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Original Article

Xifeng Huashi Formula presents therapeutic effect on diarrhea predominant irritable bowel syndrome through regulating brain-gut axis and SCF/C-KIT signaling pathway

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Abstract: Diarrhea predominant irritable bowel syndrome (D-IBS) is a chronic functional gastrointestinal disease, which correlated with abnormal brain-gut axis, visceral hypersensitivity, unstable psychology and abnormal motility. In recent years, it was demonstrated that interstitial cells of Cajal (ICC) are involved in the pathogenesis of D-IBS including gastrointestinal hormone, visceral hypersensitivity, gastrointestinal motility and 5-HT system. Therapeutic effect of Xifeng Huashi Formula (XHF) on D-IBS was demonstrated. However, the mechanism remains unclear. In the present study, D-IBS rats induced by acetic acid were observed for behavior change. Change of ICC in colon was detected by electron microscope. Besides, the expression levels of brain-gut axis related proteins neuropeptide Y1 (NYP) and vasoactive intestinal peptide (VIP) in colon and hypothalamus were measured using Western blot and real-time PCR, respectively. In addition, concentration of SCF/C-KIT signaling pathway related proteins stem cell factor (SCF) and tyrosine kinase receptor (C-KIT) were also evaluated. We explored the mechanism of XHF through brain-gut axis and SCF/C-KIT signaling pathway so as to provide experimental basis for rational drug use and novel therapeutic method against D-IBS in clinical.

Keywords: Diarrhea predominant irritable bowel syndrome, Rou-gan Xi-feng decoction, brain-gut axis, SCF-C-KIT signaling pathway, interstitial cells of Cajal

Introduction

Irritable bowel syndrome (IBS) is a chronic and functional gastrointestinal disease, among which diarrhea-predominant Irritable bowel syndrome (D-IBS) is the most common in clinical. There is a tendency to increase in the morbidity of IBS due to the development of the society, the accelerating rhythm of life and changed diet structure [1]. Although serious illness or death caused by IBS has not been identified, negative effects on patients' life quality and social communication exist. Besides, IBS also consume large amounts of health care resources directly or indirectly. The etiology and pathogenesis of IBS remains unclear at present. So far the pathogenesis of IBS was associated with brain-gut axis anomalies, high visceral sensitivity abnormal, psychological factors, abnormal motility and complete intestinal infection. In recent years, the role of interstitial cells of Cajal (ICC) in IBS has aroused concern. Ahad Eshraghian *et al.* assumed that ICC associated with internal high sensitivity, gastrointestinal hormone, 5-HT receptor and gastrointestinal peristalsis might involve in the pathogenesis of IBS [2]. Some clinical research and system evaluation indicated that traditional Chinese medicine harbors characteristic and the superiority with a good prospect in the treatment of D-IBS [3, 4]. It has been a hot topic and ultimate goal in the study of IBS to develop a safe and effective therapeutic method. Thus, in the present study, we aimed to search an effective and safe drug from traditional Chinese medicine and explore its possible mechanisms based on previous studies.

Presently, treatments against D-IBS mainly include drug therapy and psychological behavioral therapy. Drugs for the regulation of intestinal movement and visceral sensation, CCK receptor antagonist and antidiarrheal are usually applied as drug therapy. However, above therapeutics receive unsatisfactory curative effect due to the one or two blocked pathological link by single drug. Besides, the high recurrence rate becomes the main bottleneck in the treatment of D-IBS. Nevertheless, the overall adjustment therapy using traditional Chinese medicine achieves multiple targets and links with low recurrence rate and side effects, suggesting the advantages of Chinese medicine as a promising agent against D-IBS. In the previous study, the curative effect of traditional Chinese medicine Rougan Xifeng Decoction on the D-IBS mouse was demonstrated. Rougan Xifeng Decoction downregulated the IL-8 and 5-HT levels and improved the defecation patterns. Subsequently, we originated Xifeng Huashi Formula (XHF) and presented favorable curative effect on D-IBS patients in clinical. However, the mechanism of XHF remains unclear, which need further exploration. Thus, in the present study, we focused on the ICC to investigate the brain-gut axis and visceral hypersensitivity. We hoped to provide a promising agent and theoretical foundation for the clinic treatment of D-IBS.

Materials and methods

Modeling

Totally, 66 male Wistra rats were purchased from Shanghai Super-B&K laboratory animal Corp. Ltd. Rats were adaptively fed for 2 weeks after the birth. D-IBS modeling rats were treated with continuous clysters for 2 weeks. Silicone tube connected with syringe was inserted into the anus, then 0.01 mol acetic acid were injected into colon through the tube. The silicone tube was slowly pulled out, followed with the oppression on anus for 15 s. Then rats were kept in cages with free access to standard food and water.

