Mechanism of Action of Tongxieyaofang Ultrafine Granular Powder in Treating Visceral Hypersensitivity in Rats with Diarrhea-Predominant Irritable Bowel Syndrome: A Focus on Enteric Glial Cells

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Abstract [**Objectives**] To explore the mechanism of action of Tongxieyaofang ultrafine granular powder in treating visceral hypersensitivity in rats with diarrhea-predominant irritable bowel syndrome (IBS-D) based on enteric glial cells (EGCs). [**Methods**] Eighty-four healthy male Wistar rats of SPF grade were selected and randomly assigned to seven groups, each comprising 12 rats: a normal control group, a model control group, a traditional Tongxieyaofang granular powder group (4.060 g/kg), three Tongxieyaofang ultrafine granular powder groups at low, medium, and high doses (1.015, 2.030, and 4.060 g/kg of raw drug, respectively), and a pinaverium bromide group (0.018 g/kg). With the exception of the normal control group, all other groups were subjected to an IBS-D visceral hypersensitivity sensitivity model in rats developed by the chronic water avoidance stress method. Three days post modeling, the rats received continuous oral gavage administration for 8 d. Following the treatment period, serum and colon tissue samples were collected from each group. The BDNF level in the serum was quantified using ELISA. Additionally, the protein expression levels of GFAP, BDNF, and TrkB in colon tissues were assessed via Western blot assay. [**Results**] Compared to the normal control group, the serum BDNF levels in the model control group were significantly elevated ($P<0.01$). In contrast, each treatment group exhibited a significant reduction in serum BDNF levels relative to the model control group ($P<0.01$). Furthermore, the protein expression levels of GFAP, BDNF, and TrkB in colon tissue were significantly higher in the model control group compared to the normal control group ($P<0.05$, $P<0.01$). Conversely, these protein expressions were significantly decreased in each treatment group compared to the model control group ($P<0.05$, $P<0.01$). [**Conclusions**] Tongxieyaofang ultrafine granular powder effectively alleviates visceral sensitivity in IBS-D rats and inhibits the activation of EGCs, speculating that its mechanism of action involves the suppression of abnormal EGC activation.

Key words Diarrhea-predominant irritable bowel syndrome (IBS-D), Tongxieyaofang ultrafine granular powder, Enteric glial cells (EGCs), Visceral hypersensitivity

1 Introduction

Irritable bowel syndrome (IBS) is a prevalent functional gastrointestinal disorder characterized primarily by abdominal discomfort, recurrent abdominal pain, and alterations in bowel habits. Additionally, patients frequently exhibit psychological symptoms, including anxiety and depression[1]. The progression of IBS is chronic and characterized by frequent recurrences, which substantially diminish patients' quality of work and life. The prevalence of IBS in China has been rising annually. Notably, in South China, the prevalence of IBS is 5.9%, with diarrhea-predominant irritable bowel syndrome (IBS-D) being the most prevalent, constituting approximately 47.1% of IBS cases[2]. The pathogenesis of IBS-D is complex. Notably, chronic visceral hypersensitivity is a key factor contributing to the occurrence of abdominal pain symptoms in IBS and represents its primary pathophysiological characteristic[3-4]. Studies have demonstrated that enteric glial cells (EGCs) are significantly associated with the development of visceral hypersensitivity[5]. Brain-derived neurotrophic factor (BDNF) and its receptor, the tyrosine kinase receptor B (TrkB), play essential roles in modulating pain perception. The BDNF/TrkB signaling pathway constitutes a critical mechanism underlying the pathogenesis of visceral hypersensitivity[6].

Tongxieyaofang was initially documented in *Danxi Xinfa* and is recognized as a classical prescription for addressing diarrhea caused by liver depression and spleen deficiency. It exhibits distinct therapeutic properties and advantages in the treatment of IBS-D, demonstrating significant clinical efficacy, a low recurrence rate, and minimal toxicity and side effects for patients[7-9]. Ultrafine granular powder is a novel form of traditional Chinese medicine (TCM) granular powder, characterized by advantages such as portability, rapid dissolution, reduced dosage requirements, and uniform stability[10]. Previous investigations conducted by our research team have demonstrated that the therapeutic efficacy of Tongxieyaofang ultrafine granular powder is comparable to that of conventional granular powder[11-13]. The present study aims to establish an IBS-D visceral hypersensitivity sensitivity model in rats developed by the chronic water avoidance stress method and to evaluate the effects of Tongxieyaofang ultrafine granular powder on visceral hypersensitivity in this model.

