Int J Clin Exp Med 2019;12(7):8161-8171
www.ijcem.com /ISSN:1940-5901/IJCEM0094371

*Original Article*

# Huosha Oral Solution improves symptoms of irritable bowel syndrome with diarrhea by inhibiting activation of ERK signaling pathway and apoptosis of interstitial cells of Cajal in rats

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Received March 25, 2019; Accepted June 11, 2019; Epub July 15, 2019; Published July 30, 2019

**Abstract:** Objective: To investigate the mechanisms by which Huosha Oral Solution inhibits apoptosis of interstitial cells of Cajal (ICC) and ERK pathway activation in a rat model of irritable bowel syndrome with diarrhea (IBS-D). Methods: Sixty newborn male Sprague-Dawley (SD) rats were purchased, from which 50 rats were randomly assigned to the model group for IBS-D modeling. The model group received interventions including maternal deprivation, chronic restraint, and diarrhea induced by senna leaf gavage. The remaining 10 rats were assigned to the normal group without intervention. Rectal dilatation was performed in the model group and the normal group, and the abdominal withdrawal reflex (AWR) scores under different pressures were evaluated. The 44 successfully modeled rats were randomized into 4 groups to receive oral gavage of 10 mL/kg of either Huosha Oral Solution at different concentrations or normal saline: the high-dose group (Huosha Oral Solution, 1 g/mL), the medium-dose group (Huosha Oral Solution, 0.75 g/mL), the low-dose group (Huosha Oral Solution, 0.5 g/mL), and the model group (normal saline, 10 mL/kg); there are 11 rats in each group. The fecal water content before and after gavage was evaluated and the form of feces was scored using the Bristol Stool Scale. Colon tissue from each group of rats was harvested and frozen at -80°C. The level of the neurotransmitter 5-hydroxytryptamine (5-HT) in the colon tissue of each group was determined by ELISA. The expression level of tyrosine kinase receptor c-kit protein (c-kit) in colon mucosa from each group was determined by immunohistochemistry. The protein levels of cleaved caspase-3, ERK1, p-ERK1, MEK1 and p-MEK1 in colon tissue from each group were determined by Western blot. The apoptosis of ICC in the colon from each group was detected by flow cytometry. Results: Six rats died during the modeling process; 44 rats were successfully modeled with a final success rate of 88.00% (44/50). The AWR scores at the pressures of 20, 40, 60, and 80 mmHg in the model group were significantly higher than those of the normal group (all $P<0.05$). Compared with the normal group, the Bristol Stool Scale scores, fecal water content, colonic 5-HT levels, apoptosis rate of ICC, as well as protein expression levels of p-ERK1, p-MEK1, and cleaved caspase-3 in the colon tissues significantly increased in the medium-dose, the low-dose, and the model groups (all $P<0.05$); while the c-kit protein expression in the colon mucosa decreased (all $P<0.05$). However, no significant differences were detected between the normal group and the high-dose group with respect to those parameters (all $P<0.05$). Compared with the model group, the Bristol Stool Scale scores, fecal water content, colonic 5-HT levels, apoptosis rate of ICC, as well as protein expression levels of p-ERK1, p-MEK1, and cleaved caspase-3 in the colon tissue significantly decreased in the high-dose, the medium-dose, and the low-dose groups (all $P<0.05$); while the c-kit protein expression in the colon mucosa increased (all $P<0.05$). The higher the dose, the more significant the effect ($P<0.05$). Conclusion: Huosha Oral Solution alleviates the symptoms of irritable bowel syndrome with diarrhea; it also inhibits the apoptosis of interstitial cells of cajal (ICCs) and ERK pathway activation in the rat model.

**Keywords:** Huosha Oral Solution, model of irritable bowel syndrome with diarrhea, interstitial cell of Cajal, apoptosis, ERK pathway, activation

## Introduction

Irritable bowel syndrome (IBS) is a common chronic bowel dysfunction. The predominant symptoms of patients with IBS include abdominal pain, discomfort while defecating, and changes in bowel habits [1]. In clinical practice, the disease is mainly divided into four types:

IBS with diarrhea (IBS-D), IBS with constipation (IBS-C), mixed IBS (IBS-M), and un-subtyped IBS (IBS-U) [2]. In recent years, the incidence of the disease has been increasing, which imposes a serious negative impact on people's quality of life, as well as physical and mental health [3]. However, there are currently no medications that can treat this disease effectively. Interstitial cells of cajal (ICC) are a type of neuron-like cell that plays an important role in the enteric nervous system [4]. ICC has been shown to be significant as a bridge between intestinal smooth muscle and the enteric nervous system. The changes of function and distribution of ICCs are the pathophysiological basis for the development of many functional disorders [5]. Studies have confirmed that in IBS-D patients, the number of ICCs is significantly reduced, accompanied by greatly altered morphology and function, resulting in significantly decreased gastrointestinal rhythm and contractility. It is speculated that ICC may be involved in the pathogenesis of IBS-D [6]. In recent years, more and more studies have found that traditional Chinese medicine has remarkable effects in protecting and renovating ICCs. Many traditional Chinese medicine ingredients have been proven to inhibit the inflammation of gastric and intestinal diseases, restore ICC function, and alleviate the symptoms of functional disorders in the digestive tract [7, 8]. In addition, more and more systematic reviews and clinical studies have found that traditional Chinese medicine has certain advantages in the treatment of IBS-D [9, 10]. Huosha oral solution is a proprietary traditional Chinese medicine used in the treatment of stomach and spleen disorders and diarrhea [11]. Studies have confirmed that the multiple traditional Chinese medicine ingredients in Huosha oral solution have significant therapeutic effects such as analgesia, anti-bacteria and anti-diarrhea [12]. However, the effects of Huosha Oral Solution on IBS-D have never been studied before. In this study, we investigated the effects of Huosha oral solution on IBS-D treatment and ICC by establishing an IBS-D rat model, in an effort to elucidate the underlying mechanisms of traditional Chinese medicine for treatment of IBS-D.

