate (300 mg/kg) for each rat.

Then the rats were fixed to board after their muscles were entirely relaxed in a dorsal recumbent position with their hindlimbs externally rotated. After disinfected with 70% ethanol, a longitudinal skin incision about 3–5 cm was made to expose the abdominal contents. The entire intestine was aseptically removed immediately. Mesenteric lymph nodes were taken away and grounded quickly through a 200-mesh sieve to make a single-cell suspension prepared for flow cytometry. Blood was collected by abdominal aortic puncture, and serum was prepared for biochemical analyses.

Serum Concentration of D-Xylose

Following 7 days of treatment presence or absence SBP, all the rats were anesthetized for tissue collection. As the indicator of intestinal absorption capacity, the serum D-xylose content was tested by the phloroglucinol method.[14]

Dertermination of IL-10 and IL-17 Levels by ELISA

Blood samples were collected, and the levels of IL-10 and IL-17 were measured using an ELISA kit according to the manufacturer's protocol. The experiment was repeated 3 times.

Detection of Treg Cells Content by Flow Cytometry

Cell suspension (100 μL) and 2 μL locker were added to every sample, then the surface antibody CD4 or CD25 was added to the cells and incubation at 4 ℃ for 30 min in the dark. Afterwards, the cells were washed with PBS, and 250 μL of membrane rupture agent was added to every sample and incubated at 4 ℃ for 20 min; then, the cells were washed and resuspended into 100 μL, the intracellular antibody Foxp3 was added to every sample. The samples incubated in the dark for a further 30 min, then the cells were washed and filtered with staining buffer. Levels of CD4CD25Foxp3 cells were determined by multiparameter flow cytometry. Data were analyzed with flow cytometry analysis software (FlowJo, USA).

Statistical Analysis

SPSS 22.0 software was used for analysis. The measurement data were expressed as mean ± standard deviation ($\bar{x} \pm s$). The *t*-tests and one-way ANOVA comparisons were performed. $P<0.05$ was considered statistically significant.

RESULTS

General Morphology Observation and Food Intake Testing

No rats died in all groups during the experiment. In the control group, the vitality, activity, diet, and fur color of the rats were healthy, and the weight of rats increased day by day. During the modeling process, *Radix et Rhizoma* Rhei-administered rats developed diarrhea, loose stool, loose hair and dullness. Moreover, the body weight and food intake of rats decreased. After treatment with SBP, the body weight and food intake of rats in 3 treatment groups increased (Figures 2A and 2B).