LC-MS analysis of Rougan Xifeng formula extract

The analysis of RXF was conducted using a high-performance liquid chromatography coupled with electrospray mass spectrometry system (HPLC/ESI-MS, Agilent 1100 HPLC system, Agilent Technologies, MA, USA). A chromatographic column (GS-120-5-C18-BIO, 5 μm, 250 × 4.6 mm i.d., 35°C) was employed to perform the separation. Acetonitrile as linear gradient elution B was used from 5% at 0 min to 40% at 60 min (v/v) with linear gradient elution A (0.1% formic acid water). The target wavelength was set at 210 nm simultaneously with the DAD switched on. The ratio of split and mass spectrometer was adjusted at 1:3 accompanied with 10 mL of injection volume and 1.0 mL/min of flow rate. The negative ion mode was set as followed: ultra high-purity helium (He) as collision gas, 35 psi of N_2 as nebulizer gas, 10 L/min of N_2 as drying gas, drying at 350°C, 3500 V. Mass scanned from 100-2200 m/z, target mass at 500 m/z with 100% compound stability and trap drive level.

Grouping and drug delivery

Rats were randomly and averagely allocated into 5 groups including normal group, D-IBS group, D-IBS with low dose medicine (3 g/kg), D-IBS with high dose medicine (9 g/kg) and D-IBS with PB (0.018 g/kg). Normal and D-IBS groups were delivered with distilled water. Distilled water and drugs were delivered through intragastric administration with the volume at 10 mL/kg for 2 weeks continually.

Morphologic observation

The growth rate of body mass and loss stools rate were recorded and calculated during modeling period. The growth rate of body mass was calculated as (recorded weight - initial weight)/initial weight %. Loss stools rate was calculated as loss stools/total stools %.

Abdominal withdrawal reflex

Abdominal withdrawal reflex was evaluated after modeling. Rats were kept with free access to water only for 24 h. After the anesthesia with 30 g/L of sodium pentobarbital through intraperitoneal injection, rats were inserted with 8F catheter anal connected with an air pump. Each rat was given a 5-min expansion for 3 times (1.0, 1.5 and 2.0 mL, respectively) with the interval for 30 s. Evaluation of abdominal withdrawal reflex was classified into 5 degrees as followed: stable mood of rat during stimulation (0 point); unstable mood with occasionally

twisted head (1 point); minor contractions of the dorsal muscle (2 points); forcibly contractions of the dorsal muscle with the lifted abdomen from ground (3 points); strong contractions of the abdominal muscle (4 points).

Electric analysis

Rats were kept with free access to water only for 24 h after the last delivery. Rats were inserted with an 8F catheter anal after the anesthesia with 30 g/L of sodium pentobarbital through intraperitoneal injection. A silver bipolar electrode was inserted to the inguinal ligament. After the adaptation for 30 min, each rat was given a 5-min expansion for 3 times (1.0, 1.5 and 2.0 mL, respectively) with the interval for 30 s. The contraction frequency of external oblique muscle within 5 min was recorded. The muscle activity increased 100 μV more than the base line was identified as a meaningful abdominal contraction.

Histological examination

After the modeling, every 3 rats were randomly selected from D-IBS and normal groups. Rats were sacrificed and tissues were isolated from transverse colon, descending colon and rectum, respectively. Tissues were fixed with 40 g/L formaldehyde solution and stained with wood grain-eosin. The pathological histology change of intestinal mucosa was observed through a microscope. Additionally, tissues were fixed with 4% paraformaldehyde, embedded with paraffin and cut into slices for HE staining. Morphometric analysis was performed by optical microscope (LEICA DMLB, Germany).

Western blot

Tissue samples were cut into pieces of 3 mm × 3 mm and lysed with 1 mL of pre-cold Lysis Buffer containing 10 μL phosphatase inhibitor, 1 μL protease inhibitor and 5 μL 100 mM PMSF. Tissue homogenate was centrifuged at 1000 rpm for 5 min. The supernatant containing overall protein was collected. Protein was quantified using Bradford method. Then each 15 μL sample were run on 5% SDS-PAGE gel and blots were electrophoretically transferred to a nitrocellulose filter membrane (Millipore, Shanghai, China). The membrane was blocked with 5% skim milk at room temperature for 2 h and incubated overnight with antibodies against neuropeptide Y1 (NPY, Abcam), vasoactive intestinal peptide (VIP, Abcam), proteins stem cell factor (SCF, Abcam), tyrosine kinase receptor (C-KIT, Abcam) and GAPDH (CST), respectively. After the incubation with goat anti-mouse or anti-rabbit secondary antibodies (Beyotime, Shanghai, China) for 2 h, blots were detected using Enhanced chemiluminescence (ECL, Thermo Scientific, Shanghai, China) and observed visually. Images were generated using G: BOX chemiXR5.

