RESEARCH ARTICLE | FEBRUARY 28 2019

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AIP Conf. Proc. 2079, 020028 (2019)
https://doi.org/10.1063/1.5092406

The Effects of Wumei Pill on Intestinal Flora and Neurotransmitters in Rats with Diarrhea-predominant Irritable Bowel Syndrome (IBS-D)

Xiaojie Ding, Xiling Sun, Xiaofei Yu, Shutian Zhao, Yi Yang, Wenjuan Xu, Kejun Wang , Xiaolan Zou and Zhengping Dong[a)]

School of Integrated Chinese Traditional and Western Medicine, Binzhou Medical University, Yantai 264003, Shandong, China

[a)] Corresponding author: dongzhengping@sina.cn

Abstract. The objective is to investigate the effects of Wumei Pill on intestinal flora and neurotransmitters in rats with diarrhea-predominant irritable bowel syndrome (IBS-D). Method: The model was established by means of acetic acid enema plus restraint stress. The rat fecal moisture content, fecal trait score, and abdominal wall withdrawal reflex (AWR) score were used to determine whether the model was successful. Evaluated the Wumei Pills effect on rat fecal moisture content, fecal trait score, AWR score, Bifidobacterium/Enterobacteriaceae (B/E) value, and hippocampal tissue glutamate (Glu) and γ-aminobutyric acid (γ-GABA), dopamine (DA), 5-hydroxytryptamine (5-HT) content. Results: (1) the rat IBS-D model was successfully prepared; (2) Wumei Pill could reduce the fecal moisture content, fecal trait score, AWR score (P<0.05), and alleviate the symptoms of rat diarrhea; (3) Wumei Pills can increase the rats B/E value (P<0.05) increase the intestinal colonization resistance in rats. (4) Wumei Pills can reduce the contents of Glu, γ-GABA, DA and 5-HT in the hippocampus of rats (P<0.05), and have a certain regulatory effect on neurotransmitters in the rat brain. Conclusion: Wumei Pill has a significant therapeutic effect on IBS-D, and the mechanism may be related to the regulation of intestinal flora and neurotransmitters.

INTRODUCTION

Diarrhea-predominant irritable bowel syndrome (IBS-D) is an irritable bowel syndrome with diarrhea as the main clinical manifestation. At present, IBS-D is considered to be the result of a combination of multiple pathogenesis mechanisms [1-3]. With the development of microecology and the application of molecular biology techniques, the correlation between the "intestinal flora-brain-gut axis" and IBS-D has become a hot topic [4, 5]. There is a two-way connection between the gut microbiota and the brain-gut axis. The interaction between the gut microbiota and the brain is through the two-way traffic path of the neuroendocrine network system between the brain's gut axis and by means of various neurotransmitters. Realized [6]. IBS-D belongs to the category of "diarrhea" in Chinese medicine. Wumei Pills was mentioned in No.338 article of Zhang Zhongjing's Treatise on Febrile Diseases. The article states that Wumei Pills "Zhu Jiu Li (meaning: mainly for treating the chronic diarrhea)". "Jiu Li" is also called the chronic diarrhea. And the Wumei Pill is an effective prescription for the treatment of chronic diarrhea. Clinical studies have shown that Wumei Pills have a very good therapeutic effect on IBS-D [7, 8]. The experimental study on the action mechanism of Wumei Pills for IBS-D has not been reported yet. In this study, a rat IBS-D model was prepared to compare the effects of Wumei Pills and probiotics on IBS-D in rats. And the possible action mechanism of Wumei Pills was investigated from the perspective of intestinal flora and neurotransmitters

2018 International Conference on Biotechnology and Bioengineering (8th ICBB)
AIP Conf. Proc. 2079, 020028-1–020028-7; https://doi.org/10.1063/1.5092406
Published by AIP Publishing. 978-0-7354-1807-3/$30.00