## Materials and methods

### *Animals*

A total of 60 one-day-old newborn SD male rats were purchased from the Experimental Animal Center of the Academy of Military Medical Sciences. Fifty rats were randomly selected as the model group for IBS-D modeling (interventions including maternal deprivation, chronic restraint, and diarrhea induced by senna leaf gavage). The remaining 10 rats were used as the normal group (without any intervention).

### *IBS-D modeling*

The two-day-old newborn SD rats in the model group were separated from the mother rats for 3 hours every day for 20 days and then reared normally until they were weaned and separated from the mother rats on day 50. Since the 50th day, the rats were gavaged with senna leaf decoction at 20 mL/kg at 8 o'clock in the morning for 20 consecutive days. At 1 hour after gavage, the rats were fixated with the medical tape to the front and rear limbs, chest and abdomen for 3 h, so the rats could not move freely. The rats in the normal group were separated from the mother on the 50th day without any intervention. Both groups were then subjected to rectal dilation, and abdominal withdrawal reflex (AWR) under different pressures and were assessed to verify whether the modeling was successful. The wet weight and dry weight of the feces of all rats were evaluated and the fecal forms were scored using the Bristol Stool Scale. All animal experiments were approved by the Animal Experimental Ethics Committee of Xi'an Central Hospital.

### *Preparation of senna leaf decoction*

A total of 200 g senna leaves were soaked in 1 L distilled water for 30 min, then decocted for 10 min. The decoction was filtered with two layers of gauze. The concentration was calculated by subtracting the dry weight of residue from the dry weight of senna leaves, then divided by the volume of remaining liquid. Distilled water was added to bring the concentration to 0.2 kg/L.

### *Abdominal withdrawal reflex (AWR) score*

On the 70th day of age, the rats of both the model group and the normal group were subjected to rectal dilation. All rats were fasted for 18 hours before experiment. Then the rats were fixated on a wooden board after shallow anesthesia with ether. The paraffin-lubricated air balloon was slowly inserted 1 cm into the

anus and connected with the sphygmomanometer through the T stopcock. The rats were then placed in a transparent plastic box. After the rat was awakened and completely calmed, the balloon was dilated to pressure values of 20, 40, 60, and 80 mmHg, respectively. Each rectal dilation lasted for 20 s, and each pressure value was repeated for 3 times. An average AWR score at each pressure value was calculated and recorded.

AWR score upon rectal dilation was rated as follows: 0 point: the rat did not respond to rectal dilation, and the mood was basically stable; 1 point: the rat occasionally twisted its head, and the mood became unstable; 2 points: the abdominal muscles and back muscles contracted mildly; 3 points: the abdomen was lifted off the surface of the wooden board; 4 points: the abdomen was arched and the pelvis was lifted off the wooden board [13].

*Animal grouping and drug administration*

Forty-four rats with successful modeling were randomly divided into 4 groups: the high-dose group, the medium-dose group, the low-dose group and the model group, with 11 rats in each group. On the 71st day of the experiment, the rats in the high-dose group were gavaged with a volume of 10 mL/kg Huosha Oral Solution (composed of Pogostemon Cablin, amomum villosum, tangerine peel, atractylodes rhizome, Cortex Magnoliae Officinalis; prepared by the People's Hospital of Fujian Province); and the concentration of Huosha Oral Solution is 1 g/mL. The medium-dose group was given the same volume of Huosha Oral Solution at the concentration of 0.75 g/mL. The low-dose group was gavaged at the concentration of 0.5 g/mL. Rats in the model group and the normal group were gavaged with a volume of 10 mL/kg normal saline once daily for 14 consecutive days.

*Bristol Stool Scale scores*

After the last dose of Huosha Oral Solution, the feces of all groups were collected. By restraining the animal and massaging the lower abdomen, the rat discharged a fecal pellet which was immediately packed in a microcentrifuge tube. The Bristol Stool Scale was used to rate the fecal forms [14]. Fecal water content was calculated using the formula: fecal water content (%) = (fecal wet weight - fecal dry weight)/fecal wet weight * 100%.