Real-time PCR

Trizol reagent (Invitrogen, USA) was used to extract total RNA from grinded colon and hypothalamus tissues. Reverse transcription was conducted using First strand synthesis of cDNA kit (Thermo, USA) and real-time PCR analysis was performed using SYBR Green Real-time PCR Master Mix (TOYOBO, Japan) according to the standard protocol.

Statistical analysis

Data were expressed as mean ± standard deviation and analyzed using *t*-test. GraphPad Prism 5.0 software was used to perform and analyze the data. Real-time PCR data was analyzed using ABI Prism 7300 SDS Software. $P<0.05$ was considered as statistic difference. All the data were analysis by Chemstation software.

Results

The identification of nine compounds in XHF

The mixed Chinese medicinal materials in XHF separated by HPLC/ESI-MS in positive and negative-ion mode were shown in **Figure 1**. Nine compounds 1-9, with the retention time at 0.77 min, 2.05 min, 2.84 min, 4.50 min, 3.22 min, 3.54 min, 3.59 min, 4.03 min, and 7.39 min, were identified as levistilide A (1), miltirone (2), 5-hydroxymethylfurfural (3), luteolin (4), byakangelicol (5), 6-gingerol (6), 5,6,7,8,3,4-heptemthoxyflavone (7), friedelin (8), and chlorogenic acid (9), on the basis of the observation of the pseudomolecular ion peak at *m/z* 381.0747 $[M + H]^+$ (1), *m/z* 283.1405 $[M + H]^+$ (2), *m/z* 127.0388 $[M + H]^+$ (3), *m/z* 287.1091 $[M + H]^+$ (4), *m/z* 315.0727 $[M + H]^+$ (5), *m/z* 329.0881 $[M + Cl]^-$ (6), *m/z* 431.1201 $[M - H]^-$ (7), *m/z* 461.1306 $[M + Cl]^-$ (8), *m/z* 353.0879 $[M - H]^-$ (9), in HPLC/ESI-MS chromatogram, in

