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Traditional Medicine Research 2024;9(12):68. https://doi.org/10.53388/TMR20240425004

Huoxue Tongjiang decoction-resisted reflux esophagitis by activation stem cell factor/c-kit/interstitial cell of cajal pathway and regulating the T-helper 17/regulatory T-cells balance in rats

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Author contributions
Liu Y designed the study and drafted the manuscript. Tang YP revised the manuscript and helped coordinate support and funding. Li PC, Liu L, and Liu X participated in the experiments. Liu SY performed the statistical analysis. Yang L guided the experiments. All authors read and approved the final manuscript.

Competing interests
The authors declare no conflicts of interest.

Acknowledgments
This work was supported by the National Natural Science Foundation of China (No. 81573737 and 82074213), the science foundation of Tianjin Municipal Health Bureau (No. 2023169 and 2021045), and the Tianjin Municipal Health Commission Science and Technology Project (No. TJWJ2022QN057).

Peer review information
Traditional Medicine Research thanks all anonymous reviewers for their contribution to the peer review of this paper.

Abbreviations
HXTJD, Huoxue Tongjiang decoction; RE, reflux esophagitis; LES, lower esophageal sphincter; ICC, interstitial cells of Cajal; Cx43, connexin43; SCF, stem cell factor; Th17, T helper 17; Treg, regulatory T; IL-6, interleukin 6; IL-17, interleukin 17; IL-10, interleukin 10; GERD, gastroesophageal reflux disease; SMC, smooth muscle cells; L-HXTJD, low concentration group of HXTJD; H-HXTJD, high concentration group of HXTJD; TCM, traditional Chinese medicine; OD, optical density; MS, mass spectrometry; Foxp3, forkhead box P3; CD25, cluster of differentiation 25; CD4, cluster of differentiation 4.

Citation
Liu Y, Tang YP, Li PC, et al. Huoxue Tongjiang decoction-resisted reflux esophagitis by activation stem cell factor/c-kit/interstitial cell of cajal pathway and regulating the T-helper 17/regulatory T-cells balance in rats. *Tradit Med Res*. 2024;9(12):68. doi: 10.53388/TMR20240425004.

Executive editor: Jing Yin.
Received: 25 April 2024; **Accepted:** 26 July 2024; **Available online:** 29 July 2024.

Abstract
Background: Huoxue Tongjiang decoction (HXTJD) is an effective prescription for treating reflux esophagitis (RE). We investigated the effects of HXTJD on esophageal motility and mucosal inflammation in a rat RE model. **Methods:** Chemical composition of HXTJD was analyzed by ultrahigh-performance liquid chromatography Q-Orbitrap mass spectrometry (MS). The change rates of mean contraction tension forces, mean amplitudes, and mean frequencies for the lower esophageal sphincter (LES) were recorded using the isolated tissue bath system, mechanical tension transducer, and PowerLab physiological recorder. After weighing the stomach, the phenol red labeling method was used to measure the gastric emptying rate. The LES ultrastructure was observed through transmission electron microscopy. Immunofluorescence and western blotting were used to detect the number of interstitial cells of Cajal (ICC) and the expression levels of c-kit protein, connexin43 (Cx43), and stem cell factor (SCF). Flow cytometric analysis and enzyme-linked immunosorbent assay were conducted to detect the percentages of T helper 17 (Th17) cells and regulatory T (Treg) cells and the serum concentrations of interleukin 6 (IL-6), interleukin 17 (IL-17), and interleukin 10 (IL-10) in the rats. **Results:** We identified 28 chemical constituents in HXTJD. Regarding esophageal motility, we revealed that HXTJD increased the mean contraction tension forces, mean amplitudes, and mean frequency change rate of LES and the gastric emptying rate; decreased stomach weight; and improved the LES ultrastructure. Additionally, HXTJD increased the number of ICC-positive cells, and c-kit, Cx43, and SCF expression levels. Regarding esophageal inflammation, HXTJD significantly decreased the percentage of Th17 cells, and IL-6 and IL-17 concentrations, and increased the percentage of Treg cells and IL-10 concentration. **Conclusion:** HXTJD was found to be efficacious in the rat RE model. It may promote esophageal motility and alleviate the inflammatory response by activating the SCF/c-kit/ICC pathway and regulating the Th17/Treg cell balance.

Keywords: Huoxue Tongjiang decoction; reflux esophagitis; SCF/c-kit/ICC pathway; Th17/Treg cell; esophageal motility; mucosal inflammation

Highlights

Huoxue Tongjiang decoction (HXTJD) increased the mean contraction tension forces, mean amplitudes, and mean frequency change rate of lower esophageal sphincter (LES) and the gastric emptying rate; decreased stomach weight; and improved the LES ultrastructure. HXTJD increased the number of interstitial cells of Cajal (ICC)-positive cells, and c-kit, connexin43 (Cx43), and stem cell factor (SCF) expression levels. These findings suggest that HXTJD activate the SCF/c-kit/ICC signaling pathway to augment esophageal motility. Additionally, HXTJD significantly decreased the pathological score, percentage of T helper 17 (Th17) cells, and interleukin 6 (IL-6) and interleukin 17 (IL-17) concentrations, and increased the percentage of regulatory T (Treg) cells and the interleukin 10 (IL-10) concentration. These findings suggest that HXTJD can regulate the Th17/Treg cell balance to alleviate mucosal inflammation.

Medical history of objective

HXTJD is a traditional Chinese medicine (TCM) composed of seven herbs. Both *Bletillae Rhizoma* and *Salviae Miltiorrhizae Radix et Rhizoma* are the main medicines, which were first recorded in the *Shennong Bencao Jing* from the Qin and Han Dynasties and have the effects of promoting blood circulation and treating bleeding. Modern pharmacological studies have shown that they can protect the gastric mucosa and promote mucosal repair. In addition, *Aurantii Fructus* and *Cyperi Rhizoma* can promote the functioning of the body. Modern pharmacological studies have shown that both can promote the secretion of gastrointestinal hormones and regulate gastrointestinal motility.

Background

Gastroesophageal reflux disease (GERD) develops when the reflux of stomach contents triggers troublesome symptoms or complications in the esophagus or beyond. Reflux esophagitis (RE) is a common type of GERD [1]. According to epidemiological statistics, the overall RE burden has continued to worsen, with the number of prevalent cases increasing by 77.53% from 441.57 million in 1990 to 783.95 million in 2019 [2]. The RE incidence rate is approximately 6.4% in China. With changes in the dietary structure and an accelerated pace of life, the RE incidence is also increasing each year. RE is likely to remain a common reason for primary care consultation [3]. RE has a complex mechanism. LES relaxation, LES basal pressure, and insufficient gastric motility are considered key factors in disease occurrence [4, 5]. Acid-suppressive and motility-promoting therapies are commonly used treatment methods in clinics, but the long-term use of acid-suppressive therapy could cause *Clostridioides difficile* infection, chronic kidney disease, and spontaneous bacterial peritonitis [6–8]. Motility-promoting drugs, such as domperidone and cisapride, can easily cause severe cardiac arrhythmia and sudden cardiac death [9–11]. Therefore, effective and safe alternative medicines are urgently required to improve RE treatment.

In China, TCM has been used to treat gastrointestinal diseases for hundreds of years. An increasing number of clinical and basic tests have demonstrated that some Chinese medicines such as *Fructus Aurantii-Magnolia* herb-pair and Banxia Xiexin decoction regulate the digestive tract's motility [12, 13]. The HXTJD is an effective TCM prescription for RE summarized by the long-term clinical practice of our research team. In previous studies, HXTJD reduced the inflammation level in the esophagus tissue and reduced esophageal mucosal damage in RE rats [14, 15]. Accordingly, the present study further observed the effects of TCM on LES contraction and esophageal inflammation, and the changes in its related pathways.