Bristol Stool Scale scores: 1 point: feces are separate hard lumps; 2 points: feces are sausage-shaped but lumpy; 3 points: feces are like sausages but with cracks on its surface; 4 points: feces are like sausages or snakes, smooth and soft; 5 points: feces are soft blobs with clear-cut edges; 6 points: feces are fluffy pieces with ragged edges, a mushy stool; 7 points: feces are watery, no solid pieces, and completely liquid.

*Tissue harvesting*

Twenty-four hours after the last gavage, all rats were anesthetized with 10% chloral hydrate via subcutaneous injection. At 6-7 cm proximal to the anus, about 1 cm of the colon was resected and cleaned with normal saline. Half of the colon tissue was flash frozen with liquid nitrogen and stored at -80°C. The other half was fixed with 4% paraformaldehyde for 24 h, dehydrated in an automatic dehydrator, and then embedded in paraffin for later use.

*Enzyme-linked immunosorbent assay (ELISA)*

RIPA lysate containing 20% PMSF was added into 1-2 mg of colon tissue and ground for 1-2 min to make the mixture into a slurry. The mixture was transferred into a EP tube and centrifuged at 12,000 r/min, 4°C for 2 min; and the supernatant was collected to determine the 5-HT level by ELISA. The procedures were in accordance with the instruction manual of ELISA kit (Shanghai Baiwo Science & Trade Co., Ltd., China). The absorbance value of each sample at a wavelength of 450 nm was read using a microplate reader (BioTek Synergy 2, USA). A standard curve was depicted with the concentration of the reference standard as abscissa and OD value as ordinate.

*Immunohistochemistry*

Paraffin sections of the rat colon in each group were deparaffinized in xylene and dehydrated in an ethanol gradient; then treated with 3% $H_2O_2$ (Bioworld, USA) at 37°C for 30 min, and washed with PBS 4 times for 2 min each. Antigen retrieval was performed by placing slides in a pressure cooker for 2 min; then immersed in 0.01 M citrate buffer, pH 6.0, at

95°C for 20 min. Slides were placed in 3% $H_2O_2$ for 15 min to quench the exogenous peroxidase activity; then blocked in normal sheep serum (Bio-Tc Biotechnology Co., Ltd., China) for 10 min. Rabbit polyclonal c-kit antibody of 1:400 dilution was added before incubation at 4°C overnight. Tissue sections were washed with PBS twice for 5 min each time; then 1:1,000 diluted HRP conjugated goat anti-rabbit secondary antibody (Beijing Bioss Biotechnology Co., Ltd., China) was added and incubated in room temperature for 30 min. Slides were incubated with DAB (Beyotime Biotechnology Co., Ltd., China) for 3 min, and rinsed with running water for 3 min before sealing. Tissue sections were observed and photographed under an optical microscope (Bosda Optical Instrument Co., Ltd., China). Five high-power fields (200X) were randomly selected from each slide. In each field, 100 cells were counted and the number of positive cells was tallied. The slice is c-kit positive if the positive cells/total cells ratio is higher than 10% and c-kit negative if the positive cells/total cells ratio is no higher than 10%.

*Western blot*

The frozen rat colon tissues were subjected to total protein extraction with RIPA kit (Beijing Solarbio Technology Co., Ltd., China), and protein concentration was determined by BCA protein quantitative kit (Lianke Biotechnology Co., Ltd., China). Protein was loaded into the SDS-PAGE gel and separated by electrophoresis; protein was then transferred to the membranes and blocked. Rabbit anti-mouse primary antibodies were added respectively: cleaved caspase-3 (1:500, Abcam, UK), ERK1 (1:1,000, Abcam, UK), p-ERK1 (1:1,000, Abcam, UK), MEK1 (1:1,000, Abcam, UK), p-MEK1 (1:1,000, Abcam, UK) and GADPH (1:1,000, Abcam, UK). Membranes were incubated at 4°C overnight. After that, membranes were washed with TBST 3 times for 10 min each, then we added horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG secondary antibody (1:2,000, Beijing Bioss Biotechnology Co., Ltd., China) and incubated at room temperature for 2 h. Membranes were washed with TBST 3 times for 10 min each and incubated with ECL western blotting substrate (Thermo Co., Ltd., Germany) for 1 min. Photographs were taken using gel documentation system (Bio-Rad Co., Ltd., U.S.A.); all immunoblotted bands were subjected to densitometry, and the ratio of the target band optical density to the internal reference band optical density was used as the relative expression level of the protein.

