Investigating the effect of Banxia Houpu decoction for the treatment of gastrointestinal dysfunction after multiple fractures: involvement of interstitial cells of Cajal

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Author contributions
Hua I, Wang IF, Huang IF designed and coordinated the study; Zhang Y, Fan MQ, Zhao WQ, Qian JS, Xu HH, Zheng Y performed the experiments, acquired, analyzed and interpreted data; Zhang Y, Zhao WQ and Fan MQ drafted the article and revised it critically. All authors contributed to the article and approved the submitted version.

Competing interests
The authors declare no conflicts of interest.

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Abbreviations
GI, gastrointestinal; ICC, interstitial cells of Cajal; SD, standard deviation.

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Abstract
Background: Fracture is a common large-organ traumatic injury and usually leads to complications in other systems. Multiple fractures are often associated with gastrointestinal (GI) dysfunction. However, no study has evaluated the treatment of GI dysfunction. The purpose of the study is to explore the efficacy and mechanism based on ICC pathway of Banxia-Houpu decoction on GI. Methods: Male Sprague-Dawley mice were randomly divided according to the fracture modeling method and intervention. We detected the “GI residual rate” and motility. Gastric antrum and jejunum tissues were dissected for hematoxylin and eosin staining to observe the structural integrity of the GI tract, and immunohistochemistry was used to detect the expression of c-kit protein. Results: Compared with the fracture group the GI residual rates in the Banxia Houpu decoction and mosapride groups were significantly low, while GI motility was significantly high. HE staining revealed significant GI tissue necrosis and inflammatory cell infiltrations in the fracture groups. The Banxia Houpu decoction and mosapride groups, exhibited less pathology. Immunohistochemical staining showed upregulated c-kit protein expression in the fracture groups. The c-kit protein level was decreased in the mosapride group. Additionally, c-kit protein expression in the Banxia Houpu decoction groups was significantly decreased in a concentration-dependent manner. Conclusion: Banxia Houpu decoction improves GI dysfunction after multiple fractures, reduces inflammation and necrosis of gastric epithelial cells, and inhibits c-kit protein expression in GI tissues of mice. Results showed revealed one of the mechanisms underlying the effects of this decoction on the GI dysfunction in mice after multiple fractures.

Keywords: Banxia Houpu decoction; c-kit; gastrointestinal diseases; multiple fractures; animal model

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Background

Incidence of fracture has increased in recent years. Every year in China, there are as many as 62 million patients due to trauma, of which seven hundred thousand to eight hundred thousand people die, and traumatic fractures account for the main part of traumatic injuries [1, 2]. Patients with multiple traumatic fractures often present with pain, stress, massive bleeding, and infections, among other symptoms [3]. Gastrointestinal (GI) dysfunction is a common complication after multiple fractures and hinders the rehabilitation of patients [4, 5]. Previous studies have shown that GI dysfunction is inextricably linked to signal transduction pathways involving interstitial cells of Cajal (ICCs) [6]. The fracture models used in this study were the same as those in our published article, which was the basis of this study [7]. Ten multiple fractures models were established based on the injury severity score (ISS) score, and five representative groups were selected for experiment. It has shown that these five common multiple fractures types could cause significant gastrointestinal dysfunction including abnormalities of the GI residual rate and motility and histopathological changes, and gastrointestinal dysfunction related to c-kit protein expression on interstitial cells of cajal.

The Banxia Houpu decoction, documented in the Synopsis of the Golden Chamber, is a famous Chinese prescription. Banxia Houpu decoction contains five Chinese herbs, Pinellia ternata (Thunb.), Makino (also called Banxia in traditional Chinese medicine) and Magnolia officinalis Rehder & E.H.Wilson (also called Houpu in traditional Chinese medicine) are multifunctional in traditional Chinese medicine. The former improves digestion and the latter removes dampness. *Poraria cocos* (also called Fuling in traditional Chinese medicine) benefits the spleen, Zingiber officinalis Rosco (also called Shengjiang in traditional Chinese medicine) warms the blood, and *Poria cocos* (L.) Britton (also called Zisu in traditional Chinese medicine) promotes ventilation and digestion. Banxia Houpu decoction contains these five herbs and plays a great and mutual role in improving gastrointestinal function.

Recent studies have shown that Banxia Houpu decoction promotes GI motility and gastric emptying, has anti-depressive and anxiolytic effects, and improves sleep disorders [4, 8, 9]. It is used for the treatment of GI and nervous system disorders, among other conditions. ICCs are present throughout the gut and transmit information required for normal GI function [10]. The c-kit gene is a key marker of ICCs [11]. We used immunohistochemistry to evaluate the expression of c-kit protein and determine whether signal transduction pathways involving ICCs play a role in the mechanism of action of Banxia Houpu decoction. Similar to Banxia Houpu decoction, Banxia Xiekin decoction regulates GI function through ICCs, which provided a basis for the present research [12].