Numerous studies have confirmed that ICC are the pacemaker cells of the gastrointestinal tract, and the current produced by these cells can be transmitted to smooth muscle cells (SMC), leading to rhythmic contraction of the gastrointestinal tract. The c-kit/SCF pathway plays a crucial role [16, 17]. Reflux was believed to cause chemical burns, and acid-induced epithelial damage and cell death promotes inflammation. A growing number of studies have found that RE is an immune inflammation-related disease. This is because the gastric juice reflux stimulates esophageal epithelial cells to secrete chemokines, thereby attracting inflammatory cells and promoting inflammation [18]. IL-6 is a key cytokine in RE pathogenesis, and it can regulate the balance of Th17 cells and Treg cells [19]. This balance has a pivotal role in inflammation and immune response. The Th17/Treg cell balance has been mostly studied in ulcerative colitis, but was recently found to be closely related to RE [20]. The imbalance between these cells can accelerate RE progress and even lead to Barrett's esophagus [21]. Considering the complex composition and diverse targets of HXTJD, we adopted ultrahigh-performance liquid chromatography Q-Orbitrap mass spectrometry (MS) to identify the chemical components of TCM, and explored the effects of TCM on esophageal motility and immune inflammation in RE rats, and proved that the effects may be exerted through the activation of the SCF/c-kit/ICC pathway and regulation of the Th17/Treg cell balance (Figure 1).

Materials and methods

Animals

60 SPF healthy male SD rats weighing 250–280 g from 6–8 weeks (provided by Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China, production license number, SCXK (Jing) 2021-0006) were selected. The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care. All experimental procedures were approved by the Experimental Animal Ethics Committee of Tianjin Nankai Hospital (Permit Number NKYY-DWLL-2023-185, December 13, 2023).

Animal groupings and models establishment

All rats were randomized into Control group, RE group, low concentration group of HXTJD (L-HXTJD), and high concentration group of HXTJD (H-HXTJD). The rats were kept at constant temperature (approximately 25 °C), exposed to light and dark alternately each for 12 h, had free access to standard pellet feed and drinking water, and acclimatized to the environment for 7 days. Each group was fasted for 24 h before modeling, and then, modified partial cardia myotomy + external pyloric partial ligation was performed for modeling. The specific steps are as follows: anesthesia: the rats were first anesthetized through isoflurane inhalation (induction of anesthesia 0.2–0.3 L/min, anesthesia was maintained at 1%–2%). Gastric vascular ligation: to prevent the cardia muscle from bleeding, a fine needle was used to sew the branch of the left gastric artery across the stomach-esophagus junction. Cardiac muscle incision: a mid-abdominal incision of approximately 25 mm, a longitudinal incision of the cardia muscle approximately 0.5 cm at the stomach-esophagus junction, and separation was performed until the mucosal layer was completely exposed. External pyloric ligation: a 4-mm-diameter metal rod was placed longitudinally on the outside of the gastric pylorus. The metal rod was ligated together with the pylorus, and then, the metal rod was withdrawn. Abdomen closure: 2×10^4 U gentamicin sulfate was dripped into the abdominal cavity. The two layers were continuously sutured with a 3/0 suture needle. The abdominal cavity was closed, and subsequently, the wound was disinfected with iodophor. After the operation, the rats were administered with glucose sodium chloride injection (5% glucose, 0.9% sodium chloride). After the rats were fasted for 24 h, they were first fed a half-amount (15 g/d) standard pellet diet for 3 days, and then fed a full-amount (30 g/d) diet. After the rats were fed for 7 days, the RE model was successfully established. The drug was administered from the 8th day. Each rat in the Control and RE groups were gavaged with normal saline, whereas each rat in the L-HXTJD and H-HXTJD groups was gavaged with the specified dose of TCM twice a day for 14 consecutive days (Figure 2).

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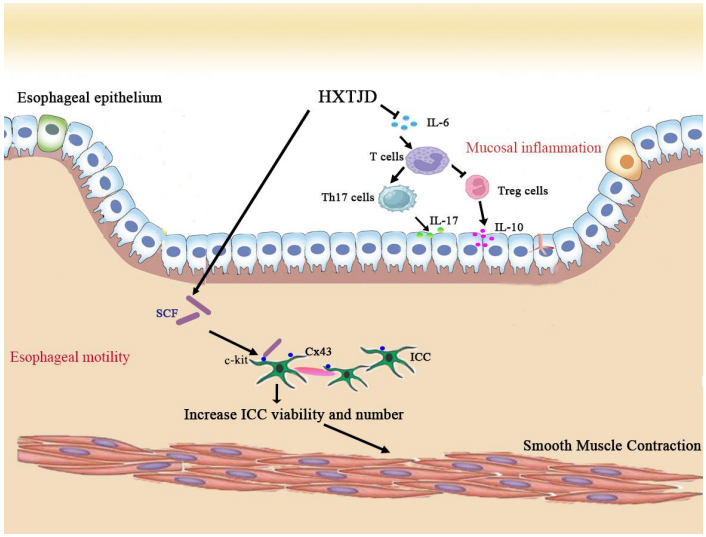

Figure 1 HXTJD resisted RE by activation of the SCF/c-kit/ ICC pathway and regulating the Th 17/Treg cells balance in rats. In terms of esophageal motility, HXTJD can increase the expression of Cx43 and activate the SCF/c-kit/ICC signaling pathway to improve esophageal motility. In terms of esophageal inflammation, HXTJD can inhibit the secretion of IL-6, regulate the balance of Th17/Treg cells and the levels of inflammatory factors related to them, and finally repair mucosal injury so as to achieve the purpose of treating esophagitis. HXTJD, Huoxue Tongjiang decoction; IL-6, interleukin 6; Th17, T helper 17; IL-17, interleukin 17; SCF, stem cell factor; IL-10, interleukin 10; ICC, interstitial cells of Cajal; Cx43, connexin43; Treg, regulatory T.

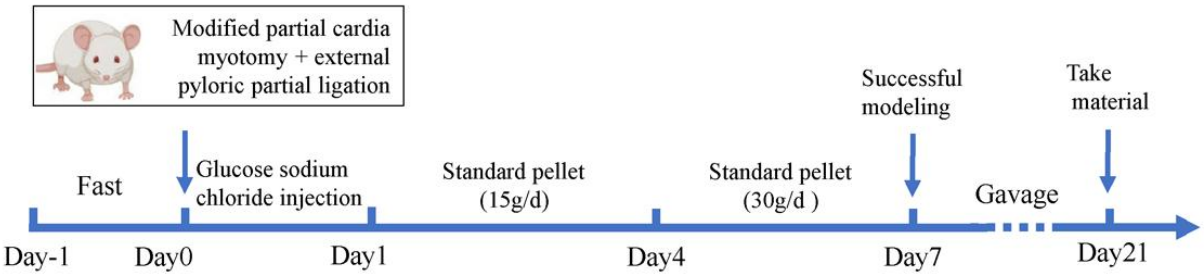

Figure 2 Illustration of the experimental design

HXTJD preparation

HXTJD was composed of *Bletillae Rhizoma* 30 g, *Salviae Miltiorrhizae Radix et Rhizoma* 30 g, *Aurantii Fructus* 15 g, *Cyperi Rhizoma* 15 g, *Curcumae Radix* 10g, *Pinelliae Rhizoma* 10 g, and *Inulae Flos* 30 g (separate packages). The dosage was divided into two groups: L-HXTJD (4.08 g/d) and H-HXTJD (8.16 g/d). The low concentration was the effective standard concentration after conversion, and the high concentration was twice the effective standard concentration. After the effective part was extracted, an aqueous suspension with a crude drug content of 1.63 g/mL of this part was formed. The suspension was refrigerated at 4 ˚C and shaken well before use.

UHPLC and MS conditions

UHPLC conditions. The chemical composition of HXTJD was analyzed on the Thermo Dionex Ultimate 3000 UPLC system (Thermo Fisher, Waltham, MA, USA), which is equipped with a binary pump, degasser, automatic sampler, and column chamber compartment. Using UHPLC HSS T3 column (2.1 × 100 mm, 1.8 μm; Waters, Milford, MA, USA) eluted with a mixture of 0.1% formic acid (A) and acetonitrile (B). Gradient was 0–1 min, maintained at 10% B; 1–8 min, linearly increasing to 80% B; 8–11 min, increased to 90% B, maintained 90% B for 1 min. The flow rate was 0.5 mL/min, and the column temperature was set at 30 ˚C.