2 Materials and methods

2.1 Experimental animals Eighty-four healthy male Wistar rats of SPF grade, weighing between 180 and 220 g, were obtained from Jinan Pengyue Laboratory Animal Breeding Co., Ltd., Shandong Province. The animals were accompanied by the quality certificate No.: 3700920005166 and the production license No.:

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SYXK (L) 20140007. They were housed in the barrier-level laboratory of Zhongshan Hospital of Traditional Chinese Medicine under the usage license No.: SYXK (Y) 2015-0109. The indoor temperature was maintained at 20 – 24 ℃, with relative humidity ranging from 40% to 70%. The animal experiments conducted in this study were approved by the Laboratory Animal Committee of Zhongshan Hospital of Traditional Chinese Medicine, under ethical approval No.: AEWC-2018031.

2.2 Drugs and reagents Tongxieyaofang consists of four traditional Chinese medicinal ingredients: Atractylodis Macrocephalae Rhizoma, Paeoniae Radix Alba, Pericarpium Citri Reticulatae, and Radix Saposhnikoviae, combined at a ratio of 3∶2∶1.5∶1. Tongxieyaofang ultrafine granular powder was supplied by Zhongshan Zhongzhi Pharmaceutical Co., Ltd., batch No.: 20160130. All TCM granular powder used in Tongxieyaofang were procured from Nanjing Tongrentang Pharmacy. The components Atractylodis Macrocephalae Rhizoma (batch No.: 20140301), Paeoniae Radix Alba (batch No.: 20140301), Pericarpium Citri Reticulatae (batch No.: 20140301), and Radix Saposhnikoviae (batch No.: 20140201) were evaluated and deemed qualified by Chief Pharmacist Zeng Congyan of the Drug Inspection Department at Zhongshan Hospital of Traditional Chinese Medicine. Pinaverium bromide (batch No.: 642647) was manufactured by Abbott Healthcare SAS, France. The BDNF ELISA kit (batch No.: 05/2017) was produced by Shanghai Enzyme-Linked Biotechnology Co., Ltd. The primary antibodies used included BDNF (0073570202, ABclonal), TrkB (GR305833-3, Abcam), GAPDH (2118S, CST), and GFAP (3670S, CST).

2.3 Instrument and equipment High-speed refrigerated centrifuge (German Eppendorf, 5910Ri); Ultra-pure water machine (Shanghai Chenxi Instrument Co., Ltd., UP PLUS-20); Vertical electrophoresis transfer system (Bio-RAD, USA, 1658033); Chemiluminescence imaging system (Bio-RAD, ChemiDocMP, USA); Multifunctional microplate reader (PerkinElmer, USA, HH3500).

2.4 Animal modeling and drug administration methods Following procurement, the rats were acclimated in the laboratory for 7 d and subsequently randomized into seven groups, each consisting of 12 rats: a normal control group, a model control group, a group receiving traditional Tongxieyaofang granular powder, three groups administered Tongxieyaofang ultrafine granular powder at low, medium, and high doses, respectively, and a pinaverium bromide group. The IBS-D visceral sensitivity model was developed in rats utilizing the chronic water avoidance stress method[14]. A custom-made box measuring 45 cm × 25 cm × 25 cm was constructed, with a small platform measuring 8 cm × 8 cm × 10 cm positioned at its center. Water at 25 ℃ was added to the box until the water level was 1 cm below the small platform. No water was added to the normal control group. The water avoidance test was conducted for 1 h at a fixed time each day over a period of consecutive 10 d. Visceral sensitivity was assessed using the abdominal wall withdrawal reflex test, and the IBS-D visceral hypersensitivity model in rats was successfully established[15].

The traditional Tongxieyaofang granular powder was prepared to a maximum final raw dosage of 4.060 g/kg. The Tongxieyaofang ultrafine granular powder was dissolved in hot water. Experimental groups were established based on dosage levels corresponding to one-quarter, one-half, and the full clinical dose, with raw dosages of 1.015, 2.030, and 4.060 g/kg[16-17], respectively. The pinaverium bromide group received a dosage of 0.018 g/kg. All rats received the appropriate dose of the drug via gavage on the 3rd day of the modeling process. The normal control group and the model control group were administered distilled water by gavage for consecutive 7 d.