*Cell culture and flow cytometry*

About 3 mm colon tissue was cut into pieces and rinsed with D-Hank's solution (Shanghai Huzhen Biotechnology Co., Ltd., China). The supernatant was discarded. After adding DMEM medium containing 0.15% collagenase, the tissue was transferred to a sterile centrifuge tube. The tube was placed on a 37°C constant temperature electromagnetic stirrer for 30 min; then centrifuged at 3,000 r/min for 5 min. The supernatant was discarded. Then DMEM low glucose medium containing 20% fetal bovine serum was added; then the tube was centrifuged at 3,000 r/min for 5 min. The cell suspension was washed with D-Hank's solution and incubated with 0.25% trypsin containing 0.02% EDTA for 5 min. The tube was centrifuged at 1,500 r/min for 5 min, and the supernatant was discarded. The cells were resuspended in DMEM medium containing 20% fetal bovine serum and inoculated into a new culture dish. After that, it was cultured for 7 days in an incubator at 37°C with 5% $CO_2$ to obtain colonic ICCs. Cell culture slides were made and washed with PBS for 2 min each time; then slides were fixed with 4% paraformaldehyde for 15 min and washed with PBS 3 times for 2 min each. Slides were permeabilized with 0.1% Triton X-100 for 15 min before being washed with PBS 3 times for 3 min each; then blocked with 3% BSA at room temperature for 30 min. Primary anti c-kit antibody was added and incubated at 4°C overnight. Then fluorescently labeled IgG secondary antibody was added and incubated for 1 h. After washing with PBS 3 times for 3 min each time, DAPI was used to stain nuclei for 2-3 min. Slides were washed again with PBS 3 times for 5 min each, then fluorescence quencher was added to the sealing liquid for sealing. For cells with good general condition after passage, apoptosis was detected using Annexin-V-FITC kit (Biovision, U.S.A.). Annexin-V-FITC/PI staining solution was made by mixing Annexin-V-FITC, PI, and HEPES buffer in a ratio of 1:2:50. A volume of 100 μL staining solution was used to resuspend $1*10^6$ cells, and the mixture was stirred and let to sit at room temperature for 15

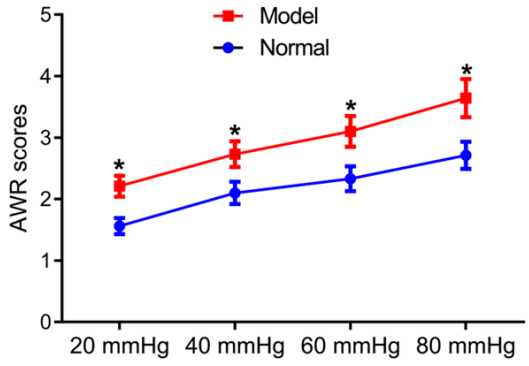


**Figure 1.** Comparison of abdominal withdrawal reflex (AWR) scores between irritable bowel syndrome with diarrhea (IBS-D) rats and normal rats under different pressures. *P<0.05 compared with the normal group.

min. Then 1 mL HEPES buffer (Procell, China) was added and stirred to mix well. FITC and PI fluorescence was excited at 488 nm; emission was detected through 525 nm and 620 nm bandpass filters to evaluate cell apoptosis.

*Statistical analysis*

The data were analyzed with SPSS 21.0 software. Quantitative data are expressed as mean ± standard deviation ($\bar{x}$ ± sd). The comparison was analyzed by one-way ANOVA, followed by Bonferroni's post-hoc comparisons. For the Bristol Stool Scale scores, the Kruskal-Wallis H test was used for comparison of all groups, followed by Wilcoxon rank sum test to compare between groups. *P* value less than 0.05 was considered statistically significant.

## Results

*IBS-D modeling*

During the modeling process, 6 rats died due to inability to adapt to the intervention and external environment. Forty-four rats were successfully modeled and the overall success rate was 88.00% (44/50). The 44 successfully modeled rats were randomly divided into 4 groups to receive a volume of 10 mL/kg oral gavage of either Huosha Oral Solution at different concentrations or normal saline: The high-dose group (Huosha Oral Solution, 1 g/mL), the medium-dose group (Huosha Oral Solution, 0.75 g/mL), the low-dose group (Huosha Oral Solution, 0.5 g/mL), and the model group (normal saline); there are 11 rats in each group.

*Comparison of AWR scores between IBS-D model rats and normal rats*

On the 70th day, the AWR scores under 20, 40, 60, and 80 mmHg pressure in the model group were significantly higher than those in the normal group (P<0.05), indicating that modeling was successful. See **Figure 1**.

*Comparison of Bristol Stool Scale scores and fecal water content*

Compared with the normal group, the Bristol Stool Scale scores and fecal water content in the medium-dose group, the low-dose group, and the model group significantly increased (all P<0.05). There were no significant differences in the Bristol Stool Scale scores and fecal water content between the normal group and the high-dose group (both P>0.05). The Bristol Stool Scale scores and fecal water content in the high-dose group, the medium-dose group, and the low dose group were significantly lower than those in the model group (all P<0.05); and the higher the dose, the more significant the difference. See **Figure 2**.

*Comparison of colonic 5-HT levels measured by ELISA*

The colonic levels of 5-HT in the medium-dose group, the low-dose group, and the model group significantly increased compared with the normal group (all P<0.05). There was no difference between the normal group and the high-dose group (P>0.05). The colonic levels of 5-HT in the high-dose group, the medium-dose group, and the low-dose group were significantly lower than those in the model group (all P<0.05); and the higher the dose, the more significant the difference. See **Figure 3**.