MS conditions. MS was carried out on the Thermo Q-Exactive Orbitrap Mass Spectrometer (Thermo Fisher, Waltham, MA, USA) equipped with an electric spray ion source. The MS condition was set as following: capillary temperature, 320 ˚C; spray voltage, 3.8 kV in positive, 3.1 kV in negative ion modes; sheath gas (N_2) flow rate, 45 Arb; collision energy of 40 eV. Full scan mass spectrum was recorded in m/z 70–1,000 at seven spectra/s. The MS/MS experiment was set to data dependent scanning. All data collection was controlled by Thermo Xcalibur 4.0.27.

Confirmation of constituents for HXTJD

Apply the BATMAN-TCM database (http://bionet.ncpsb.org/batman-tcm) to search for the medicinal constituents of HXTJD, and compare and confirm the results with the MS analysis [22].

Histological staining and scoring

The 1-cm tissue at the esophagus-stomach junction was cut, soaked, and fixed in formalin, embedded in paraffin and sliced, stained with hematoxylin and eosin, and observed using a light microscope (LEICA DM4000B, LEICA, Wetzlar, Germany). The pathological score was based on the RE diagnostic criteria established by the Society of Digestive Endoscopy of the Chinese Medical Association in December 1999 [23] (Table 1).

Muscle strip preparation and esophageal smooth muscle contraction detection

Before the experiment, the male Wistar rats were fasted for 12 h. The abdomen was opened quickly after excessive anesthesia, and the entire esophagus was removed. In a petri dish filled with 4 ˚C pre-cooled Krebs solution (Prepared from $CaCl_2$, KCl, $MgCl_2$, NaH_2PO_4, NaCl, $NaHCO_3$ and glucose, Sinopharm Chemical Reagent Co., Ltd., Shanghai, China), the LES was cut to form 8 × 3 mm muscle strips. Then, the muscle strip was immediately hung longitudinally in a bath

Table 1 Pathological grading of RE

Lesions	Grade		
	Mild	Moderate	Severe
Squamous epithelial hyperplasia	+	+	+
Mucosal lamina propria papillae extension	+	+	+
Inflammatory cell infiltration within the epithelial cell layer	+	+	+
Mucosal erosion	−	+	−
Ulceration	−	−	+
Barrett's esophageal changes	−	−	+/−

Mild (1 score), Moderate (2 score), Severe (3 score).

containing 10 mL of Krebs solution (37 ˚C). A mixed gas of 95% O_2 and 5% CO_2 was blown into the bath forming 60–90 bubbles per min. One end of the strip was fixed on the ventilating hook at the lower part of the bath, and the other end was connected to a mechanical tension transducer. After applying 0.6 g preload, the Krebs solution was changed every 15 min. Then, 1 mL of 500 mmol/L KCl solution was added after the muscle strip was stable for 60 min. Muscle strip contraction was recorded using a PowerLab physiological recorder (ADInstruments PL3508, ADInstruments GmbH, Sydney, Australia), and data were analyzed using LabChart 7.2 (ADInstruments GmbH, Sydney, Australia). The mean contraction tension forces, mean amplitudes, and mean frequencies within 3 min after balance were recorded. The basic value before KCl was added, and the effect value after KCl was added. The Equation (1) for calculation was as follows:

$$\text{Change Rate} = (\text{Effect Value} - \text{Basic Value})/\text{Basic Value} \times 100\% \quad (1)$$

Stomach weight and gastric emptying

Before the experiment, the rats were fasted without water for 24 h. Each rat was gavaged with 2 mL of phenol red solution (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) (50 mg/dL). After 30 min, the rats were sacrificed, and the abdominal section was opened. The pylorus and cardia were ligated, and the entire stomach was removed and weighed. Then, a cut was made along the greater curvature of the stomach. The stomach contents were rinsed with normal saline (Cisen Pharmaceutical Co., Ltd., Jining, China), and the volume was diluted to 20 mL. Then, 20 mL sodium hydroxide solution (Sigma-Aldrich Corporation, St Louis, MO, USA) (0.5 mol/L) was added, and the mixture was stirred evenly. After the mixture was allowed to stand for 1 h, 5 mL supernatant was collected, and 0.5 mL trichloroacetic acid solution (Shanghai Yien Chemical Technology Co., Ltd., Shanghai, China) (20%) was added to the supernatant and centrifuged at 3000 g/min for 10 min. The supernatant was collected, and the absorbance (optical density, OD) was measured at 560 nm by using an ultraviolet spectrophotometer (Shimadzu UV1800, Shimadzu GmbH, Tokyo, Japan). Another 2 mL of phenol red solution was added to the mixture. Then, 18 mL of physiological saline, 20 mL of sodium hydroxide solution, and 4 mL of trichloroacetic acid solution were added in sequence and stirred well. The OD value, which is the standard phenol red OD, was determined. The Equation (2) for calculation is as follows:

$$\text{Gastric Emptying Rate} = (1 - \text{Measured Phenol Red OD}/\text{Standard Phenol Red OD}) \times 100\% \quad (2)$$

Transmission electron microscopy

The LES of each rat was cut into 5 × 3 × 3 mm^3 slices, placed in a fixative containing 2% paraformaldehyde and 3% glutaraldehyde, incubated at 4 ˚C for 24 h, rinsed with phosphate-buffered saline (PBS) thrice, fixed with 1% sosmium tetroxide at 4 ˚C for 2 h, and stained with saturated uranyl acetate for 3 h in a dark box at room temperature. After the slices were rinsed with distilled water, they were dehydrated in different alcohol concentrations and embedded in propylene oxide. The slice was cut into 0.3 × 0.2 mm and stained with 1% toluidine blue. The selected area of the slice was pasted on grids of 200 meshes and stained with lead citrate and uranyl acetate. At last, the stained sections were observed under a transmission electron microscope (Hitachi HT7700, Hitachi, Tokyo, Japan).

Immunofluorescence

The LES tissue was fixed in 4% paraformaldehyde solution for 48 h, dehydrated, embedded, sliced, and baked into slices for 3 h. After the prepared white tablets were deparaffinized, the specimens were washed 3 times with PBS for 5 min each time. After 20 min of antigen retrieval, the specimens were placed at room temperature and washed with PBS thrice for 5 min each time. Then, 50 μL hydrogen peroxide was added dropwise, and after 20 min of reaction, the specimen was washed thrice with PBS for 5 min each time. Then, the specimens were blocked with normal goat serum for 30 min at room temperature, incubated with the rat primary antibody (c-kit, AffinitY, Melbourne, Australia, 1:200 dilution; Cx43, Proteintech, Chicago, IL, USA, 1:200 dilution) overnight at 4 ˚C, and washed with PBS thrice for 5 min each time. Then, the specimens were incubated with the secondary antibody (Alexa Fluor 594, Bioss, Beijing, China) in a light-proof humidified box for 1 h and rinsed thrice in PBS for 5 min each time. The sections were stained with the 4',6-diamidino-2-phenylindole dihydrochloride (Solarbio, Beijing, China) dye and washed thrice with PBS for 5 min each time, and the slides were mounted. Five immunofluorescence sections of c-kit and Cx43 were created for each group, and 8 fields were selected (× 400 times) for each c-kit section under a fluorescence microscope (LEICA DM4000B, LEICA, Wetzlar, Germany), and the number of ICC-positive cells was counted.

Western blotting

The LES was removed and homogenized through mechanical disruption in a radioimmunoprecipitation assay buffer containing protease inhibitors. The lysate was incubated for 30 min on ice and centrifuged at 12,000 g/min for 10 min. The BCA protein analysis kit was used to determine the protein content in the supernatant. After the lysate was diluted with the buffer, it was heated to 95 ˚C for 10 min. Based on the difference in molecular weights, the proteins were separated through 8%–10% SDS-PAGE electrophoresis and transferred to a polyvinylidene fluoride membrane. The membrane was blocked in 5% skim milk phenol for 2 h and incubated with the first rabbit antibody (c-kit, Affinity, Melbourne, Australia; Cx43 and SCF, Proteintech, Chicago, IL, USA) overnight at 4 ˚C. The first antibody was removed by shaking the membrane with TBST thrice, 5 min each time. The membrane was incubated with the second antibody for 1.5 h and removed by shaking the membrane with Tris-Buffered Saline (Wuhan Servicebio Technology Co., Ltd., Wuhan, China) Tween thrice, 5 min each time. The protein was visualized using the enhanced chemiluminescence reagent. Image J software was used for semiquantitative analysis.