MATERIALS AND METHODS

Materials

Materials Experimental Instruments and Medicines

Wumei Pills are manufactured by Kunming Chinese Medicine Co., Ltd. (GYZZ No.Z53020892) and probiotics are provided by US SWANSON Health Foods (60 capsules/bottle). The ELISA kits glutamic acid (Glu), γ-aminobutyric acid (gamma-GABA), dopamine (DA), and 5-hydroxytryptamine (5-HT) were purchased from Shanghai Bluegene Biotech CO., Ltd and the DNA extraction kits were purchased from Germany Qiagen. Test consumables include 10% chloral hydrate, 4% acetic acid (Tianjin Bodi Chemical Co., Ltd.), capillary glass tubes, gavage needles, catheter with balloon (Germany Braud), 7500 Real-Time PCR System (American ABI Company), and HR/T16M refrigerated centrifuge (Hunan Hexi Instrument & Equipment Co., Ltd.).

Experimental Animals

SPF SD rats, male, 32 in total, 3-4 weeks old. Purchased from Jinan Pengyue Experimental Animal Breeding Co., Ltd. with production license number: SCXK (Lu) 20140007. All animals were quarantined as required prior to testing. Environmental conditions for laboratory animal feeding and management: room temperature 20 ~ 26 °C, daily temperature difference ≤ 4 °C, relative humidity 40 ~ 70%, light and dark alternation time is 12/12h. Animals were housed in standard rat cages, 4 rats per cage. During quarantine and experimental period, rats ate foods and drank water freely. The feed is for the growth of SPF-class large and small rats and is produced by Beijing Keao Xieli Feed Co., Ltd. Drinking water is urban tap water that has been disinfected by high temperature.

Method

Experimental Grouping

SD rats were randomly divided into normal group, model group, probiotic group, and Wumei Pill group by random number table. Each group had 8 rats. After the quarantine period, the other three groups except the normal group were established with IBS-D model.

Rat IBS-D Model Preparation

the method of using acetic acid enema plus restraint stress is adopted in modeling [9]. 24 hours before the experiment, rats were prohibited for eating, but not prohibited for drinking. After ether anesthesia, the silicone tube which connects the syringe was inserted into the anal (8cm into the anal). Then, 1mL of 40mL/L acetic acid was poured into the colon, and then the silicone tube was slowly pulled out. After that, the anal was oppressed and the tail was raised up for 30s. And then, the colon was rinsed with 0.01mol/L PBS1mL. When all steps were finished, the rats were put back into the cages where they could eat and drink water freely. On the 7th day, restraint stress was exerted. The rats were placed in a special transparent cylindrical drum which can restrict its acts but not restrict its breath. 3h later, the rates were taken back to the cages. The rat fecal moisture content, rat fecal trait score, and abdominal wall withdrawal reflex (AWR) score were used to determine whether the model was successful [9,10].

Dosage Regimen

Dosing after successful modeling. The normal group and the model group were given normal saline, probiotic group was given probiotics, and the Wumei pill group was given Wumei pill. The dosage was calculated according to the weights with the formula of: dB = dA x KB/KA (dB is the daily dose per kilogram of the rat, in units of mg; dA is the daily dose per kilogram of an adult, in mg; the adult weight is calculated as 60 kg; KB = 0.71, KA = 0.11 is a constant). Continuous gavage for 14d. The weight of adult is calculated as 60kg. The adult dosage of probiotics is 1 capsule/day. When this is converted to dosage of rat, it shall be 0.11 capsule/kg rat/day; the Wumei pill adult dosage is 18g/day, and the adult weight is calculated as 60kg, converted into rats, it shall be 1936.4 mg/kg rat/day.