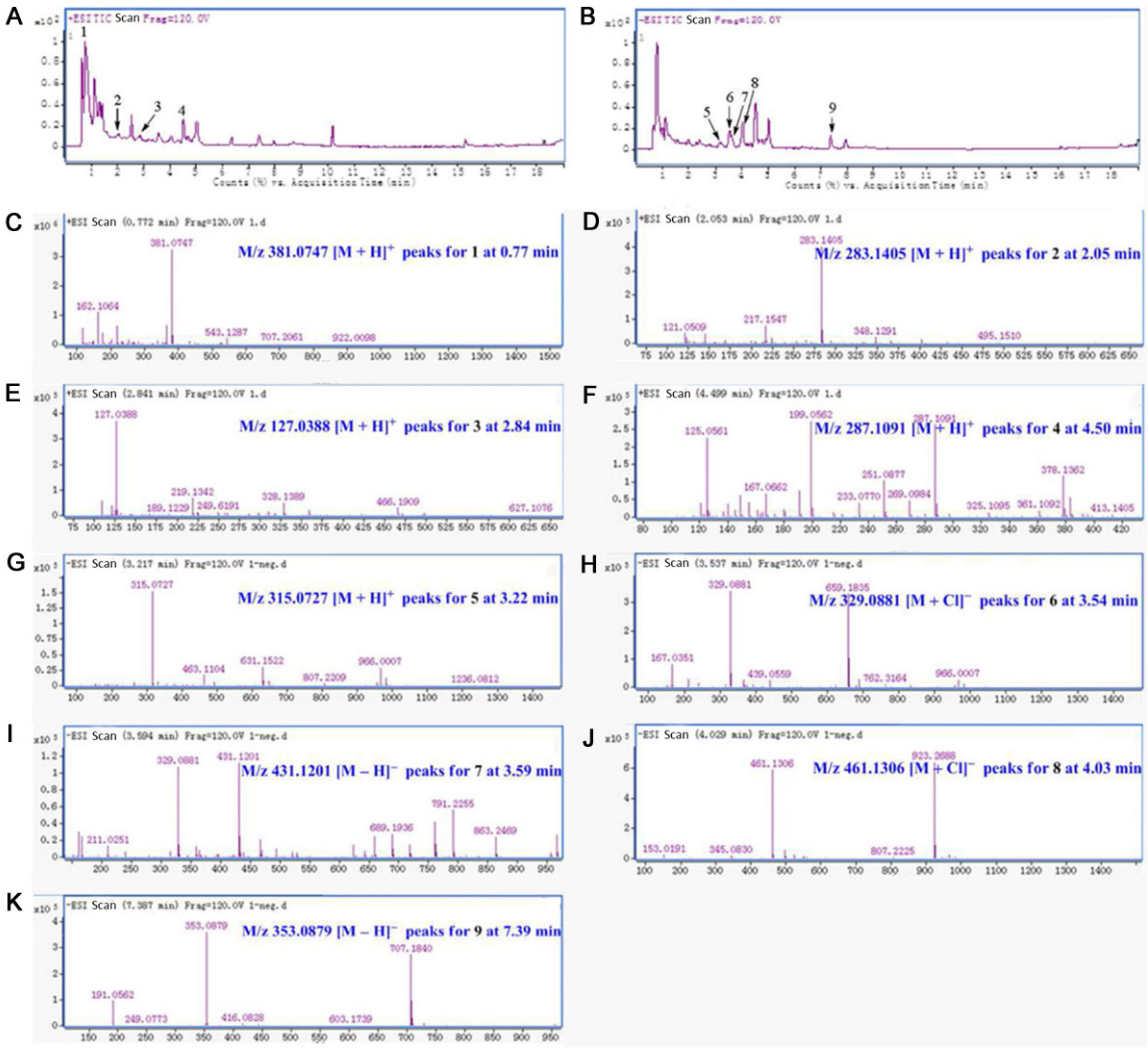

Figure 1. HPLC/ESI-MS chromatogram of the aqueous extract in positive and negative mode (A and B). (C) ESI-MS spectra of $[M + H]^+$ ion of compound 1 (retention time: 0.77 min). (D) ESI-MS spectra of $[M + H]^+$ ion of compound 2 (retention time: 2.05 min). (E) ESI-MS spectra of $[M + H]^+$ ion of compound 3 (retention time: 2.84 min). (F) ESI-MS spectra of $[M + H]^+$ ion of compound 4 (retention time: 4.50 min). (G) ESI-MS spectra of $[M + H]^+$ ion of compound 5 (retention time: 3.22 min). (H) ESI-MS spectra of $[M + Cl]^-$ ion of compound 6 (retention time: 3.54 min). (I) ESI-MS spectra of $[M - H]^-$ ion of compound 7 (retention time: 3.59 min). (J) ESI-MS spectra of $[M + Cl]^-$ ion of compound 8 (retention time: 4.03 min). (K) ESI-MS spectra of $[M - H]^-$ ion of compound 9 (retention time: 7.39 min).

accordance with the molecular weights of nine compounds (levistilide A, miltirone, 5-hydroxymethylfurfural, luteolin, byakangelicol, 6-gingerol, 5,6,7,8,3,4-heptemthoxyflavone, friedelin, and chlorogenic acid). A total of 9 compounds were unambiguously identified by comparing the retention times and the MS data with the reference standards.

Histological examination exhibited cell damage due to D-IBS model

HE staining exhibited damaged tissues and cells in transverse colon, descending colon and rectum (**Figure 2**). Cells in normal transverse and descending colon presented smooth mucosa surface. Gland and smooth muscle were orderly arranged in rectum. However, damaged and necrotic columnar epithelial cells were observed in D-IBS transverse and descending colon with inflammatory cell infiltration in mucous layer. Besides, congestion and edema were detected in submucosa of rectum, indicating the cell and tissue damaged caused by D-IBS. In addition, electron microscope observed the morphologic change of ICC. As shown in **Figure 3**, compared with normal ICC, ICC in D-IBS rats presented decreased cyto-