Flow cytometric analysis

Treg and Th17 cells were screened from the peripheral blood through flow cytometry. The Treg cells were positively sorted on the basis of cluster of differentiation 4 (CD4), forkhead box P3 (Foxp3), and cluster of differentiation 25 (CD25). Gated and FACS analyses were performed on the peripheral blood $CD4^+$, $Foxp3^+$, and $CD25^+$cells. The Th17 cells were positively sorted on the basis of IL-17A and CD4.

Gated and FACS analyses were also performed on the peripheral blood IL-17A^{+} and CD4^{+} cells. The IL-17A^{high}/CD4^{high} double-positive cells were used to determine Th17 cell percentages. In addition, the cells were detected using a NovoCyte flow cytometer (ACEA Biosciences, San Diego, CA, USA) and analyzed using NovoExpress software.

Enzyme-linked immunosorbent assay

The experiment was conducted using the enzyme-linked immunosorbent assay kit (IL-6, IL-17, Cloud-Clone Corp, Houston, TX, USA; IL-10, ABclonal, Boston, MA, USA). The OD value was measured at 450 nm by using the microplate reader (ST36, Shanghai Kehua Bio-engineering Co., Ltd., Shanghai, China). A standard curve was drawn, and IL-17, IL-10, and IL-6 concentrations in the rat serum were calculated by referring to the standard curve.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 8.0.2 software. The Shapiro Wilk normality test was used as the normal distribution test. When it conforms to normal distribution, the measured data were expressed as the mean ± standard deviation ($\bar{x} \pm s$). When it conforms to non normal distribution, the measured data were expressed as the median (interquartile range) [M (P25, P75)]. When the normal distribution was satisfied and the variance was homogeneous, one-way ANOVA was applied for multigroup comparisons. The significance of the distinctions between groups was assessed using Student's t-test. The Kruskal Wallis test was used as the non normal distribution. Between groups, $P < 0.05$ was considered statistically significant.

Results

Identification of the chemical constituents in HXTJD

The chemical constituents in HXTJD were analyzed using ultrahigh-performance liquid chromatography Q-Orbitrap MS. Under optimized UHPLC and MS conditions, the main constituents were well separated and detected (Figure 3). The search was conducted on the BATMAN-TCM database, resulting in 215 candidate chemical constituents. The results were compared and confirmed with MS analysis, we identified 28 chemical constituents (Table 2).

Effects of HXTJD on esophageal pathology

To explore the effect of HXTJD on esophageal mucosal inflammation in RE rats, the esophageal pathology of the rats was evaluated. In the Control group, very little squamous epithelial proliferation was observed in the esophageal tissue, with neatly arranged cells and clear layers. Only a small number of scattered inflammatory cells was observed in the epithelial layer. The RE group exhibited a large amount of squamous epithelial hyperplasia, obvious nipple extension, and extensive inflammatory cell infiltration in the epithelial layer. The L-HXTJD group exhibited a small extent of squamous epithelial hyperplasia, reduced nipple extension, and less inflammatory cell infiltration in the epithelial layer. The mucosal epithelial damage was repaired in the H-HXTJD group, with a relatively neat arrangement of cells and a small amount of squamous epithelial hyperplasia. The nipple extension range was further reduced, and only a small number of scattered inflammatory cells were visible in the epithelial layer. A significant difference in the pathological score was noted between the Control and RE groups (Figure 4). The esophageal pathological score decreased after the TCM treatment. Thus, HXTJD alleviates the rat RE syndrome and repairs the mucosa.

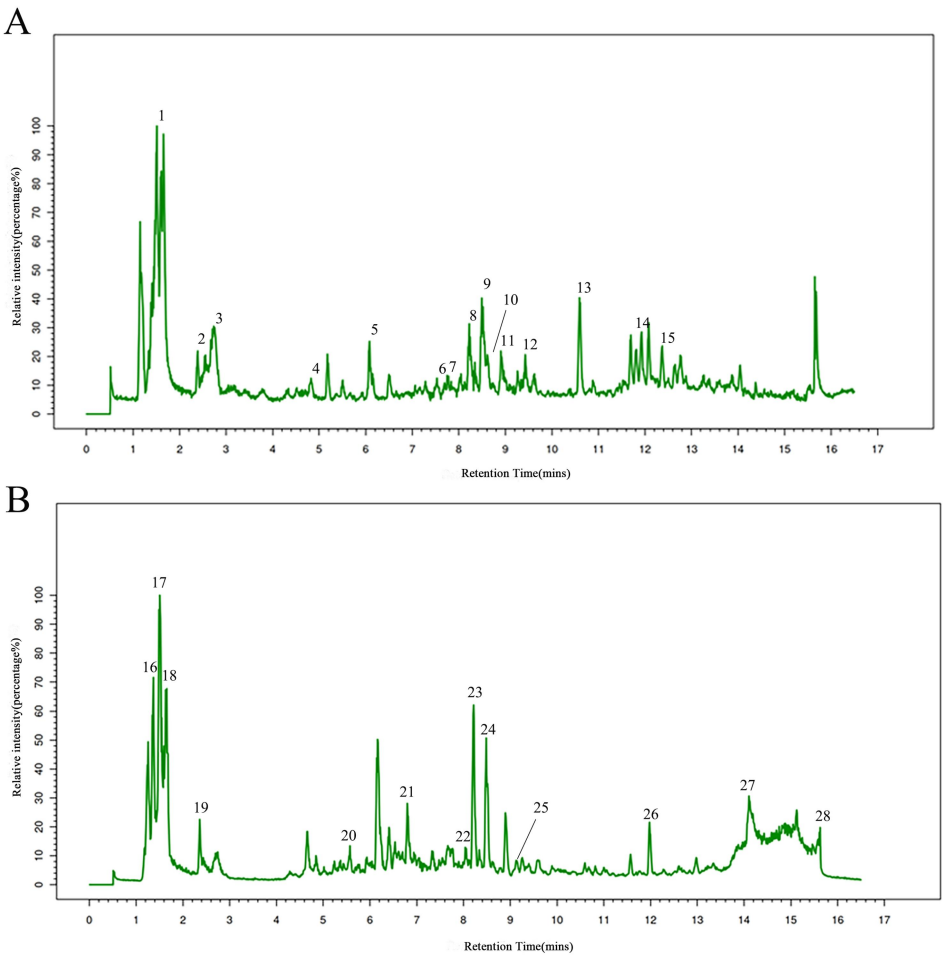

Figure 3 Total ion chromatogram monitored in positive (A) and negative (B) ion modes for HXTJD