Detection Indicators and Methods

Feces moisture content: 6h defecation points: put a piece of clean filter paper in the cage to observe the feces droppings of rats within 6h; 6h loose stool rate: points sticky on paper / defecation points x 100%; 24h Feces moisture content: Weighing the wet weight and dry weight of feces in each group of rats (weighing the wet weight of the feces first, and then put the feces in the drying box for more than 2 hours, and weighing and recording, this is the dry weight), the feces moisture content = (wet weight - dry weight) / wet weightx100%

Stool trait scores: Model rats were scored according to the Bristol Stool Scaling (see Table 1) and the scores were recorded. The rat fecal traits were measured at 6-8 am in the morning. At this time, the rats had more fecal matter and were easy to collect and observe. The two collaborated. One seized the rat and fixed its head and limbs vertically. One used the index finger and the middle finger to massage on the abdomen of the rat. And then, the rat feces was collected on a piece of filter paper for observation.

TABLE 1. Bristol stool scaling and scoring

Scale	Stool trait	Score
Scale-1	Scattered dry balls, like nuts, are difficult to drain	1 score
Scale-2	Sausage-like, many pieces	2 scores
Scale-3	Sausage-like, cracked surface	3 scores
Scale-4	Sausage-like or snake-like, smooth, soft	4 scores
Scale-5	Soft blobs with clear edges (easily drained)	5 scores
Scale-6	Soft sheet, frizzy, or mushy	6 scores
Scale-7	Liquid stool samples, no fixed ingredients	7 scores

Abdominal Wall Retraction Reflex (*AWR*) *Score*

the rats were prohibited for eating but not prohibited for drinking 18 hours before testing. After chloral hydrate anesthesia, the 8F catheter with a paraffin-oil coated balloon was inserted through the anus (balloon end was 1cm away from the anus, and fixed at the tail end of the rat 1cm outside the anus), place the rat in a self-made transparent plastic barrel cage (20cm×8cm×8cm), to limit the rat to only move back and forth, cannot turn around. The test was started 30min after the rat becoming adapting to the environment. Record the minimum amount of water that causes rat AWR, namely the minimum capacity threshold. Each threshold was repeated for three times, respectively 1.0, 1.5, and 2.0 mL, each of which lasted for 30 s with an interval of 5 min. The obtained data were averaged. AWR score: 0 points: the mood of the rats is basically stable when the colorectal distension is stimulated; 1 point, became unstable when given stimulation, and occasionally twisted the head; 2 points, the abdomen muscles contracted slightly but the abdomen did not raise above ground; 3 points, abdominal muscles strongly contract and lift the abdomen off the ground; 4 points, abdominal muscles contracted strongly, the abdomen bowed and the abdomen, perineum lifted off the ground.

Collection of Rat Intestinal Flora Samples and Detection of B/E Values

Aseptic collection of 0.3 g colon stools, divided into two, and stored in sterile cryovials, and one was for DNA extraction (operating reference DNA extraction kit), Real-time fluorescence quantitative PCR detection of B / E values (target gene PCR amplification primer sequences shown in Table 2); the other one was for being stored in -80°C refrigerator.

TABLE 2. Target gene PCR amplification primer sequences

Strain	PCR amplification fragment length (bp)	Primer sequence(5'-3')
Bifido	233	GATTCTGGCTCAGGATGAACGC;22bp
		CTGATAGGACGCGACCCCAT;20bp
Ecoli	190	CATTGACGTTACCCGCAGAAGAAGC;25bp
		CTCTACGAGACTCAAGCTTGC;21bp

Determination of Glu, γ-GABA, DA, and 5-HT Contents in Hippocampus of Rat Brain

Rat hippocampus was dissected from ice, cold saline was used to rinse and remove blood, and the filter paper was applied to wrap it. And then, it was weighed and added with cold saline. After homogenized in a homogenate tube, 10% brain homogenate was prepared. The supernatant was centrifuged at 3,000 rpm for 15 minutes, and the contents of GLU, γ-GABA, DA, and 5-HT in the hippocampus of the rat were measured using an ELISA kit

Statistical Methods

SPSS 19.0 statistical software was used for data analysis. The measurement data were analyzed using the t-test of the sample mean. Comparisons between groups were analyzed by analysis of variance. The results were expressed as mean±standard deviation ($\bar{x}$±s). When $P<0.05$, it was deemed as that the difference was statistically significant.