Material and methods

Animal model

The experiment passed the animal experimental ethics review, and the experimental animal license number was (SYXK(Zhe)2018-0012). Experiments were approved by the ethics committee for research on laboratory animal use of the institution (I AUC-20210111-01).

Male C57BL/6 mice were obtained from Zhejiang Chinese Medical University Animal Experimental Center. In total, 150 male C57BL/6 mice, weighing 250g ± 50g, were randomly divided into groups (n = 5 per group; Table 1). The mice were acclimated by adaptive feeding for a week before the experiment. The mice were assigned to multiple fracture groups following the method used in the China National Health Service Survey, which was the first comprehensive nationwide survey of traumatic fractures conducted in China [2]. The most common types of multiple fractures were modeled. To model multiple fractures of long bones, the mice were given intraperitoneal anesthesia. After anesthesia induction, surgical vascular clamps were used to forcefully clamp both ends of the long bones simultaneously. When a sound indicative of bone fracture was heard, X-ray was used for confirmation. Using this method, closed fractures of the lower limbs and extremities were modeled. Pelvic, rib, and spinal fractures were created using vascular forces applied forcefully to the pelvis, ribs, and lumbar vertebral body, respectively. After fracture induction, an electrical lamp was used to irradiate the mice under anesthesia and keep them warm until consciousness was regained. After the mice regained consciousness, they were placed into individual mouse cages.

For modeling of the spinal fractures, the mice were placed in the left lateral decubitus position. Then, the fourth and fifth lumbar vertebrae were exposed in the right ventral posterolateral position; a vascular forceps was used to exert an external force on the lumbar vertebral body. The images of confirmed fractures were performed as described in our previous study [7].

Banxia Houpu decoction is composed of *Pinellia ternata* (Thunb.) Makino (Banxia) (15 g), *Magnolia officinalis* Rehder & E.H.Wilson (Houpu) (9 g), *Poraria cocos* (Fuling) (12 g), *Zingiber officinalis* Rosco (Shengjiang) (15 g) and *Poria cocos* (L.) Britton (Zisu) (6 g) at the ratio of 5:3:4:5:2 (Table 2). The five traditional Chinese medicines are provided by the Outpatient Department of Zhejiang Chinese Medicine University. The Chinese herbal components are placed in a decoction container and a 5-fold greater quantity of herbs is soaked in cold water for 2 h. Then, the mixture is boiled, gently fried for 30 min under intense heat, and filtered. A 2-fold greater quantity of water is added, and the mixture is then fried and boiled for a further 20 min and filtered. The filtrate was combined twice and concentrated to 30, 50, and 90 g/L. Mosapride citrate tablets (serial number: Sinophosphorat H20090158; Shanzhi Yabao Pharmaceutical, Yuncheng, China) were used for mosapride treatment.

Mice without Banxia Houpu decoction or mosapride treatment were administered saline, while 30, 60, and 90 g/L of Banxia Houpu decoction was administered to the low-, medium-, and high concentration-groups, respectively. Mosapride (0.15 g/L) was administered to the mosapride group. All mice were treated for 10 consecutive days.

Gastrointestinal function evaluation

A semi-solid nutritional paste was used to determine the “GI residual rate” and GI motility of mice. We dissolved 5 g of shuttle methyl cellulose in 125 mL of distilled water. Then, 8 g of milk powder, 4 g of sugar, and 4 g of starch were added successively to the mixture, stirring after each addition for complete mixing. Thereafter, 1.5 g of activated carbon was added to the mixture. Finally, almost 150 mL of the mixture (containing 150 g of the nutrient paste and activated carbon mixture) was stored at 4 °C until further use. Fasting was initiated 12 h before the measurements, although water was allowed until 2 h before the measurements. Each mouse was injected with 2 mL of paste by using a gavage needle, inserted along the esophagus almost 3 cm from the incisors, for 10 consecutive days. On day 11, 30 min after the final administration, the mice in each group were administered the semi-solid nutritional paste to determine the GI residual rate and GI motility.

After the mice were executed, their abdominal cavities were opened to remove the small intestines and stomachs. The stomach was weighed after obtaining a swab using filter paper. After killing the animals and opening the abdominal cavity, the small intestine was cut from the ileocecal region, removed and placed on white paper without traction. The distance between the pyloric sphincter and the first point of the content that corresponded to the “propulsion distance” of the activated carbon. The distance from the pyloric sphincter to the end of the ileum corresponded to the length of the small intestine. The GI residual rate and motility were calculated using the following equations: GI residual rate (%) = [(total weight of stomach – net weight of stomach)/weight of semi-solid nutritional paste] × 100%; and GI motility (%) = (propulsion distance of activated carbon/length of small intestine) × 100%.