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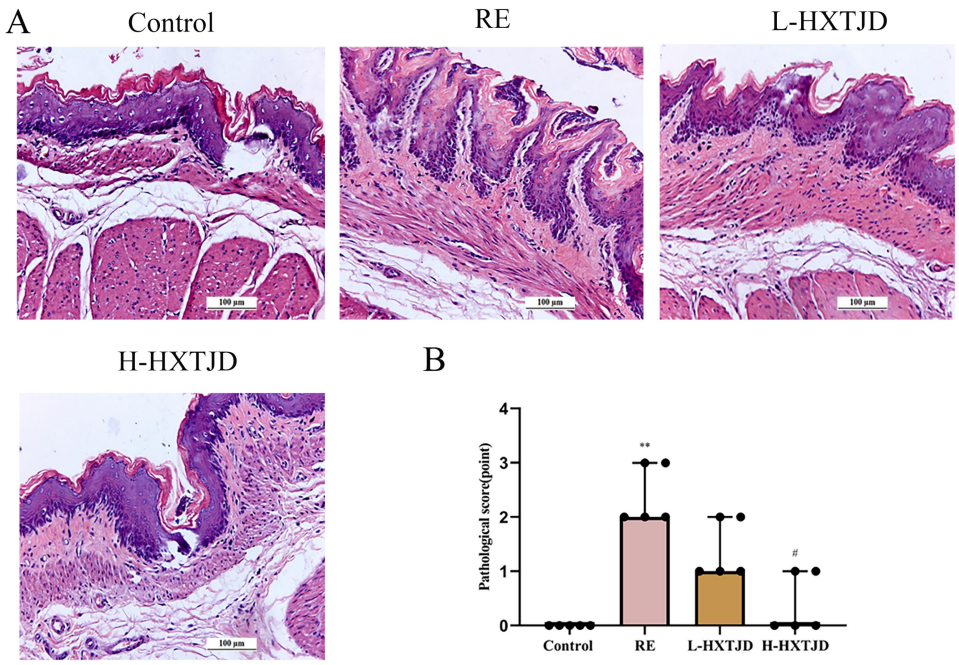

Figure 4 Effects of HXTJD on the gastroesophageal junction pathology. $^{**}P < 0.01$, compared with Control group; $^{\#}P < 0.05$, compared with RE group; n = 5. Scale bar = 100 μm. HXTJD, Huoxue Tongjiang decoction; L-HXTJD, low concentration group of HXTJD; H-HXTJD, high concentration group of HXTJD; RE, reflux esophagitis.

Table 2 Identified constituents of HXTJD

No.	Compound	RT (min)	CAS	Molecular formula	Error (ppm)	Exact mass
1	Melezitose	1.50	597-12-6	$C_{18}H_{32}O_{16}$	5.55	504.16903
2	Niacinamide	2.41	98-92-0	$C_6H_6N_2O$	1.52	122.04801
3	Linamarin	2.80	554-35-8	$C_{10}H_{17}NO_6$	8.135	247.10558
4	Monotropein	4.80	5945-50-6	$C_{16}H_{22}O_{11}$	2.773	390.11621
5	Myricetin 3,3-digalactoside	6.1	28454-81-1	$C_{27}H_{30}O_{18}$	8.372	642.14321
6	Astragalin 7-rhamnoside	7.53	38784-79-1	$C_{27}H_{30}O_{15}$	8.271	594.15846
7	Eriodictyol-7-glucoside	7.76	38965-51-4	$C_{21}H_{22}O_{11}$	4.029	450.11621
8	3-o-methylquercetin	8.35	1486-70-0	$C_{16}H_{12}O_7$	2.134	316.0583
9	Hesperetin 7-neohesperidoside	8.50	13241-33-3	$C_{28}H_{34}O_{15}$	1.698	610.18976
10	Iridin	8.60	491-74-7	$C_{24}H_{26}O_{13}$	4.035	522.13733
11	Neoline	8.84	466-26-2	$C_{24}H_{39}NO_6$	8.239	437.27772
12	Mecheliolide	9.41	68370-47-8	$C_{15}H_{20}O_3$	0.223	248.14124
13	Leucodin	10.61	17946-87-1	$C_{15}H_{18}O_3$	0.433	246.12559
14	Costunolide	11.88	553-21-9	$C_{15}H_{20}O_2$	9.383	232.14632
15	Reynosin	12.41	28254-53-7	$C_{15}H_{20}O_3$	3.684	248.14124
16	7, 8-dhmc	1.38	2107-77-9	$C_{10}H_8O_4$	11.128	192.04226
17	Palmatine	1.43	3486-67-7	$[C_{21}H_{22}NO_4]^+$	9.655	352.15488
18	Ethyl gallate	1.67	831-61-8	$C_9H_{10}O_5$	7.375	198.05282
19	Orcinol	2.37	504-15-4	$C_7H_8O_2$	1.222	124.05243
20	Perillic acid	5.59	7694-45-3	$C_{10}H_{14}O_2$	0.104	166.09937
21	Medicocarpin	6.81	52766-70-8	$C_{22}H_{24}O_9$	3.593	432.14203
22	Aba-ge cpd	8.06	21414-42-6	$C_{21}H_{30}O_9$	2.497	426.18897
23	Hesperidin	8.24	520-26-3	$C_{28}H_{34}O_{15}$	3.636	610.18976
24	Syringaldehyde	8.41	134-96-3	$C_9H_{10}O_4$	3.151	182.05791
25	Ovalitenin b	9.18	64280-21-3	$C_{19}H_{18}O_4$	5.601	310.1205
26	Lusianthridin	11.99	87530-30-1	$C_{15}H_{14}O_3$	2.02	242.09429
27	Emodic acid	14.08	478-45-5	$C_{15}H_8O_7$	11.542	300.027
28	4-hydroxy-3-methoxybenzenemethanol	15.68	498-00-0	$C_8H_{10}O_3$	1.108	154.06299

CAS, Chemical Abstracts Service.

Effects of HXTJD on the LES muscle contraction, stomach weight, and the gastric emptying rate

The effect of HXTJD on the LES contraction was observed in the RE rats. After 1 mL KCl (500 mmol/L) was added, the change rates of mean contraction tension forces, mean amplitudes, and mean frequencies were lower in the RE group than in the Control group (Figure 5A–5D). After the HXTJD treatment, the aforementioned change rates increased, and the H-HXTJD group exhibited greater change rates than the L-HXTJD group. Interestingly, HXTJD affected not only the LES contraction ability but also the gastric contraction of the RE rats. As shown in Figure 5E, 5F, compared with the Control group, the stomach weight increased in the RE group, and the gastric emptying rate was significantly reduced. After the HXTJD treatment, stomach weight decreased, and the gastric emptying rate increased. Thus, HXTJD increases the LES contractility in the RE rats as well as stomach contraction, thereby improving the esophageal and gastric motility.

Effects of HXTJD on the LES ultrastructure

The ultrastructure of LES in each group is shown in Figure 6. ICC in the Control group had a huge nucleus, less cytoplasm, rich organelles, more mitochondria, a rough endoplasmic reticulum, and Golgi complexes. The connections between ICCs and between ICC and SMC were tight. ICC in the RE group had an irregular polygonal shape, the cytoplasm was dissolved, the number of mitochondria was reduced, the mitochondria were severely vacuolated, the rough endoplasmic reticulum was deregulated, and the gaps were enlarged. This indicated that RE causes damage to the ICC of the LES in rats. In the L-HXTJD group, the ICC had large nuclei, extended branches, mild swelling of mitochondria, and a small amount of rough endoplasmic reticulum in the cytoplasm, and the gaps shrank. In the H-HXTJD group, the mitochondria in the ICC were slightly swollen and rich in the rough endoplasmic reticulum, and the gaps were reduced. In summary, HXTJD significantly improves the RE-induced damage of the LES ultrastructure, and the efficacy of H-HXTJD group was better than that of the L-HXTJD group.

Effects of HXTJD on Cx43, c-kit, and SCF expression and the number of ICC-positive cells

Western blotting and immunofluorescence were performed to detect the effects of HXTJD on c-kit, Cx43, and SCF expression in the LES of the RE rats (Figure 7A–7F). C-kit, Cx43, and SCF expression was significantly lower in the RE group than in the Control group. Cx43, c-kit, and SCF expression increased after the HXTJD treatment. Additionally, the number of ICC-positive cells was counted, as shown in Figure 7G. The number of ICC-positive cells was lower in the RE group than in the Control group. The number increased after the HXTJD treatment. Thus, we speculate that HXTJD increases c-kit, Cx43, and SCF expression in the LES of the RE rats as well as the number of ICC-positive cells.

Effects of HXTJD on the percentages of Th17 and Treg cells

The percentages of Th17 and Treg cells were detected through flow cytometry (Figure 8). Compared with the Control group, the percentage of Th17 cells increased significantly in the RE group, whereas the percentage of Treg cells decreased. We continued to observe the effect of HXTJD on the Th17 and Treg cells. The percentage of Th17 cells decreased, whereas that of Treg cells increased after the medication. Therefore, the Th17/Treg cell imbalance is serious issue in the peripheral blood of the RE rats. HXTJD improves the inflammatory response by reducing the percentage of Th17 cells, while increasing the percentage of Treg cells.