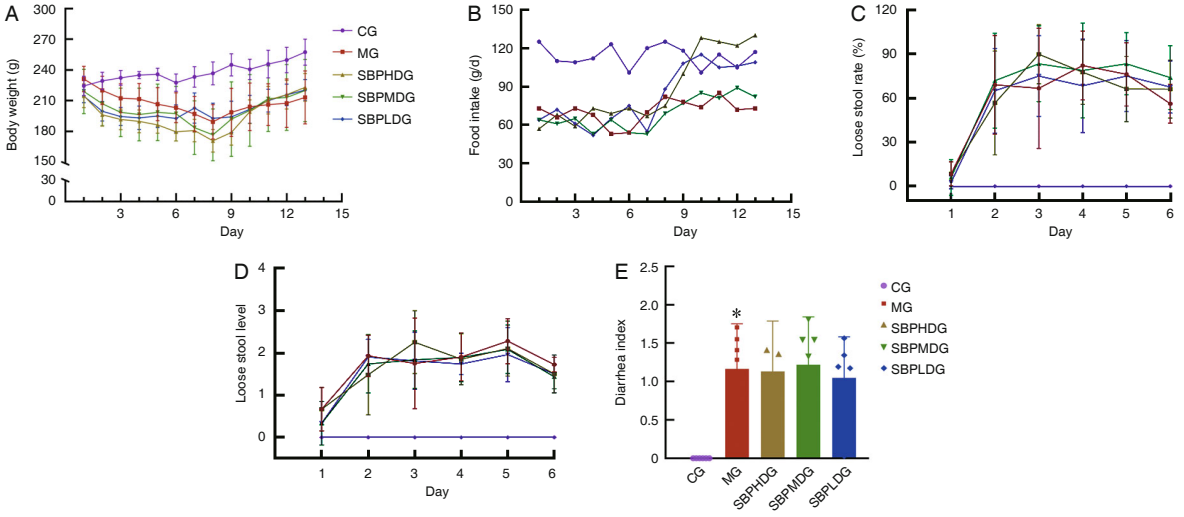

Figure 2. Body Weight, Food Intake and Diarrhea in Rats in Each Group

Notes: Trend of body weight of rats for 14 days (A); (B) changes in food intake of rats for 14 days; loose stool rate (C) and loose stool levels (D) of rats during the modeling period; (E) diarrhea index of rats during the modeling period. CG: control group; MG: model group; SBPHDG: high-dose SBP group; SBPMDG: medium-dose SBP group; SBPLDG: low-dose SBP group. The same below. *$P<0.05$ *vs*. control group

Comparison of Loose Stool Rate and Diarrhea Index

During the modeling process, compared with the control group, the loose stool rate and diarrhea index of rats in the model group increased (P<0.05, Figures 2C–2E). There was no significant difference in diarrhea between the treatment groups.

Open Field Test

On the 7th day, compared to the control group, the total movement distance of rats in the model group was significantly reduced (P<0.05), and there was no significant difference between the model and treatment groups. On the 14th day, after treatment with SBP, there was no significant difference in the total distance of movement compared with model group (P>0.05, Table 1).

Table 1. Comparison of the Total Distance of Exercise on the 7th and 14th Day by Open Field Test (cm, $\bar{x} \pm s$)

Group	n	Day 7	Day 14
CG	6	4893 ± 708.9	2774 ± 456.6
MG	6	3049 ± 304.4*	1804 ± 623.2*
SBPHDG	6	2710 ± 660.6	2266 ± 814.8
SBPMDG	6	2883 ± 762.0	2358 ± 971.0
SBPLDG	6	3059 ± 372.0	2301 ± 539.2

Note: *P<0.05 *vs*. control group

Serum D-xylose Content

Compared to control group, serum D-xylose level in the model group was significantly reduced (P<0.05). However, after treatment with SBP, the serum D-xylose level in SBPHDG and SBPMDG increased compared to the model group (P<0.05, Figure 3A).

Serum IL-17 and IL-10 Levels

Compared to control group, the serum IL-17 level was significantly increased in the model group (P<0.05). The serum IL-17 level was significantly decreased in SBPHDG compared with the model group (P<0.05). After the treatment with SBP, the expression of IL-10 in SBPHDG was higher than that the model group (P<0.05, Figures 3B and 3C).

Flow Cytometry for Detection of Treg Cells

Compared with the control group, Treg cells in the model group increased (P<0.01). After the treatment with medium- and high-dose SBP, there was a significant increase in Treg cells compared with the model group (P<0.01, Figure 4).

DISCUSSION

FD is a heterogeneous disorder, involving multiple pathogenetic mechanisms. Developing treatments for FD has been challenging. Diet, probiotics, and antibiotics are often used in the clinic. These methods have high recurrence rates and side effects. So far, there is no favorable treatment. SBP is a classical Pi-tonifying Chinese herbal formula, and studies proved the curative effect of SBP on FD without noticeable side effects.[15-17] In our research, we found the underlying mechanism of the treatment effect of SBP on Pi-deficiency induced FD through testing the cytokines involved in the differentiation