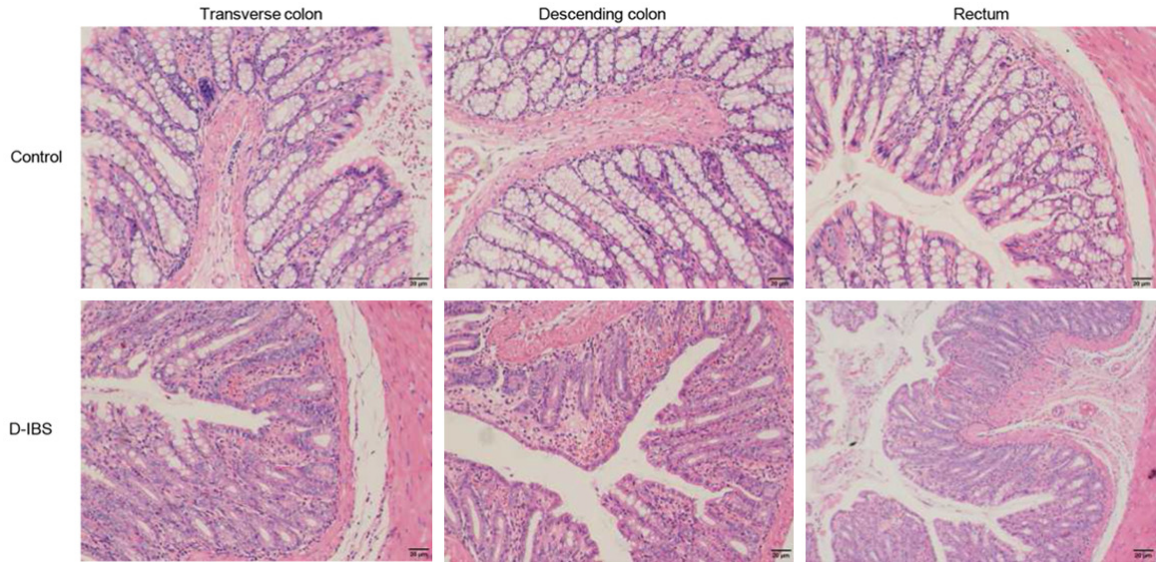

Figure 2. HE staining exhibited damaged tissues and cells in transverse colon, descending colon and rectum. Cells in normal transverse and descending colon presented smooth mucosa surface. Gland and smooth muscle were orderly arranged in rectum. However, damaged and necrotic columnar epithelial cells were observed in D-IBS transverse and descending colon with inflammatory cell infiltration in mucous layer. Besides, congestion and edema were detected in submucosa of rectum, indicating the cell and tissue damaged caused by D-IBS.

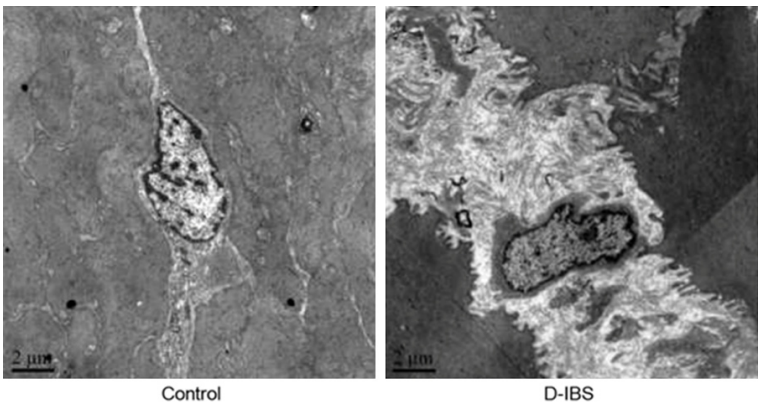

Figure 3. The cellular morphology of ICC in colon tissues was observed using electron microscope, respectively. Compared with normal ICC, ICC in D-IBS rats presented decreased cytoplasm and organelle with interstitial edema. The lesion in colon tissues of D-IBS rats indicated the successful modeling.

plasm and organelle with interstitial edema. Besides, D-IBS rats showed significant differences in body weight gain and loose stool rate compared with control rats before week 2, while no statistics difference was detected in XHF and pinaverium groups. These lesion in colon tissues and changes in D-IBS rats indicated the successful modeling.

XHF presented therapeutic effect on D-IBS

The abdominal withdrawal reflex and abdominal electrical activities were detected to evaluate the therapeutic effect of XHF on D-IBS rats. After the delivery of XHF and pinaverium on week 2, significant differences were observed in weight and loose stool rate as shown in **Figure 4A** and **4B**. Compared with model rats, D-IBS rats treated with high dose of XHF present a higher weight growth with significant difference in week 4. Besides, lower loose stools rate was detected in XHF and pinaverium groups compared with model group in week 4. In addition, the scores of abdominal withdrawal reflex were obtained in XHF and pinaverium groups with the decreased expansion volume in week 4 (**Figure 4C**). Further, frequented abdominal electrical activities accompanied with increased expansion volume were attenuated in XHF and pinaverium groups. These findings indicated that XHF presented similar effect as pinaverium on D-IBS, suggesting the therapeutic effect of XHF on D-IBS.