Effects of HXTJD on IL-6, IL-17, and IL-10 concentrations

We studied the effects of HXTJD on the percentage of Th17 and Treg cells. The changes in the levels of inflammatory factors associated

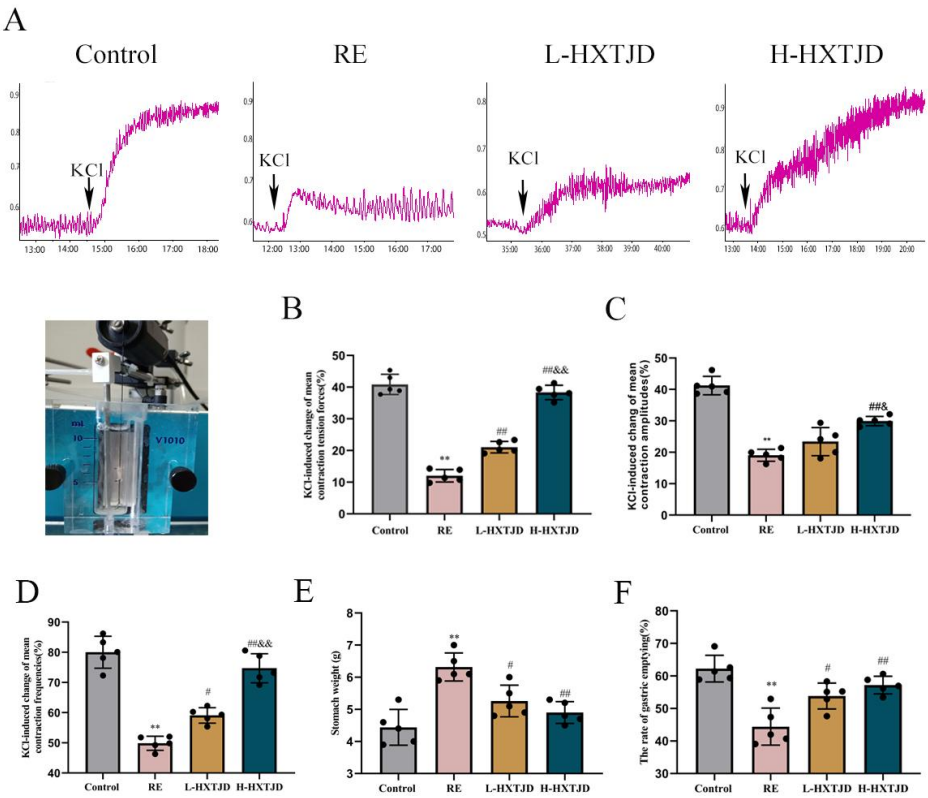

Figure 5 Effects of HXTJD on LES contraction, stomach weigh and gastric emptying rate. (A) Effects of HXTJD on the contraction of LES for different groups. (B) Mean contraction tension forces. (C) Mean contraction amplitudes. (D) Mean contraction frequencies. (E) Stomach weigh. (F) Gastric emptying rate. $^{**}P < 0.01$, compared with Control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with RE group; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$, compared with L-HXTJD group; n = 5. HXTJD, Huoxue Tongjiang decoction; L-HXTJD, low concentration group of HXTJD; H-HXTJD, high concentration group of HXTJD; RE, reflux esophagitis.

Traditional Medicine Research 2024;9(12):68. https://doi.org/10.53388/TMR20240425004

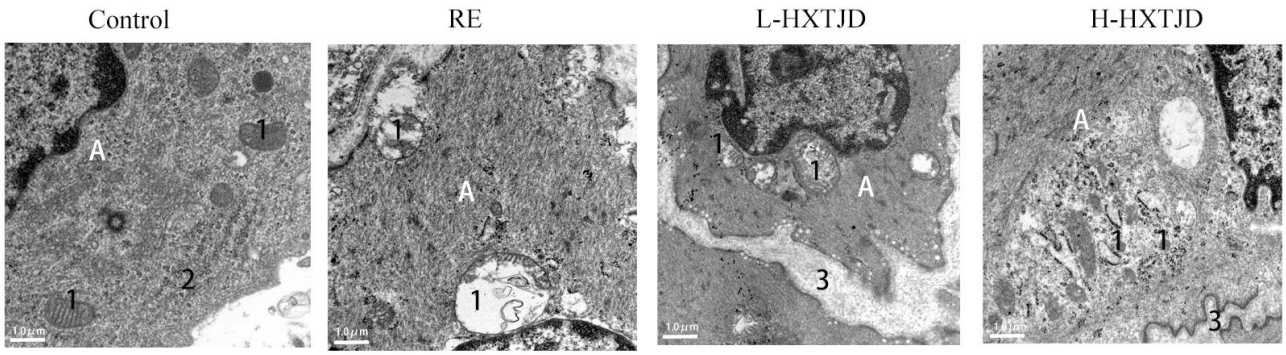

Figure 6 Effects of HXTJD on the ultrastructure of smooth muscle tissue. A represents the ICC, (1) mitochondria, (2) endoplasmic reticulum, (3) gaps. (×5,000). Scale bar = 1.0 μm. HXTJD, Huoxue Tongjiang decoction; L-HXTJD, low concentration group of HXTJD; H-HXTJD, high concentration group of HXTJD; RE, reflux esophagitis.