2.5 Specimen collection Following the completion of the final modeling procedure, the rats were anesthetized with pentobarbital sodium at a dosage of 40 mg/kg, after which a laparotomy was performed. Blood samples were obtained from the abdominal aorta. The collected blood was allowed to stand at 4 ℃ for 2 h, then centrifuged at 3 500 rpm for 15 min. The resulting supernatant was stored at – 80 ℃ for subsequent analysis. Thereafter, the colon tissue was excised, thoroughly rinsed with normal saline to remove contents, and stored at – 80 ℃ for future use.

2.6 Main measurement indicators

2.6.1 Determination of BDNF level in rat serum via ELISA. The prepared serum stored at – 80 ℃ was analyzed strictly following the methods and procedures outlined in the ELISA kit manual. The optical density (OD) value of the standard sample was measured, the linear regression equation of the standard curve was calculated, and the BDNF level in the sample was determined based on its OD value.

2.6.2 Determination of expression levels of GFAP, BDNF and TrkB proteins in the colon tissue of rats via Western blot assay. 50 mg of colon tissue were excised and added to RIPA lysis buffer. The tissue was homogenized twice at 4 ℃ for 90 sec each time, followed by incubation on ice for 30 min. Subsequently, the sample was centrifuged at 12 000 rpm, 4 ℃ for 15 min. The supernatant was collected, and protein concentration was determined using the BCA assay. The samples were prepared at a consistent concentration and mixed with the protein loading buffer at a 4∶1 ratio, followed by denaturation at 97 ℃ for 10 min. 50 μg of protein samples were loaded and subjected to SDS-PAGE under constant pressure. Following electrophoresis, the proteins were transferred onto a PVDF membrane and blocked with a rapid blocking solution for 15 min. After blocking, the membrane was incubated with primary antibodies against GFAP (1∶1 000), BDNF (1∶1 000), TrkB (1∶1 000), and GAPDH (1∶1 000) overnight at 4 ℃. The following day, the membrane was washed three times with PBST for 5 min each. Subsequently, fluorescently labeled secondary antibodies—rabbit (1∶10 000) and mouse (1∶10 000)—were applied and incubated at room temperature for 1 h, followed by three additional washes with PBST for 5 min each. The ECL exposure solution (solution A∶solution B = 1∶1) was thoroughly absorbed and mixed. The bands were then immersed in the exposure solution and developed using a developer.

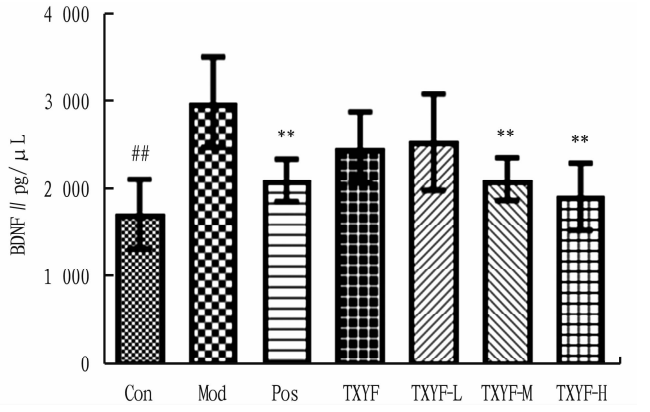
2.7 Statistical processing methods Statistical analyses were conducted using SPSS 23.0. All data are presented as mean ± standard deviation ($\bar{x} \pm s$). One-way analysis of variance (ANOVA) was employed when the assumption of homogeneity of variance was satisfied; otherwise, non-parametric tests were applied. Independent *t*-tests were utilized for pairwise comparisons between

groups. A P-value less than 0.05 was considered indicative of statistical significance.