*Comparison of c-kit expression in colon mucosa measured by immunohistochemistry*

The c-kit protein expression in the medium-dose group, the low-dose group, and the model group significantly decreased compared with the normal group (all P<0.05). There were no significant differences between the normal group and the high-dose group (P>0.05). The c-kit protein expression in the high-dose group, the medium-dose group, and the low-dose group were significantly higher than the model group (all P<0.05); and the higher the dose, the more significant the difference. See **Figure 4**.

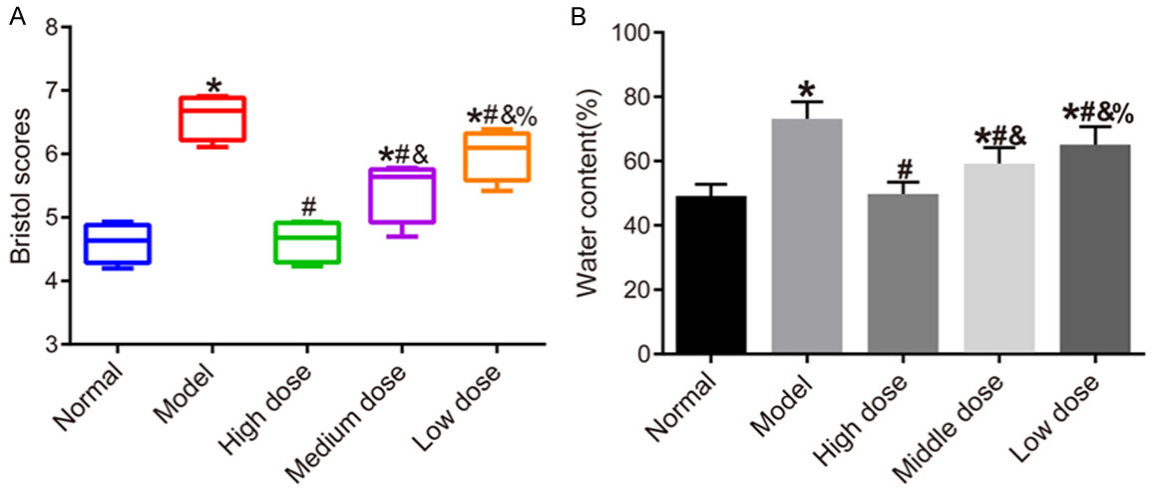


**Figure 2.** Comparison of Bristol Stool Scale scores and fecal water content of each group. A: Bristol Stool Scale scores in each group. B: Histogram of the fecal water content in each group. *P<0.05 compared with the normal group. #P<0.05 compared with the model group. &P<0.05 compared with the high-dose group. %P<0.05 compared with the medium-dose group.

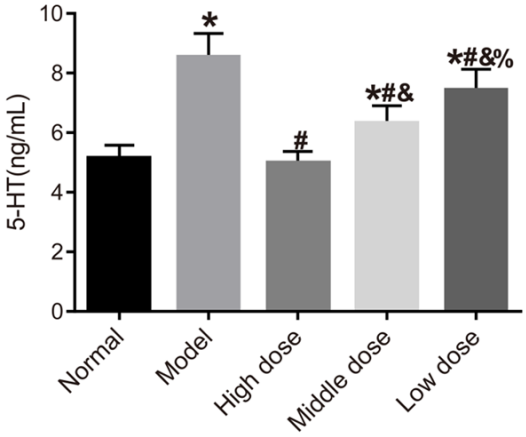


**Figure 3.** Colonic 5-HT levels in each group. *P<0.05 compared with the normal group. #P<0.05 compared with the model group. &P<0.05 compared with the high-dose group. %P<0.05 compared with the medium-dose group.

*Comparison of protein expression levels of cleaved caspase-3, p-ERK1, ERK1, p-MEK1, and MEK1 in the colon measured by Western blot*

Compared with the normal group, the colonic protein expression levels of cleaved caspase-3, p-ERK1, and p-MEK1 in the high-dose group, the medium-dose group, the low-dose group, and the model group significantly increased (all P<0.05). There were no significant differences with regard to the ERK1 and MEK1 levels between groups (all P>0.05). The levels of cleaved caspase-3, p-ERK1, and p-MEK1 in the high-dose group, the medium-dose group, and the low-dose group were significantly lower than those in the model group (all P<0.05); and the higher the dose, the more significant the difference. See **Figure 5**.

*Comparison of apoptosis of ICC in colon measured by flow cytometry*

Immunofluorescence was used to detect the expression of c-kit (ICC marker) in the cultured cells. The isolated cell bodies and processes are c-kit stained, and the cells are connected to each other to form a network structure. See **Figure 6**. The results of flow cytometry showed, the apoptosis of ICC in the medium-dose group, the low-dose group, and the model group significantly increased compared with the normal group (all P<0.05). There was no significant difference between the normal group and the high-dose group (P>0.05). The apoptosis of ICC in the high-dose group, the medium-dose group, and the low-dose group were significantly lower than the model group (all P<0.05); and the higher the dose, the more significant the effect. See **Figure 7**.