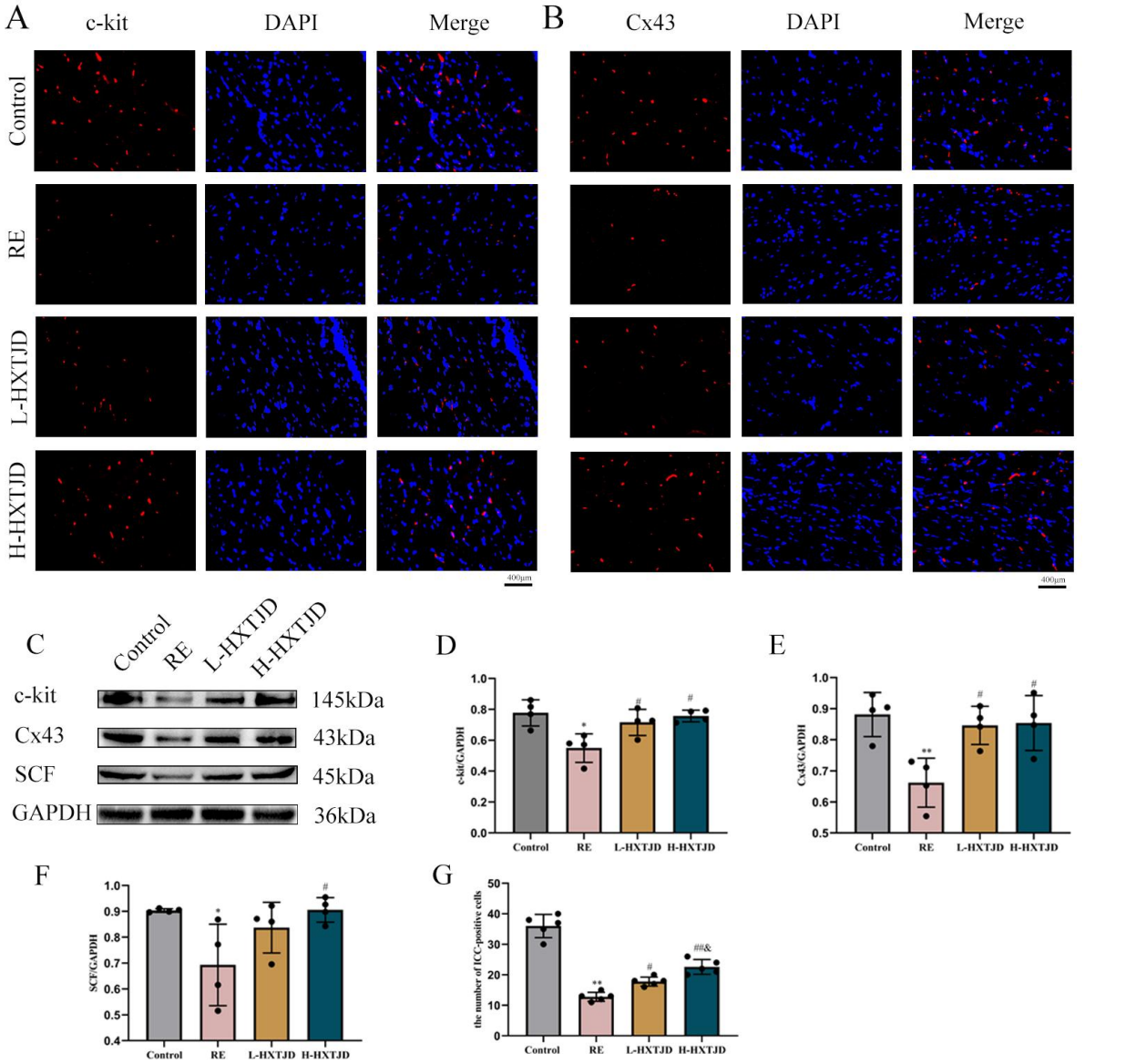

Figure 7 Effects of HXTJD on the expression of c-kit, Cx43, SCF and the number of ICC-positive cells. (A, B, G) Quantitative Immunofluorescence tested effects of HXTJD on the expression of c-kit, Cx43 and the number of ICC-positive cells. (C–F) Western blotting tested effects of HXTJD on the expression of c-kit, Cx43 and SCF. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with Control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with RE group. $^{\&}P < 0.05$, compared with L-HXTJD group. Scale bar = 400 μm. HXTJD, Huoxue Tongjiang decoction; L-HXTJD, low concentration group of HXTJD; H-HXTJD, high concentration group of HXTJD; SCF, stem cell factor; Cx43, connexin43; DAPI, the 4',6-diamidino-2-phenylindole dihydrochloride; ICC, interstitial cells of Cajal; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RE, reflux esophagitis.

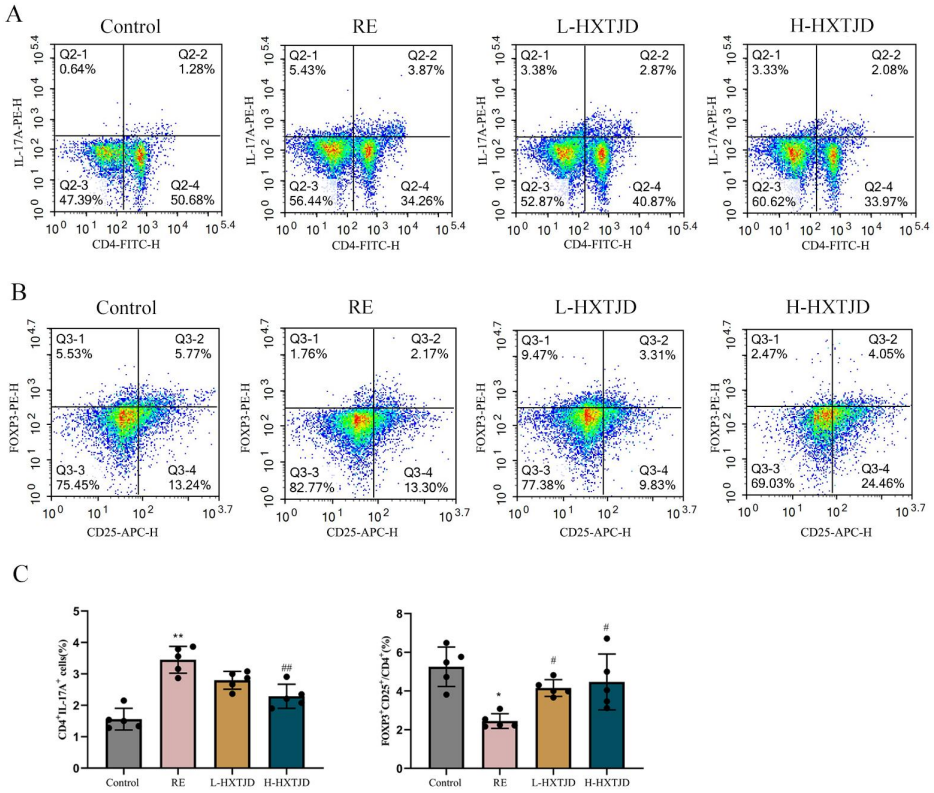

Figure 8 Effects of HXTJD on the percentage of Th17 cells and Treg cells. (A) Effects of HXTJD on the percentage of $CD4^+$ $IL\text{-}17A^+$ Th17 cells in the blood of rats. (B) Effects of HXTJD on the percentage of $CD25^+$ $Foxp3^+$ $CD4^+$ Treg cells in the peripheral blood of rats. (C) Data are expressed as mean ± standard deviation. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with Control group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with RE group; $^{\&}P < 0.05$, compared with L-HXTJD group; n = 5. HXTJD, Huoxue Tongjiang decoction; L-HXTJD, low concentration group of HXTJD; H-HXTJD, high concentration group of HXTJD; RE, reflux esophagitis; IL-17A, interleukin 17A; Foxp3, forkhead box P3; CD25, differentiation 25; CD4, differentiation 4; APC-H, allophycocyanin-height; PE-H, phycoerythrin-height; FITC-H, fluorescein isothiocyanate-height.

with the aforementioned cells were also explored (Figure 9). Compared with the Control group, serum IL-6 and IL-17 concentrations significantly increased, whereas the serum IL-10 concentration decreased in the RE group. We continued to observe the effects of HXTJD on these cytokines. IL-6 and IL-17 concentrations decreased. However, the IL-10 concentration increased. This change trend was consistent with the Th17 and Treg cells. Thus, HXTJD affects the percentages of Th17 and Treg cells and change the key cytokines related to these cells, which can reduce IL-17 concentrations and increase the IL-10 concentration, so as to alleviate the inflammatory response of the RE rats.

Discussion

RE is a chronic and refractory disease because of its prolonged, difficult to heal, repeated symptoms. At present, the RE incidence rate is significantly increasing in China and Western countries. Reports of the side effects of drugs for disease treatment are not uncommon, and this has promoted our search for new alternative therapies. TCM has long been used for the treatment of gastrointestinal diseases. However, few studies have explored the mechanism underlying the influence of TCM on RE inflammation and motility. We used the bioinformatics platform BATMAN-TCM to search for effective ingredients in HXTJD and compared them with MS analysis results to identify 28 effective ingredients. Summarizing the compound categories, it was found that most of the compound structures belong to flavonoids, terpenes and alkaloid. Modern pharmacological studies have shown that compounds of the above categories often have anti-inflammatory, prokinetic, and antioxidant stress effects [24, 25]. This study used a rat RE model to prove that the effects of TCM on immune inflammation and esophageal motility have a multi-target and multi-level regulation.

RE is a chronic inflammatory disease of the esophageal mucosa that is caused by multiple factors [26]. We found that the pathological score of RE rats increased, and decreased after TCM treatment, which indicated that HXTJD can alleviate mucosal inflammation.

Numerous studies have demonstrated that inflammation of RE esophageal mucosa is related to the esophageal motility disorder [27]. Similarly, the LES contraction was severely impaired in the RE rats, and the change rates of mean contraction tension forces, mean amplitudes, and mean frequencies were significantly reduced. HXTJD restored the LES contraction in the RE rats, and the effect of H-HXTJD was excellent. So, we speculate that HXTJD affects the contraction of the LES muscle and increases esophageal motility in the RE rats. Whether esophageal motility impairment is related to delayed gastric emptying has been investigated. Lundell found that they were associated, possibly because delayed gastric emptying causes prolong postprandial gastric distention, maintains the stomach gastric contents, increases the risk of gastroesophageal reflux, and finally damages the esophagus [28, 29]. We here weighed the whole stomach and used phenol red gavage to determine the gastric emptying rate. The stomach weight increased in the RE rats, and the gastric emptying rate reduced. However, both these parameters improved after treatment with HXTJD, so HXTJD was speculated to affect gastric emptying and increase gastric motility in the RE rats.