RESULTS

General Conditions of Rats during the Experiment

No rats died during the experiment. Normal group: The rats had good mental status, normal activities, and a sensitive response. The fecal traits were normally granular and the fur was neat and shiny. Model group: After 3 days from the start of the modeling, some rats began to suffer from mental retardation, wilting, reduced activity, arched backs, unsmoothed fur and less luster, diarrhea, loose stools, and loose stools near the anus. No recovery was found at the end of the trial. In the probiotic group, the symptoms were the same as those in the model group during the modeling period. From the 3rd day after intragastric administration, the diarrhea of some rats gradually decreased, the mental state of the rats increased, the activity gradually increased, and the coat was tidier but less glossy. At the end of the experiment, there was still a small part of the rat's feces traits. In Wumei Pills group, the symptoms were the same as those in the model group during the modeling period. Rats gradually relieved diarrhea from the third day after gavage, and the fecal traits changed from unshaped to half-shaping. The mental state of the rats gradually increased and the activities gradually increased. At the end of the experiment, there was still a small part of the rat's feces traits.

Determination of Fecal Moisture Content in Each Group

After the establishment of the model, compared with the normal group, the 6h defecation point, 6h defecation rate, and 24h feces moisture content in the model group, probiotic group, and Wumei Pill group all increased significantly and had obvious differences ($P<0.05$); After administration, compared with the model group, the detection levels of the indicators decreased to varying degrees, with significant differences ($P<0.05$). This indicates that the probiotics and the Wumei Pills can ease the diarrhea of the rats. See Table 3 for details.

TABLE 3. Feces moisture content changes of all groups of rats (n=8, $\bar{x}$±s)

Group	After Modeling			After Administration		
	6h Defecation Points	6h Stool Rate	Feces Moisture Rate	6h Defecation Points	6h Stool Rate	Feces Moisture Rate
Normal group	5.75±1.58	2.08±5.89	29.92±2.69	5.13±1.36	1.56±4.42	31.31±4.00
Model group	12.75±2.71*	54.03±9.49*	63.05±6.05*	13.13±2.53*	54.81±8.94*	62.80±7.53*
Probiotics group	13.38±2.62*	53.03±10.53*	64.98±6.72*	10.00±1.41△	38.02±6.18△	48.45±7.09△
Wumei Pill group	13.63±2.88*	57.06±7.05*	65.02±5.72*	9.75±1.91△	30.66±11.00△	47.23±6.54△

Note: compared with normal group, *$P<0.05$; compared with model group, △$P<0.05$

Rat fecal trait scores: Rat fecal trait scores were essentially the same in all groups before modeling, and there was no statistical difference. After modeling, compared with the normal group, the fecal trait scores of the model

group, the probiotic group, and the Wumei Pill group were all significantly higher (P<0.05); after the completion of the administration, compared with the model group, the fecal trait score of each group decreased to varying degrees with significant differences (P<0.05), indicating that the probiotics and Wumei Pills could all alleviate the diarrhea symptoms of the model rats. See Table 4 for details.

TABLE 4. Fecal trait scores of all groups of rats (n=8, $\bar{x}$±s)

Group	Before Modeling	After modeling	After administration
Normal group	3.25±0.71	3.63±0.92	3.38±0.74
Model group	3.38±0.52	6.13±0.83*	6.38±0.74*
Probiotics group	3.00±0.76	6.38±0.74*	5.25±1.04△
Wumei Pill group	3.38±0.52	6.50±0.76*	5.00±0.76△

Note: compared with normal group, *P<0.05; compared with model group, △P<0. 05

AWR scores: After modeling, compared with normal group, the AWR scores of the model group, probiotic group, and Wumei Pill group were significantly higher than that of the normal group (P<0.05). After the completion of administration, compared with the model group, the AWR scores of each volume decreased to some extent, and some of them had significant differences (P<0.05), indicating that probiotics and Wumei Pills were able to reduce the organ sensitivity of model rats. This is shown in Table 5 for details.