Histopathological observation

The mice were executed 15 min after the final drug administration. Then, the abdominal cavity was opened, and the entire stomach and a
segment of the jejunum were removed and stored in 10% neutral formaldehyde solution to pre-fix the tissues. Then, each sample was embedded in paraffin and sectioned into 2–3 μm, followed by staining with HE (hematoxylin and eosin). After HE staining, the tissues were evaluated for histopathological changes.

**Immunohistochemical analysis**

After opening the abdominal cavity, a small segment of the antrum and jejunum was removed. Tissue samples were fixed with 4% buffered paraformaldehyde for 48 h at normal temperature. Samples were then embedded in paraffin and sectioned to 2–3 μm. Each sample was incubated with 100 μL PBS-diluted (1:1,000) rabbit monoclonal antibody [EPR22566-344] against c-Kit (ab256345, Abcam) overnight at 4 °C and then incubated with 40 μL PBS-diluted (1:1,000) Horseradish peroxidase (HRP) secondary antibody goat anti-rabbit (ab0101, Abcam) at room temperature for 30 min. Finally, the cells were incubated with 2-(4-aminophenyl)-6-indolecarbamidine DAPI staining solution for 5 min and observed under a light microscope.

**Statistical analysis**

The data were normally distributed. Data are presented as mean ± standard deviation (SD) and were analyzed using one-way analysis of variance. *P* value < 0.05 was considered to indicate a significant difference. Analyses were performed using SPSS software.

**Results**

**GI function evaluation**

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The efficacy of Banxia Houpu decoction and mosapride is shown in Figures 1, 2. Compared to the negative control (NC) group, the NC-mos, NC-low, NC-med, and NC-high groups had lower GI residual rates (each $P < 0.05$) and higher GI motility (each $P < 0.05$).

Compared to the NC group, the "pure model" groups had a higher GI residual rate (each $P < 0.05$) and lower GI motility (each $P < 0.05$). The GI function differed among fracture groups. Groups A (closed limb fractures) and E (spinal fractures) had the mildest and most severe injuries, respectively.

Compared to the "pure model" groups, the mos and low-, medium-, and high-concentration Banxia Houpu decoction groups had lower GI residual rates (each $P < 0.05$) and higher GI motility (each $P < 0.05$) than the non-pure-model A-E groups. The low- and medium-concentration Banxia Houpu decoction groups (A–E) showed no differences from the mosapride groups in GI residual rate or motility (each $P > 0.05$), whereas there were statistically differences between the mosapride and high-concentration Banxia Houpu decoction groups (each $P < 0.05$). The therapeutic effect of mosapride was intermediate between that of the low- and medium-concentrations of Banxia Houpu decoction, whereas the therapeutic effect of the high-concentration Banxia Houpu decoction was better than that of mosapride.

**Histopathological evaluation of mouse stomach and jejunum**

The histopathological analysis revealed structural changes and necrosis of jejunum tissues after multiple fractures. HE staining showed that the mucosa of the jejunum tissues in the NC group was smooth intact and normal, while the multiple fracture groups showed the jejunum tissues was incomplete, with the loss of mucosa, and revealed widespread cell necrosis accompanied by inflammatory cell infiltration in no treatment groups A–E. Otherwise, above changes were different between groups. The necrosis was much milder in groups A (closed limb fractures), B (limbs fracture with pelvic fracture), and C (limb fractures with multiple rib fractures) than the other groups, including groups D (pelvic fracture with multiple rib fractures) and E (spinal fractures). However, mosapride could obviously decrease the necrosis of jejunum. Banxia Houpu decoction treatment also ameliorated the pathological changes of jejunum, especially the medium- and high-concentration had even better effect than the mosapride in deceasing the necrosis of the epithelium (Figure 3).

**Immunohistochemical analysis of c-kit protein expression**

Figure 1 GI residual rates of the mouse groups (mean ± SD). $P < 0.05$, $\Delta\Delta P < 0.01$ compared to NC group. $* P < 0.05$ (other fracture groups vs. pure model group). $** P < 0.05$ (other fracture groups vs. mosapride group).

Figure 2 GI motility of the mouse groups (mean ± SD). $* P < 0.05$, $\Delta P < 0.01$ compared to NC group. $* P < 0.05$ (other fracture groups vs. pure model group). $** P < 0.05$ (other fracture groups vs. mosapride group).
Many studies have reported that c-kit expression was associated with gastrointestinal dysmotility diseases, so we used the immunohistochemical analysis of c-kit protein expression in jejunum. Yellow-brown particles were rarely observed in the cytoplasm of the mucosa cells in jejunum tissue of NC group, suggesting the low c-kit protein expression (Figure 4), while the yellow-brown substance was increased in the multiple fracture groups, particularly groups C (limb fractures with multiple rib fractures), D (pelvic fracture with multiple rib fractures), and E (spinal fractures). After the treatment of mosapride, the expression of c-kit was decreased in the mucosa cells of jejunum tissue, compared with the no treatment groups. Banxia Houpu decoction had the same efficacy, especially the medium- and high-concentration, suggesting that the Banxia Houpu decoction has concentration-dependent effects in decreasing the expression of c-kit in jejunum.