XHF regulated brain-gut axis in D-IBS

Expression levels of gastrointestinal hormones NPY and VIP in colon were measured by Wes-

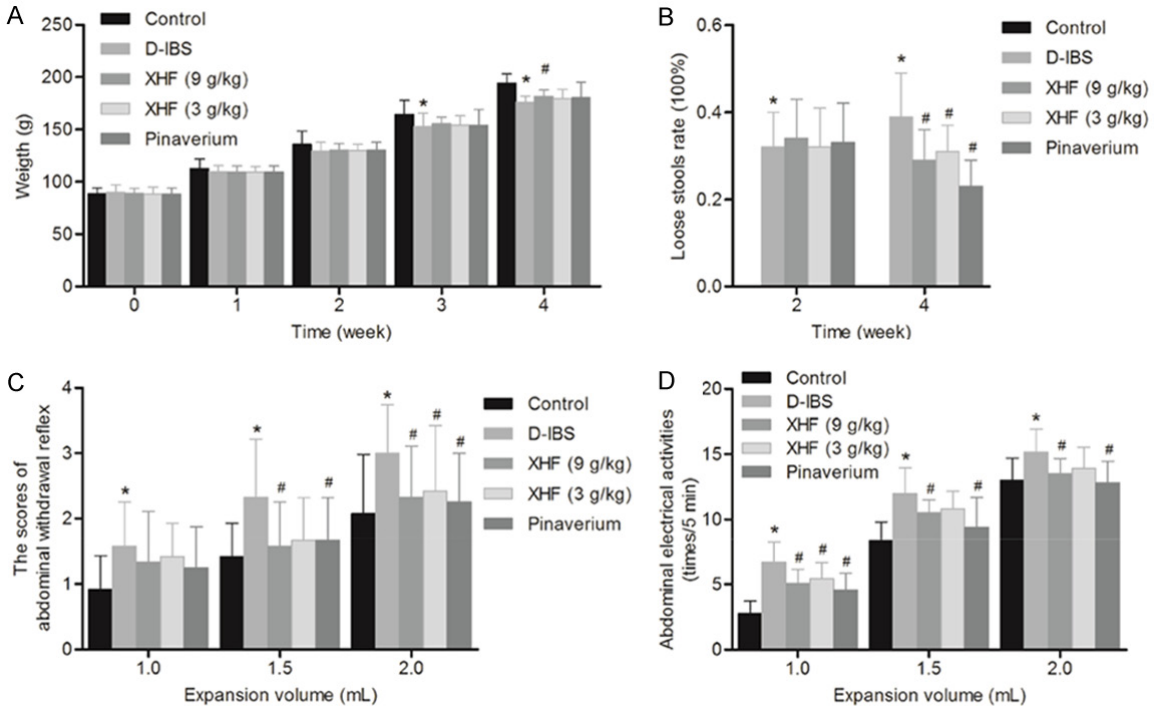

Figure 4. The weight growth, loose stools rate, abdominal withdrawal reflex and abdominal electrical activities were detected to evaluate the therapeutic effect of XHF on D-IBS rats. A: D-IBS rats showed a significant decrease in the body weight gain. Rats treated with XHF or pinaverium in week 2 present attenuated weight gains in week 3 and week 4. *P<0.05, compared with control group. #P<0.05, compared with D-IBS group. B: XHF and pinaverium groups presented lower loose stools rate in comparison to the D-IBS group. *P<0.05, compared with control group. #P<0.05, compared with D-IBS group. C: The abdominal withdrawal reflex was detected on week 4. D-IBS rats gained a high score with significant difference compared with the normal rats, while XHF groups showed attenuated scores with a dose-dependent manner. *P<0.05, compared with control group. #P<0.05, compared with D-IBS group. D: Similar result was obtained in the analysis of abdominal electrical activities. XHF and pinaverium groups showed lower reflex times in comparison to the D-IBS rats with significant difference. *P<0.05, compared with control group. #P<0.05, compared with D-IBS group.

tern blot and real-time PCR, respectively. Decreased NPY and increased VIP accompanied with damaged ICC in D-IBS rats suggested the correlation between D-IBS and gastrointestinal hormones (**Figure 5A-C**). Significant differences were detected in both XHF and pinaverium groups. Decreased NPY and increased VIP levels were attenuated dramatically in XHF groups with a dose-dependent manner compared with D-IBS group. Compared with the pinaverium group, XHF groups showed a trendy to close, indicating the regulation effect of XHF on gastrointestinal hormones NPY and VIP. In addition, the concentrations of NPY and VIP in hypothalamus were also measured (**Figure 5D-F**). Similarly, up-regulated VIP and down-regulated NPY in D-IBS rats were attenuated in XHF and pinaverium groups with significant difference, corresponding to the results obtained from colon tissues. These findings indicated the important role of brain-gut axis in D-IBS. Regulated gastrointestinal hormones NPY and VIP in XHF and pinaverium groups suggested the therapeutic effect of XHF on D-IBS through brain-gut axis.