Subsequently, the rat LES ultrastructure was observed. The number of ICC and SMC decreased in the RE group, and the organelles also exhibited varying degrees of damage, such as swelling and vacuolation of mitochondria, deregulation of the rough endoplasmic reticulum, and enlargement of gaps. After the HXTJD treatment, the number of ICC and SMC restored; mitochondria swelling and vacuolation improved; the number of rough endoplasmic reticulum increased; and gaps reduced. Therefore, HXTJD is believed to repair the LES ultrastructure damage in the RE rats.

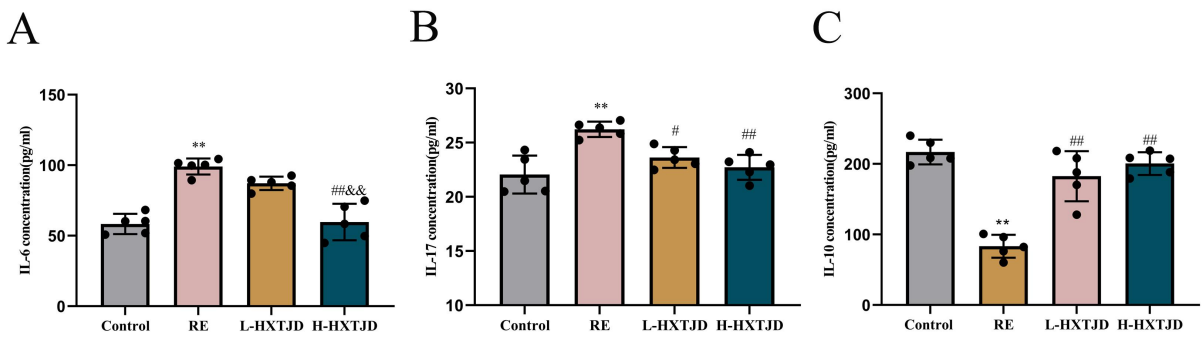

Figure 9 Effects of HXTJD on inflammatory factors concentrations in serum. (A) Effects of HXTJD on IL-6 concentration in serum. (B) Effects of HXTJD on IL-17 concentration in serum. (C) Effects of HXTJD on IL-10 concentration in serum. $^{**}P < 0.01$, compared with Control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with RE group; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$, compared with L-HXTJD group; n = 5. HXTJD, Huoxue Tongjiang decoction; L-HXTJD, low concentration group of HXTJD; H-HXTJD, high concentration group of HXTJD; RE, reflux esophagitis; IL-6, interleukin 6; IL-17, interleukin 17; IL-10, interleukin 10.

To further confirm the action mechanism of TCM in alleviating esophageal motility abnormalities, we explored motility-related crucial cells and proteins in LES. ICC is pacemaker cell that periodically spontaneously depolarize the esophagus and gastrointestinal tract and generate a pacemaker potential, which is the basis of muscle activity. Almost all gastrointestinal motility disorders cause injury and/or loss of ICC [30, 31]. Shafika first found that the decrease in ICC was chiefly concentrated in the gastroesophageal junction in GERD patients. They believed that this decrease was a critical reason for the decrease in the LES pressure and distal esophageal motility [32]. ICC can specifically express the c-kit receptor, and therefore, the c-kit protein is a specific ICC marker [33]. SCF, produced by SMC and neurons, is a natural ligand of the c-kit receptor. The specific combination of SCF and the c-kit receptor activates the c-kit/SCF signaling pathway and promotes ICC proliferation. Therefore, the activation of the SCF/c-kit signaling pathway is crucial for the normal development, maturation, and phenotype maintenance of ICC [34–37]. When the c-kit receptor is blocked, the number of ICC decreases, thus affecting the regeneration and maintenance of the ICC network [38]. The number of ICC and their branches are reduced, and the reticular structure is loose, thereby leading to intestinal dyskinesia [39]. We here observed that c-kit and SCF expression decreased, the ICC structure was loose, and the number of ICC-positive cells decreased in the RE group, which was consistent with the changes in ICC in the LES ultrastructure. After treatment with HXTJD, c-kit and SCF expression significantly increased, and the number of ICC-positive cells also increased. The results demonstrated that HXTJD increases esophageal motility by activating the SCF/c-kit/ICC pathway.

Furthermore, we explored the causes of gap enlargement in the LES ultrastructure and found a vital connexin. Cx43 widely exists in ICC or between ICC and SMC. Cx43 is the most critical connexin affecting the ICC function and the SMC contraction. The network of ICC, SMC, and neurons form the basic functional unit of esophageal gastrointestinal motility [40]. Studies investigating Hirschsprung's disease have found that the lack of Cx43-based signal transduction between ICC and SMC may partly cause gastrointestinal motility disorders [41, 42]. Some scholars have investigated the order of the decrease in Cx43 and ICC in the gastrointestinal tract. These studies found that with an increase in the age of the normal mice, the decrease in Cx43 expression appeared before ICC injury or loss [43]. In this study, Cx43 expression significantly decreased in the RE group and was restored after the HXTJD treatment. This was consistent with the changes in gaps in the LES ultrastructure observed. Therefore, the study demonstrated that the decrease in Cx43 expression in the RE rats leads to increased gaps, weakens intercellular signal transduction, and inhibits the SCF/c-kit/ICC signaling pathway, thereby affecting the normal development and function of ICC. HXTJD increases Cx43 expression to enhance intercellular signal transduction, activates the SCF/c-kit/ICC signaling pathway, restores the ultrastructure of the smooth muscle, and increases esophageal motility.

Enhanced LES contraction effectively inhibits the number of gastric juice reflux and reduces esophageal mucosal inflammation. In this study, HXTJD reduced the pathological score of the esophagus. An increasing number of scholars recently believe that cytokines mediate the occurrence of inflammatory injury. Our studies have reported that nuclear factor-κB (NF-κB) is a crucial pathway associated with esophageal inflammation. Activation of this pathway can release inflammatory factors such as tumor necrosis factor a [15]. This is consistent with the results of Souza Rhonda Frances. The author reported that reflux induces the activation of hypoxia inducible factor 2 alpha, thereby boosting the transcriptional activity of activated NF-κB and causing an increase in proinflammatory cytokine levels and T cell migration [44]. IL-6 is a crucial proinflammatory cytokine in RE and can induce the Th17/Treg cell imbalance [45]. Th17 and Treg cells are the third and fourth subgroups of T cells, respectively. The Th17/Treg cell imbalance is known to exist in diseases such as autoimmunity, inflammation, and cancer, especially ulcerative colitis [20, 46, 47]. However, the Th17/Treg cell imbalance has recently been shown to be closely related to RE patients [21]. Therefore, we here discussed the relationship between IL-6 and the Th17/Treg cell balance in RE and explored the effect of HXTJD on this relationship. In this study, the IL-6 concentration increased, the percentage of Th17 cells increased significantly, whereas that of Treg cells decreased in the RE rats. The key factors related to these cells also changed with the same trend, that is, the serum IL-17 concentration increased, and the IL-10 concentration decreased. After treatment with TCM, the results were reversed, which lead to a decrease in IL-6, IL-17 levels, and Th17 cells, and an increase in Treg cells and IL-10 levels. The study demonstrated that HXTJD inhibits Th17 cells proliferation and promotes Treg cells proliferation by reducing IL-6 secretion, regulating the Th17/Treg cell balance, reducing IL-17 secretion, increasing IL-10 secretion, and alleviating the inflammatory response of the RE rats.

Conclusion

There are 28 effective ingredients in HXTJD. HXTJD is an effective prescription for the RE treatment. HXTJD increased Cx43 expression, activated the SCF/c-kit/ICC pathway, and enhanced LES contraction, which thus increased esophageal motility. Moreover, HXTJD inhibited IL-6 secretion and regulated the Th17/Treg cell balance and the levels of inflammatory factors related to these cells, thereby ultimately alleviating esophageal inflammation.

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