3 Results and analysis

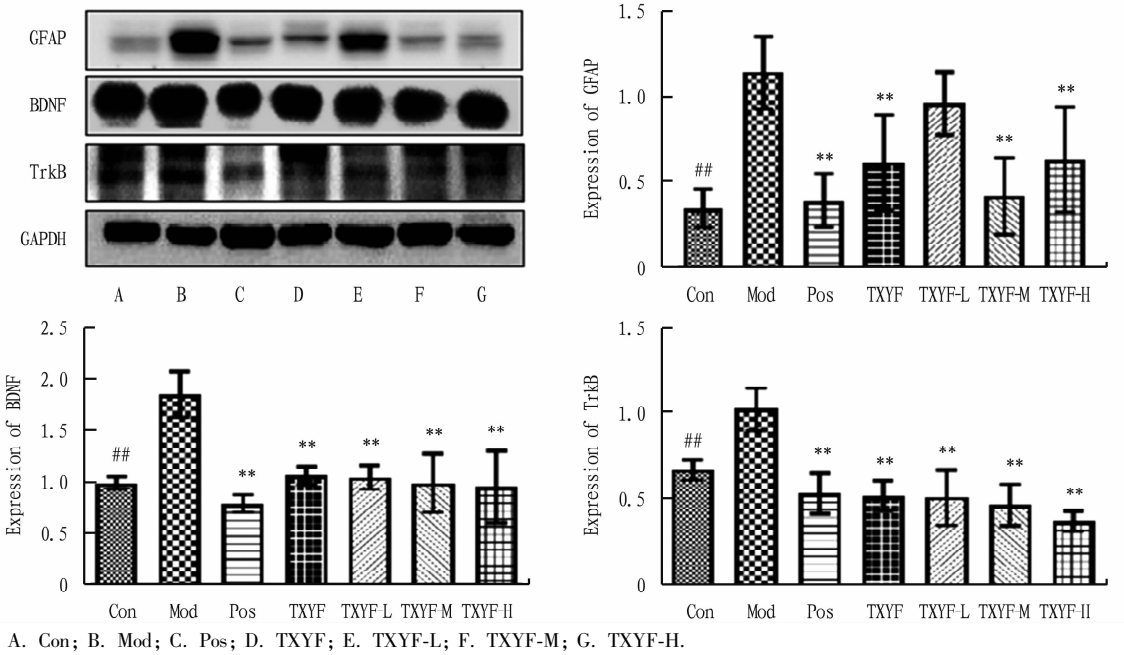
3.1 Effect on BDNF level in the serum of IBS-D rats with visceral hypersensitivity Compared to the normal control group, the serum BDNF levels of rats in the model control group were significantly elevated ($P<0.01$). Following drug intervention, the pinaverium bromide group and the medium-dose and high-dose groups treated with Tongxieyaofang ultrafine granular powder exhibited a significant reduction in serum BDNF levels compared to the model control group ($P<0.01$). Although the traditional Tongxieyaofang granular powder group and the low-dose Tongxieyaofang ultrafine granular powder group demonstrated a decreasing trend in BDNF levels, these changes were not statistically significant (Fig. 1).

3.2 Effect on expression levels of GFAP, BDNF and TrkB proteins in the colon tissue of IBS-D rats Compared to the normal control group, the expression levels of GFAP, BDNF, and TrkB proteins in the colon tissue of rats in the model control group were significantly elevated ($P<0.01$). Following drug intervention, the expression levels of GFAP, BDNF, and TrkB proteins in all treatment groups were significantly reduced compared to the model control group ($P<0.01$, Fig. 2).



NOTE Con. Normal control group; Mod. Model control group; Pos. Positive group; TXYF. Traditional Tongxieyaofang granular powder group; TXYF-L. Low-dose Tongxieyaofang ultrafine granular powder; TXYF-M. Medium-dose Tongxieyaofang ultrafine granular powder; TXYF-H. High-dose Tongxieyaofang ultrafine granular powder. Compared to the normal control group, $^{\#}P<0.05$, $^{\#\#}P<0.01$; compared to the model control group, $^{*}P<0.05$, $^{**}P<0.01$.

Fig. 1 Effect of Tongxieyaofang ultrafine granular powder on BDNF levels in the serum of IBS-D rats with visceral hypersensitivity



NOTE A. Con; B. Mod; C. Pos; D. TXYF; E. TXYF-L; F. TXYF-M; G. TXYF-H.

Fig. 2 Effect of Tongxieyaofang ultrafine granular powder on expression levels of GFAP, BDNF and TrkB proteins in the colon tissue of IBS-D rats