TABLE 5. AWR scores of all groups of rats (n=8, $\bar{x}$ ±s)

Group	After modeling			After administration		
	1ml	1.5ml	2ml	1ml	1.5ml	2ml
Normal group	0.58±0.24	1.25±0.39	2.17±0.31	0.63±0.33	1.33±0.50	2.33±0.36
Model group	1.13±0.25*	2.04±0.21*	3.08±0.35*	1.42±0.39*	2.17±0.25*	3.25±0.30*
Probiotics group	1.17±0.44*	2.21±0.47*	3.13±0.43*	0.96±0.52	1.79±0.50	2.63±0.38△
Wumei Pill group	1.13±0.35*	2.13±0.35*	3.08±0.24*	1.00±0.36△	1.79±0.31△	2.75±0.30△

Note: compared with normal group, *P<0.05; compared with model group, △P<0. 05

Detection of B/E value of rat intestinal flora: compared with the normal group, the B/E value of the model group was significantly lower with statistical significance (P<0.05); compared with the model group, the B/E values of all groups of administration were increased to different extents with significant difference (P<0.05), indicating that both probiotics and Wumei Pills had a role in enhancing the intestinal colonization resistance of model rats. See Table 6 for details.

TABLE 6. B/E value comparison of intestinal flora samples of all groups of rats (n=8, $\bar{x}$±s)

Group	B/E value
Normal group	1.16±0.04
Model group	1.02±0.06*
Probiotics group	1.21±0.01*△
Wumei Pill group	1.23±0.07*△

Note: compared with normal group, *P<0.05; compared with model group, △P<0. 05

The contents of Glu, γ-GABA, DA and 5-HT in the hippocampus of rats in each group: compared with the normal group, the contents of Glu, γ-GABA, DA and 5-HT in the hippocampus of model group rats were significantly increased with statistical significance difference (P<0.05). Compared with the model group, the levels of Glu, γ-GABA, DA, and 5-HT of all the administration groups were decreased in different degrees. Probiotics and Wumei Pills groups both had significant difference (P<0.05); this indicates that probiotics, Wumei Pills have a certain degree of regulation of Glu, γ-GABA, DA, and 5-HT in the hippocampus of model rats. See Table 7 for details.

TABLE 7. Comparison of Glu, γ-GABA, DA, 5-HT contents in the hippocampus of all groups of rats (n=8, $\bar{x}\pm s$)

Group	Glu pg/mL	γ-GABA pg/mL	DA pg/mL	5-HT pg/mL
Normal group	13.907±0.658	3.240±0.476	8.552±0.427	4.354±0.806
Model group	17.481±0.472*	6.169±0.918*	10.082±0.927*	6.501±0.853*
Probiotics group	15.919±0.723*△	4.845±0.672*△	8.988±0.882△	4.836±0.438△
Wumei Pill group	15.118±0.820*△	4.224±0.909*△	8.914±0.944△	4.682±0.490△

Note: compared with normal group, *P<0.05; compared with model group, △P<0.05.