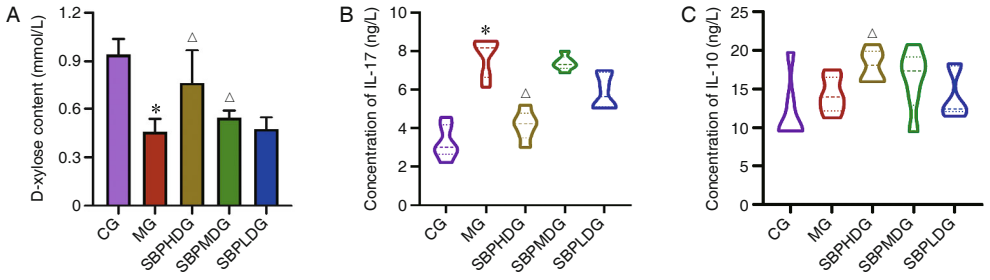

Figure 3. Contents of D-xylose, IL-17 and IL-10 in the Serum

Notes: *P<0.05 *vs*. control group; △P<0.05 *vs*. model group

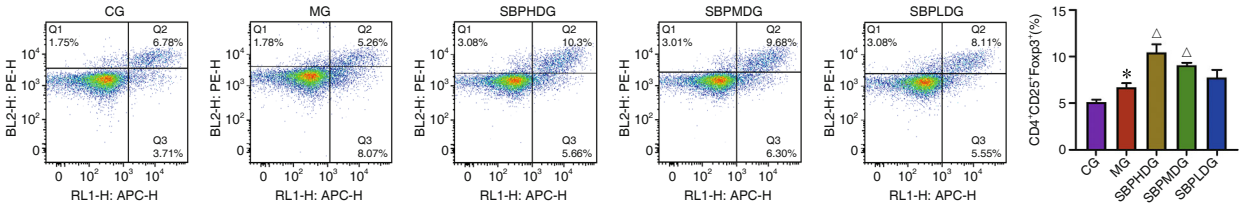

Figure 4. Flow Cytometry Detection of Proportion of Mesenteric CD4⁺CD25⁺Foxp3⁺ T Cells

Notes: *P<0.01 *vs*. control group; △P<0.01 *vs*. model group

of Treg cells and Th17 cells. In CM theory, Pi regulates transportation and transformation,[18] and Wei receives food, both organs show essential functions in the digestive process. In the present study, compared to the control group, the loose stool rate and diarrhea index in the model group were significantly increased, showing the model process through *Radix et Rhizoma* Rhei gavage successfully. The OFT can be used to measure spontaneous activity in rats. On the 14th day, the results of OFT indicated that SBP promoted the gradual healing of Pi-deficiency induced FD.

Xylose is a pentose sugar, which is quickly diffused in the small intestine after oral administration.[19,20] A study has shown that patients with Pi-deficiency diarrhea have decreased serum D-xylose content due to weakened absorption.[21] The serum D-xylose concentration is an essential objective indicator for detecting Pi-deficiency diarrhea. When the rats showed Pi-deficiency syndrome, it can reduce the rat's absorption function in the small intestine, which had a specific effect on the absorption and metabolism of D-xylose. Based on our results, our research showed that the high-dose SBP has an excellent therapeutic effect on Pi-deficiency FD.

One recent study has shown that the immune system among the rats with Pi-deficiency syndrome is dysfunctional.[22] Th17 and Treg cells balance are essential for the human immune system.[23] Th17 cells are involved in adaptive immunity, and mainly secreting the cytokine IL-17.[24-27] Treg cells secrete IL-10 to induce and maintain immune tolerance in the body, which plays an essential role in the body's immune balance.[28-31] Our experiment, showed that high-dose SBP has a treatment effect on Pi-deficiency FD rats through up-regulation of IL-10 expression and downregulation of the expression of IL-17 in rat's serum.

In conclusion, these findings suggested that high-dose SBP exhibited effects against Pi-deficiency FD, which might be attributed to its modulations on intestinal absorption function as well as adaptive immunity in mesenteric lymph nodes of rats. A better understanding of the immune regulatory mechanisms operating at FD will significantly speed up the development of diagnostic and therapeutic interventions in clinical research. This study shows the advantages of high-dose SBP in treating FD disease and enhancing the body's immune function. CM has rich drug sources, simple production processes, economical and applicable, and other advantages. CM is a promising therapy for FD in light of its safety and pleiotropic effect in enhancing immunity, reducing symptoms of diarrhea, increasing appetite, and improving mental state. The study will provide a novel strategy for enhancing immunity and reducing diarrhea symptoms in patients with FD.

Conflict of Interest

The authors declare that there is no conflict of interest.

Author Contributions

Xiao Y was involved in all aspects of research, analyzed the data, and drafted the manuscript; Zhuang K and Zhu SY performed the experiments and analyzed the data; Deng XL and Chen XY contributed to revising the manuscript; Fu NL and Chen J participated in research design and supervision of the study.

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