## Discussion

IBS-D is often classified as “diarrhea” and “abdominal pain” in traditional Chinese medicine [15]. Huosha Oral Solution used in this study is composed of traditional Chinese medi-

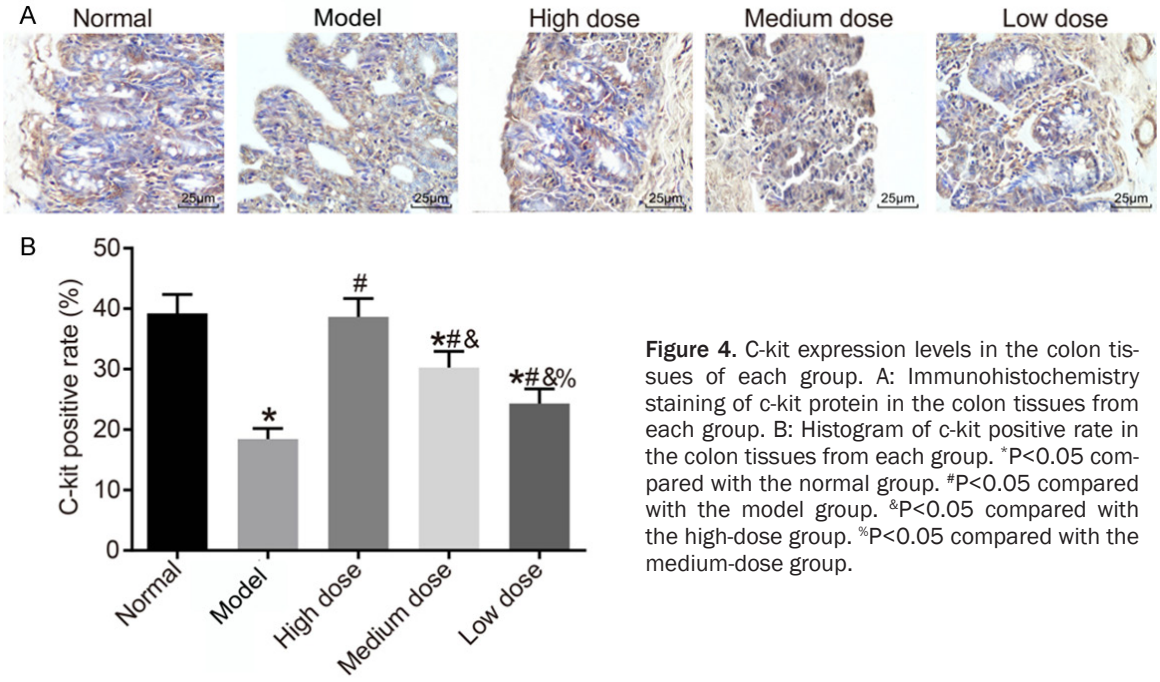


**Figure 4.** C-kit expression levels in the colon tissues of each group. A: Immunohistochemistry staining of c-kit protein in the colon tissues from each group. B: Histogram of c-kit positive rate in the colon tissues from each group. *P<0.05 compared with the normal group. #P<0.05 compared with the model group. &P<0.05 compared with the high-dose group. %P<0.05 compared with the medium-dose group.

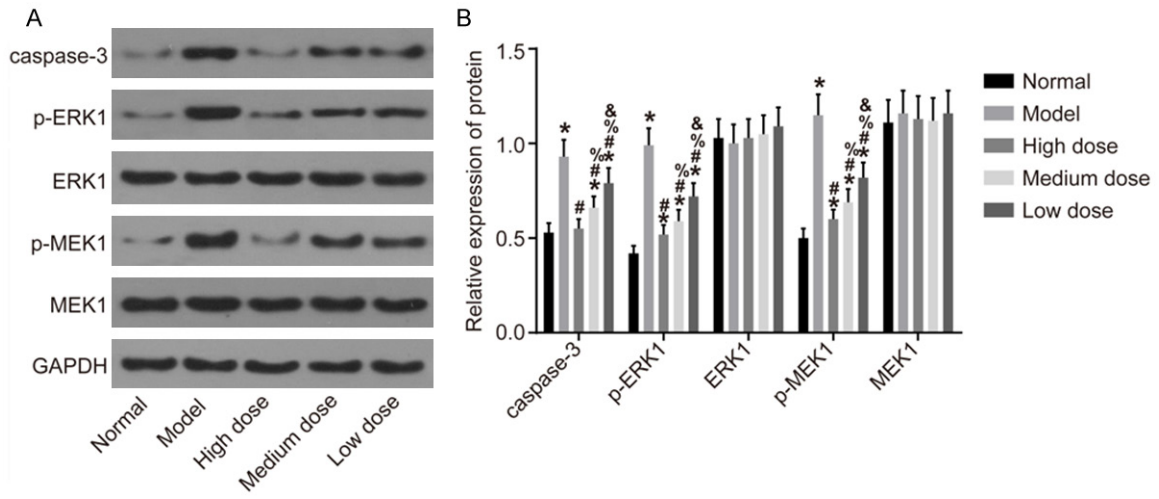


**Figure 5.** Protein expression levels in the colon tissues of each group. A: Western blot bands of each group. B: Histogram of protein expression levels from each group. *P<0.05 compared with the normal group. #P<0.05 compared with the model group. %P<0.05 compared with the high-dose group. &P<0.05 compared with the medium-dose group.