4 Discussion

IBS-D is the most prevalent subtype of IBS, characterized by a complex pathogenesis that encompasses multiple factors, including dysbiosis of the intestinal microbiota, abnormal gastrointestinal motility, visceral hypersensitivity, and psychosocial influences. Among these, visceral hypersensitivity is regarded as a central mechanism underlying the manifestation of abdominal pain symptoms in IBS-D. Visceral hypersensitivity in IBS-D involves the enteric, central, and peripheral nervous systems and results from complex, multi-level, and multidimensional interactions among these neural systems[18-19]. The enteric nervous system (ENS) consists of intestinal neurons and EGCs. The activation and proliferation of EGCs are closely associated with visceral hypersensitivity[20]. Changes in the intestinal environment can induce abnormal

activation of EGCs, resulting in the upregulation of the EGC marker GFAP. This upregulation promotes the release of pain-related factors, including SP, CGRP, and TRPV1, thereby triggering hypersensitivity responses in internal organs[21-22]. BDNF, a member of the neurotrophic factor family, plays a crucial role in pain regulation. EGCs are capable of synthesizing and secreting BDNF to support the normal functioning of sensory nerves. However, abnormal elevations in BDNF levels can result in heightened excitability of nociceptive neurons, leading to abdominal pain[23]. Studies have demonstrated that the expression of BDNF is elevated in the intestinal mucosa of patients with IBS-D. Additionally, intraperitoneal administration of BDNF has been shown to reduce the sensory threshold to colonic distension in healthy rats. An abnormal increase of BDNF within the local intestinal microenvironment may lead to excessive activation of its receptor, TrkB, thereby inducing neuronal hyperexcitability, amplified pain signaling, and visceral hypersensitivity[24-25]. Studies have demonstrated the presence of TrkB receptors on the cell membranes of EGCs. An abnormal increase in BDNF levels within the intestinal microenvironment can result in excessive activation of TrkB receptors on EGC surfaces, subsequently triggering the release of various pain factors and exacerbating visceral hypersensitivity responses[26]. Consequently, the EGCs/BDNF/TrkB signaling pathway constitutes a critical mechanism underlying the development of visceral hypersensitivity in IBS-D.

IBS-D is categorized under "abdominal pain" and "diarrhea" in TCM, with the liver depression and spleen deficiency pattern being the most prevalent. In recent years, TCM has demonstrated significant therapeutic efficacy in treating IBS-D, particularly with the use of Tongxieyaofang, which has shown notable effectiveness in alleviating abdominal pain and diarrhea associated with the liver depression and spleen deficiency pattern[7-8]. Among the components of Tongxieyaofang, Atractylodis Macrocephalae Rhizoma tonifies the spleen and harmonizes the middle energizer; Paeoniae Radix Alba calms the liver and alleviates pain; Pericarpium Citri Reticulatae promotes qi circulation and invigorates the spleen; and Radix Saposhnikoviae dispels dampness and relieves pain. The combination of these four herbs exerts synergistic effects, including tonification of the spleen, soothing of the liver, elimination of dampness, and cessation of diarrhea. This study examined the peripheral blood and colon tissues of rats using ELISA, Western blot, and other analytical techniques. ELISA results demonstrated a significant increase in BDNF levels in the peripheral blood of rats with IBS-D. Additionally, expression levels of GFAP, BDNF, and TrkB proteins were markedly elevated in the colon tissues of these rats. These findings suggest that the increased GFAP in the intestinal tract of IBS-D rats activates EGCs, which in turn promote BDNF synthesis. The abnormal elevation of BDNF subsequently activates TrkB receptors on the surface of EGCs, leading to the sustained release of pain factors and exacerbation of abdominal pain symptoms. Tongxieyaofang ultrafine granular powder significantly reduces the levels of BDNF in the peripheral blood of IBS-D rats and inhibits expression levels of GFAP, BDNF, and TrkB proteins in colon tissue. It is hypothesized that Tongxieyaofang not only decreases BDNF levels in IBS-D rats but also acts on colon tissue to reduce GFAP expression, thereby preventing the excessive activation of intestinal EGCs. This inhibition may suppress the abnormal release of the intestinal pain mediator BDNF and mitigate the overactivity of its receptor TrkB, ultimately alleviating abdominal pain and reducing visceral hypersensitivity in IBS-D rats.

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a 1 ∶ 1 ratio was significantly superior to that observed with the individual application of traditional Chinese medicine. However, this effect was found to be less potent when compared to the positive control group. Additionally, the acetic acid writhing test demonstrated that the frequency of writhing responses in the *O. fragrans* group was significantly lower than that in both the normal saline group and the compatibility group. The experimental results indicate that *B. malabaricum* flowers and *O. fragrans*, as well as their compatibility, exhibits synergistic effects, with the analgesic effect of this compatibility significantly differing from that observed with the individual application of each medicine. This study primarily explored the effects of *B. malabaricum* flowers and *O. fragrans*, as well as their compatibility, on analgesic outcomes in mice, but the underlying mechanisms of action warrant further investigation.

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