XHF regulated SCF/C-KIT signaling pathway in D-IBS

Concentrations of SCF/C-KIT signaling pathway related proteins SCF and C-KIT in colon tissues were measured using Western blot and real-time PCR (**Figure 5A-C**). Both SCF and C-KIT increased dramatically in D-IBS rats compared with the normal ones, while raised C-KIT and SCF declined after the treatment of XHF or pinaverium with significant difference. Down-regulated C-KIT and SCF in XHF-treated groups with dose-dependent manner suggested the therapeutic effect of XHF on D-IBS

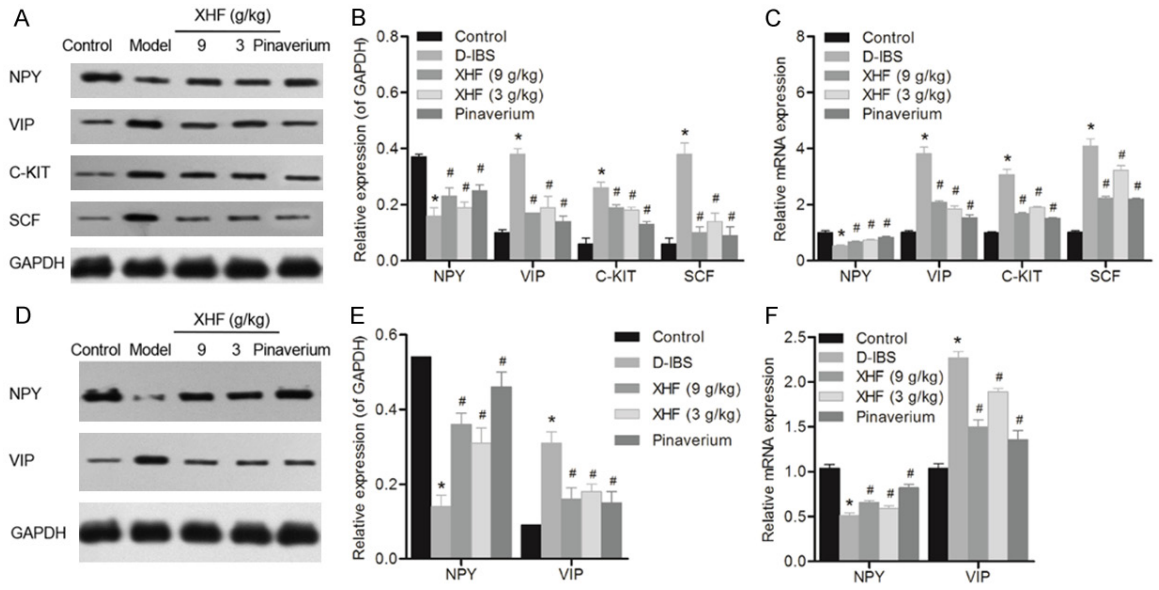

Figure 5. Brain-gut axis and SCF-C-KIT signaling pathway related proteins were measured by Western blot and real-time PCR. A and B: Concentration of NPY, VIP, C-KIT and SCF in colon was measured using Western blot. *P<0.05, compared with control group. #P<0.05, compared with D-IBS group. C: Gene expression of NPY, VIP, C-KIT and SCF were also measured using real-time PCR. *P<0.05, compared with control group. #P<0.05, compared with D-IBS group. D and E: Expression levels of NPY and VIP in hypothalamus were measured using Western blot. *P<0.05, compared with control group. #P<0.05, compared with D-IBS group. F: Gene expression of NPY and VIP in hypothalamus was also measured using real-time PCR. *P<0.05, compared with control group. #P<0.05, compared with D-IBS group.

compared with pinaverium group through the regulation of SCF/C-KIT signaling pathway.