cine ingredients that have the effects of strengthening the spleen and harmonizing the stomach, as well as stopping diarrhea and expelling dampness. The ingredients Patchouli and Amomum Villosum are warm in nature; the combination of the two can alleviate the retention of dampness in the spleen and stomach. They can also activate qi and resolve turbidity [16]. In addition, dried tangerine peel, Magnolia, and Atractylodes Rhizome have the effects of improving stomach yang, resolving qi stagnation and promoting lifting function of spleen and stomach [17]. The ingredients Alismatis Rhizoma and Tuckahoe can expel dampness and reduce fluid exudation; Massa Medicata Fermentata can dredge the spleen and stomach, as well as reduce the accumulation of phlegm and food. The formulation is supplemented with licorice to reconcile all the ingredients, which improves the functions of harmo-

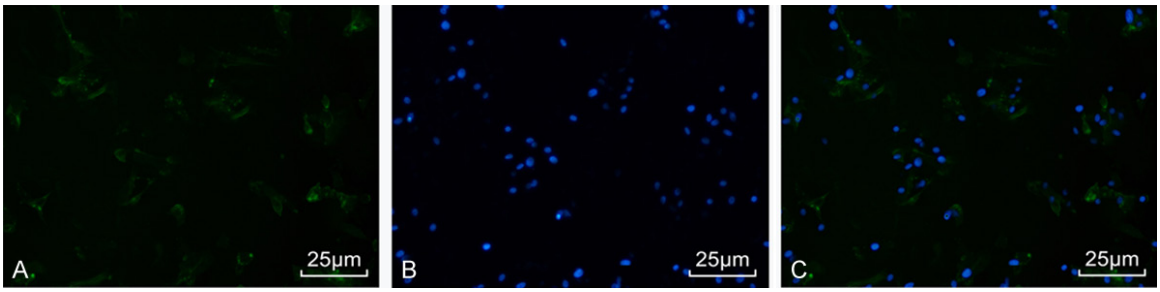


**Figure 6.** Identification of interstitial cell of Cajal. A: Immunofluorescence of c-kit protein. B: Nuclei staining with DAPI. C: Image with merged color.

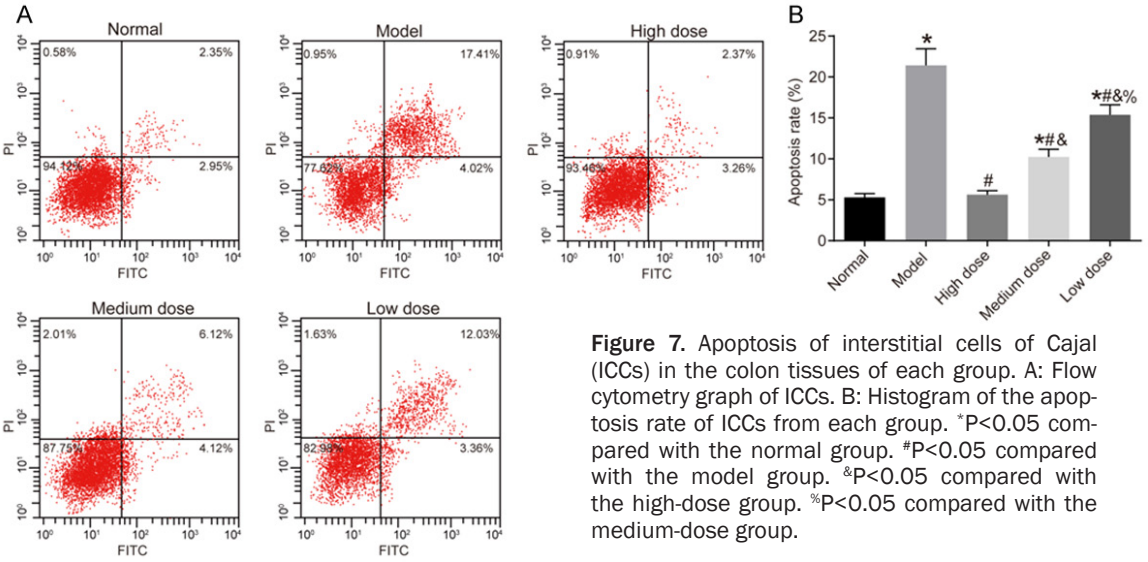


**Figure 7.** Apoptosis of interstitial cells of Cajal (ICCs) in the colon tissues of each group. A: Flow cytometry graph of ICCs. B: Histogram of the apoptosis rate of ICCs from each group. $^{*}P<0.05$ compared with the normal group. $^{\#}P<0.05$ compared with the model group. $^{\&}P<0.05$ compared with the high-dose group. $^{\%}P<0.05$ compared with the medium-dose group.

nizing the stomach, stopping diarrhea, strengthening the spleen, expelling dampness, activating qi and resolving flatulence [18]. Modern pharmacology has confirmed that many traditional Chinese medicine ingredients in Huosha Oral Solution can exert antibacterial, analgesic and antidiarrheal effects [19]. This also laid a theoretical foundation for the treatment of IBS-D with Huosha Oral Solution. In this study, we established the IBS-D rat model and found that Horsa oral solution could alleviate the symptoms of IBS-D and reduce the apoptosis of ICC.