DISCUSSION

Intestinal Flora, Neurotransmitters and IBS-D

IBS-D is closely related to intestinal dysbacteriosis [11]. The IBS-D patients suffer decrease of bifidobacteria and increase of enterobacteriaceae, which results in Bifidobacterium/Enterobacteriaceae values (B/E) values <1. And this value is an important index of dysbacteriosis in the intestinal flora [12]. Studies have shown that [13-16], Glu, γ-GABA, DA, 5-HT and other neurotransmitters have a certain regulatory role in the pathogenesis of IBS-D. At present, some neurotransmitter modulators have been used for the treatment of IBS-D and have achieved certain clinical efficacy [17]. Increased levels of Glu and gamma-GABA in the brain can cause abnormalities in the brain-gut axis, causing intestinal dysfunction. As an important regulatory factor, DA is involved in the regulation of gastrointestinal sensation, movement and gastrointestinal secretion and absorption, and plays an important role in the brain intestine axis. 5-HT is both a brain-gut peptide that is closely related to gastrointestinal tract activity and a neurotransmitter that is widely presented in the central nervous system and the gastrointestinal tract. There is a close relationship between the intestinal flora and neurotransmitters, which plays an important role in the pathogenesis of IBS-D.

Wumei Pill, Probiotics, and IBS-D

Wumei Pill consists of 10 medicinal herbs including ebony, asarum, dried ginger, berberine, cork, angelica, aconite, peony, cassia twig, and ginseng. The whole prescription combines acid, bitter, sweet, and acrid together to regulate organs. It opens by acrid and reduce by bitter, use both cold and hot materials to both treat and regulate. Professor Liu Duzhou believes that [18]: "Since this prescription applies both cold and hot materials to heal diarrhea and give nutrition to the body, and it also has acid of Wumei to help to stop diarrhea, ...it can treat the diarrhea caused by cold/hot mixed factors." "The key expert group of the Spleen and Stomach Center of the State Administration of Traditional Chinese Medicine used the name of the disease for IBS-D as "diarrhoea disease (Irritable Bowel Syndrome)". This kind of naming that takes modern disease name as a background and highlights the characteristics of traditional Chinese medicine has some advantages for the development of the integrated traditional Chinese and Western medicine [19]. Gastrointestinal diseases are characterized by upper heat and lower cold features [20]. IBS-D is a common disease of the digestive system. The disease has a long course of disease, has stagnation of liver Qi and stagnated heat, and is associated with the essence of spleen deficiency. There are often syndromes of mixed cold and hot, excessive and deficiency. Experimental studies have shown that [21], Wumei Pill has functions of anti-inflammatory, immune regulation, promote gastrointestinal function recovery, regulate intestinal flora, inhibit apoptosis, repair mucosal barrier and anti-oxidative damage

Probiotics are the most widely used microecological preparations in clinical practice. They are currently the main drugs for the regulation of intestinal flora. They are divided into bacterial preparations and fungal preparations based on the use of probiotic microorganisms. Common probiotics include: Bifidobacteria, acidophilus Bacillus, enterococci, Bacillus subtilis, Bacillus licheniformis, Bacillus cereus, caseoacid bacteria, Streptococcus thermophilus, Brachysporium, etc [22]. Probiotics used in our laboratory were provided by the US SWANSON Health Foods Company and included 16 probiotics such as Bifidobacterium longum, Lactobacillus acidophilus, Bifidobacterium bifidum, Bifidobacterium breve, and Bifidobacterium lactis. Probiotics help to regulate the intestinal flora of IBS-D patients and improve the efficacy of IBS-D patients [23, 24].

CONCLUSIONS

Experiments show that Wumei Pill has a certain therapeutic effect on IBS-D, and its mechanism of action may be related to the regulation of intestinal flora and neurotransmitters. Due to the existing of the two-way connection between intestinal microflora and neurotransmitters, whether the Wumei Pills affect the intestinal microflora through the regulation of neurotransmitters, or affect the neurotransmitters through the regulation of intestinal microflora and exert therapeutic effects is still to be further researched.

ACKNOWLEDGEMENTS

This work was supported by National Natural Science Foundation of China (81703983), Shandong Province Chinese Medicine Science and Technology Development Project (2017-232), Financial support for youth backbone teacher training program of Binzhou Medical University in 2017, and College Students' Innovation and Entrepreneurship Training Program of Binzhou Medical University (201710440134).

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