Discussion

ICC is the pacemaker cells of gastrointestinal movement. Various kinds of gastrointestinal motility diseases involve the lesion of ICC [5]. Four subtypes of ICC including ICC-MP, ICC-IM, ICC-DMP and ICC-SMP run throughout the digestive system from esophagus to the anus. Gastrointestinal motility disorders might be caused by the change of amount, structure or density of ICC due to toxic substances, toxins, viruses or nerve injury [6]. An unusual slow wave caused by decreased ICC amount could decline the contractility of intestinal transport [7].

Brain-gut axis acts a vital role in the regulation of gastrointestinal feeling and movement. It was believed that the abnormal change of brain-gut axis is one of the most important pathogenic factors in IBS. The connection between brain and guts relies on the transmission of brain-gut peptide including 5-HT, 5-HT R, NPY, CCK and VIP. Researches indicated that 5-HT plays a key role in the pathogenesis of IBS. The significant increased 5-HT on colonic mucosa was detected by Kosola *et al.* [8]. The up-regulated 5-HT in correlation to the increased EC dominated by adrenal gland affected the gastrointestinal motivation and visceral sensation ultimately through the regulation of brain-gut axis. In the present study, decreased NPY and increased VIP were detected in both colon and hypothalamus of D-IBS rats, indicating the unbalance of brain-gut axis.

Studies of other gastrointestinal hormone demonstrated the up-regulated concentrations in IBS patients through several mechanisms including MC activation and brain-gut axis [9]. Besides, these gastrointestinal hormones could affect gastrointestinal motivation and sensation indirectly through the immune system. However, the measurement of gastrointestinal hormone levels in blood and intestinal mucosa is insufficient for the comprehensive evaluation of the hormone status in patients with IBS due to the complex interaction between gastrointestinal hormones. It was demonstrated recently that 5-HT2B receptor is expressed by ICC, especially ICC-MP and ICC-DMP. Further,

the activation of C-γ5-HT2B receptor induced by ICC was identified [10]. Therefore, increased ICC level due to up-regulated 5-HT in D-IBS patient results in the aggravated gastrointestinal disorders and other IBS symptoms. Kim et al. indicated the inhibited pacemaker activity of ICC due to VIP through kinase G pathway of NO-CGMP [11]. Besides, intestinal motility regulated by NPY receptor expressed by ICC-MP was also identified [12]. A lower plasma and sigmoid colon concentration of NPY was detected in IBS patients, especially in patients with D-IBS [13]. While CCK, another gastrointestinal hormone expressed by ICC-IM, presents a higher level in IBS patients. These evidences suggested that ICC induces IBS through the interaction between gastrointestinal hormones.

IBS with visceral hypersensitivity usually occurs in peripheral nervous system, as well as in central nervous system [14]. The specific mechanism of visceral hypersensitivity remains unclear due to the various kinds of neurotransmitters participated in the regulation of visceral hypersensitivity. It was identified so far that the sensitizing message of IBS patients transfers from primary nerve to dorsal horn neurons mainly containing microglia. These cells release neuroactive substances including glutamic acid, substance P and so on, leading to the increased excitability of spinal cord, resulting in the exciting of dorsal commissural nucleus [15].

KIT is one of the membrane receptor of ICC. Its ligand SCF is a multifunctional growth factor combined with C-KIT, leading to the activity of Kit, rustling in a series of phosphorylation process. SCF act as a regulator in the proliferation, differentiation and migration process of various cells including ICC [16]. Currently the close relation between SCF/C-KIT signaling pathway and ICC was demonstrated. The former plays an important role in the development and function maintenance of ICC. Researchers have indicated the significant hyperplasia of mast cells and monocytes on local colonic mucosa due to the over expressed SCF. Meanwhile, SCF combined with its receptor C-KIT induces the hyperplasia of ICC and regulates the expression of connexin. In consequence, generated ICC results in the multiple ectopic rhythm pacemakers and easier conductive wave rhythm signal between ICC. Further, due to the damaged network connection between ICC, ectopic rhythm occurring in ICC triggers the excessive movement of bowel. In addition, the change of ICC could affect gastric sensory nerve directly because of the synapse-like contacts between ICC and gastric sensory nerve, resulting in the regulation of central nervous system. Thus, these evidences suggest that the changes of ICC induced by SCF might be involved in the pathogenesis of IBS [17].

In conclusion, we demonstrated the therapeutic effect of XHF on D-IBS through the regulation of brain-gut axis and SCF/C-Kit signaling pathway. We provided experimental basis for rational drug use and novel therapeutic method against D-IBS in clinic.

Acknowledgements

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Disclosure of conflict of interest

None.

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