One of the key pathophysiological mechanisms of IBS is the high sensitivity of the viscera. Visceral hypersensitivity is also considered to be an important cause of abdominal discomfort and bowel motility disorders in patients with gastrointestinal diseases [20]. In this study, we evaluated the IBS-D rat model using AWR, which is an important indicator of visceral hypersensitivity. It was found that the AWR score of the model group was significantly higher than that in the normal group. Furthermore, this study evaluated the Bristol Stool Scale scores and fecal water content of each group after modeling. The results showed that the Bristol Stool Scale scores and the fecal water content significantly decreased in IBS-D rats after treatment with Huosha Oral Solution. As a kind of monoamine neurotransmitter, 5-HT is mainly found in the human gastrointestinal and nervous systems [21]. Studies have shown that it plays an important regulatory role in a variety of physiological functions such as cognition, reproduction and gastrointestinal motility [22]. In recent years, more and more studies have demonstrated that 5-HT is closely related to symptoms such as diarrhea and abdominal pain in patients with IBS, and is a key signaling system for maintaining intestinal homeostasis [23]. Many scholars at home or abroad have confirmed the significant increase in 5-HT lev-

els in patients with IBS [24, 25]. In this study, the colonic 5-HT in each group was determined by ELISA. We found that the 5-HT levels in the model group were significantly higher than that in the normal group, while the 5-HT levels of IBS-D rats receiving the treatment of Huosha Oral Solution were significantly lower than the model group. Moreover, the degree of decline is closely related to the dose of Huosha Oral Solution, with the high-dose group having the greatest decline. It is suggested that Huosha Oral Solution can reduce the colonic level of 5-HT of IBS-D rats and improve intestinal homeostasis.

ICCs are mesenchymal cells first described by the Spanish anatomist Cajal in the enteric nervous system, which is mainly distributed in the intestinal wall [26]. Studies have revealed that it can act as an excitatory conduction cell and pacemaker cell for gastrointestinal rhythmic movements. It also plays an critical role in regulating gastrointestinal smooth muscle contraction and has also been shown to be associated with pathogenesis of many gastrointestinal functional disorders [27]. Zhu et al. found that the cell membrane of ICCs can specifically express c-kit protein [28]. As a proto-oncogene, the c-kit has been shown to bind to its ligands to regulate ICC growth and differentiation [29]. Zhao et al. confirmed that the decrease in c-kit expression can promote the destruction of ICC structure, which is also an important pathological basis for the onset of IBS [30]. Studies in patients with IBS have found there is a significant reduction in the number of ICCs and a great change in the morphology in patients' colons [31]. Wang et al. also found that the number and the branching of ICC in the colon wall of IBS patients decreased, and lipid deposition increased [32]. In this study, c-kit was significantly increased in the intestinal mucosa, and the apoptosis rate of ICCs was significantly reduced after administration of Huosha Oral Solution; the higher the dose, the more obvious the effects. The results provided further support that Huosha Oral Solution has remarkable therapeutic effects in IBS patients.

The ERK signaling pathway has been shown to be associated with the pathogenesis of IBS. O'Malley found that IBS patients are accompanied by activation of ERK signaling pathways and dysfunction of the immune system [33]. Li et al. also demonstrated that the visceral sensitivity of IBS rats was significantly enhanced and the phenotype of intestinal dendritic cells was remarkably altered, which may be related to the activation of ERK signaling pathway [34]. To further explore the possible mechanism of Huosha Oral Solution affecting ICC changes in IBS-D rats, we examined candidate pathways associated with the pathogenesis of IBS. The results showed that the expression of p-ERK1 and p-MEK1 was significantly up-regulated in the IBS-D model rats, suggesting that the ERK pathway was activated. When the Huosha Oral Solution was administered to IBS-D rats, the ERK pathway was inhibited, and the expression of the apoptosis-promoting factor cleaved caspase-3 was also down-regulated, which further demonstrates that Huosha Oral Solution can treat IBS-D by mediating ERK signaling pathway. In this study, we established an IBS-D rat model to prove that Huosha Oral Solution can regulate ERK signaling pathway and ICC proliferation. One limitation of this study was the failure to conduct a series of separate experiments on each component of Huosha Oral Solution and thus failed to analyze the active ingredients qualitatively and quantitatively.

In conclusion, Huosha Oral Solution can improve the symptoms of irritable bowel syndrome with diarrhea by inhibiting activation of the ERK signaling pathway and apoptosis of ICCs. Huosha Oral Solution has a significant therapeutic effect on IBS-D.

## Disclosure of conflict of interest

None.

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