Discussion

As an important organ for digesting food, absorbing nutrients and releasing waste, the normal function of the gastrointestinal tract is the basis for maintaining the normal function of the body [13]. However, patients with multiple traumatic fractures are more likely to have stomach problems such as GI dysfunction, including abdominal pain and alternating constipation and diarrhea. The GI dysfunction may be caused by bleeding into the surrounding soft tissue after multiple traumatic fractures or compressive stimulation of the peripheral sympathetic ganglia by hematoma produced during surgery, which may lead to severe abdominal distension, constipation, nausea, and vomiting, among other symptoms, resulting in a poor prognosis and low quality of life [14–16].

Therefore, this study established different models of gastrointestinal dysfunction after multiple fractures, and evaluated the gastrointestinal function. In this study, different types of multiple fractures were performed on mice to simulate the symptoms of gastrointestinal dysfunction that may occur in patients with different types of multiple fractures. The results showed that different types of multiple fractures may have different effects on GI dysfunction. Based on the severity of abnormalities of the GI residual rate and motility, histopathological changes, and c-kit protein expression, closed limb fractures were associated with the mildest abnormalities, followed by limb fracture with pelvic fracture, limb fractures with multiple rib fractures, pelvic fracture with multiple rib fractures, and spinal fractures, in order of increasingly severe abnormalities. Spinal fractures are associated with the most severe GI dysfunction because the GI tract is innervated by the sympathetic and parasympathetic nerves; therefore, spinal trauma may activate the inhibitory sympathetic reflex system, enhance the activity of the GI sympathetic nervous system, and inhibit GI peristalsis by suppressing activity of the excitatory neurons of the GI plexus [17].

We also found that Banxia Houpu decoction has a gastrointestinal effect to a great extent, significantly improved GI motility and reduced the GI residual rate in mice with multiple fractures. Banxia Houpu decoction showed significant concentration-dependent effects in all types of multiple fractures, including decreased c-kit protein expression in the jejunum and reduced inflammation and necrosis of the gastric epithelial cells (in a concentration-dependent manner) in mice with multiple fractures. In previous studies, Banxia Houpu decoction improved GI motility disorders after lumbar fracture surgery, chronic mild stress after sucrose consumption, and gastroesophageal reflex disease due to Qi Depression and Phlegm-Stagnation [2, 18, 19], among other GI diseases. These studies have demonstrated that Banxia Houpu decoction may improve GI dysfunction by regulating the GI nerves. Most importantly, this study innovatively explored the mechanism that may play a therapeutic role: involvement of interstitial cells of Cajal (ICC). ICCs are special interstitial cells present throughout the GI tract that act as pacemakers of GI slow waves and transmit information between GI nerves and smooth muscle cells. These cells play important roles in maintaining normal GI function. Modern studies have demonstrated that c-kit expression in the colon cells promotes the proliferation and migration of intestinal epithelial cells [20]. Many GI functional diseases, such as functional dyspepsia, are accompanied by changes in the number, function, and reticular structure of ICCs [13]. Stable GI function requires normal ICC function. ICCs are also important treatment targets for GI diseases [21, 22]. For example, the treatment of diabetic gastroparesis is based on signal transduction pathways involving ICCs [23]. Astragaloside IV alleviates slow-transit constipation by modulating the gut microbiota and promoting the production of butyric acid through ICC-based signal transduction pathways [24]. Non-invasive auricular vagal nerve stimulation improves GI dysmotility by revealing the expression of ICC proteins [25]. Currently, c-kit immunohistochemistry is used to measure the expression of ICC proteins [26]. C-kit has been considered as a possible therapeutic target for gastrointestinal mesenchymal tumors and relevant inhibitors are under investigation [27]. We evaluated c-kit protein expression to explore the efficacy and mechanism of action of Banxia Houpu decoction for treating GI dysfunction after multiple fractures.
Conclusions

The severity of GI dysfunction differed among different multiple fractures models. The spinal fracture and pelvic fracture with multiple rib fracture were the most severe. Banxia Houpu decoction promoted GI dysfunction, reduced inflammation and necrosis, and decreased c-kit expression, which may explain its therapeutic effects on GI dysfunction. Mosapride also improved GI dysfunction, exhibiting a therapeutic effect intermediate between those of the low- and medium-concentration Banxia Houpu decoctions.

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