Effects of a Combination of Fu Zi and Rou Gui on Intestinal Neurotransmitters and Microflora in Rats with Slow Transit Constipation

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Abstract [**Objectives**] This study was carried out to explore the combined effects of Fu Zi (Radix Aconiti Lateralis Praeparata, the secondary root of perennial herbaceous plant Acontium carmichaeli Dehx. of family Ranunculaceae) and Rou Gui (Cortex Cinnamomi, the bark of *Cinnamamunz cassia* Presl of family Lauraceae) on intestinal neurotransmitters and microflora in rats with slow transit constipation (STC). [**Methods**] Experimental rats were given loperamide hydrochloride by gavage to induce STC, and then treated with Fu Zi alone, Rou Gui alone, a combination of Fu Zi and Rou Gui (2∶1 *w/w*), and prucalopride, respectively, for 14 days. Meanwhile, the general condition, the time to first black stool and the rate of intestinal propulsion of rats in each group were observed after STC was induced and after drug treatment, and the pathological changes in rat colon were observed *via* hematoxylin-eosin (HE) staining, and the levels of colonic 5-hydroxytryptamine (HT), vasoactive intestinal peptide (VIP) and substance P (SP) were detected by ELISA, and the changes in intestinal flora were detected by 16S rRNA Real-time PCR. [**Results**] Compared with healthy rats, the time to first black stool and the rate of intestinal propulsion, colonic 5-HT and SP levels significantly decreased ($p<0.01$), while their colonic VIP level significantly increased ($p<0.01$). Compared with STC rats, the time to first black stool, the rate of intestinal propulsion, colonic 5-HT and SP levels in Fu Zi-Rou Gui (2∶1) treated rats and prucalopride treated rats significantly increased ($p<0.01$), while their colonic VIP level significantly decreased ($p<0.01$). There was no significant difference in alpha diversity between healthy rats and STC rats. However, analysis on beta diversity revealed that there were differences in microflora structure and composition between them. Compared with healthy rats, the relative abundance of Firmicutes and Proteobacteria in STC rats significantly increased, while that of Bacteroidetes decreased. Compared with STC rats, the relative abundance of Proteobacteria decreased and that of Bacteroidetes and Firmicutes increased in Fu Zi-Rou Gui (2∶1) treated rats; the relative abundance of Bacteroidetes and Proteobacteria decreased while that of Firmicutes increased in Fu Zi treated rats; the relative abundance of Proteobacteria decreased while that of Bacteroidetes increased in Rou Gui treated rats; the relative abundance of Firmicutes and Proteobacteria decreased while that of Bacteroidetes increased in prucalopride treated rats. The intestinal flora in rats of all groups was dominated by *Lactobacillus* spp. and other genera of anaerobic bacteria. Compared with healthy rats, the relative abundance of Lactobacillus spp. and Clostridium spp. in STC rats decreased, while those of *Blautia* spp. and *Ruminococcus* spp. and Allobaculum spp. increased. Compared with STC rats, the relative abundance of Lactobacillus spp. in all rats treated with drugs increased. [**Conclusions**] The combination of Fu Zi and Rou Gui (2∶1) can effectively improve intestinal motility in STC rats by regulating intestinal microbial community and the levels of colonic neurotransmitters.

Key words Slow transit constipation (STC); Fu Zi-Rou Gui; Intestinal motility; Neurotransmitters; Intestinal microflora

Slow transit constipation (STC) is a functional colonic disorder characterized by delay in transit of stool through the colon caused by various reasons[1]. Due to the lack of effective drugs, STC is usually treated by using laxatives, surgical approaches and biofeedback therapy *etc.* in modern medicine. However, laxatives may lead to metabolic disorders, dehydration, colonic melanosis and laxative colonization and other side effects, even induce cancer, or may be poorly tolerated by patients[2-4]. And for these reasons, Chinese traditional medicine is getting more and more attention in STC treatment and research.

Previous studies have shown that the occurrence of intestinal dysmotility which is the main pathological change of STC is closely associated with intestinal flora disorders. The homeostasis of gut flora helps to improve the intestinal immune barrier and maintain intestinal motility. The changes in intestinal flora will alter gastrointestinal motility, mainly by affecting enteric neurotransmitters such as 5-HT, VIP and SP.

In Chinese traditional medicine, it is believed that STC is a type of constipation. According to yin and yang theory, the normal physiological function of the large intestine depends on yang energy, which is active and responsible for moving and warming in human body. In this study, STC rats were treated with a combination of Fu Zi (Radix Aconiti Lateralis Praeparata, the secondary root of perennial herbaceous plant Acontium carmichaeli Dehx. of family Ranunculaceae) and Rou Gui (Cortex Cinnamomi, the bark of *Cinnamamunz cassia* Presl of family Lauraceae), then the changes of 5-hydroxytryptamine (HT), vasoactive intestinal peptide (VIP) and substance P (SP) and intestinal flora were measured to reveal the roles of the combination in improving intestinal motility in STC rats.

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Materials and Methods

Materials

Animals 35 male and 35 female specific-pathogen-free (SPF) Sprague Dawley (SD) rats [SYXK(Chuan) 2015-010], 4 to 6 weeks old, weighing (200 ± 20) g, were purchased from Chengdu Dossy Experimental Animals Co., Ltd., kept in individual cages, five rats per cage, at a temperature 20 – 26 ℃ and a relative humidity 40% – 70%, with free access to diet and water, exposed to light all day and night.

Experimental drugs and preparation Prucalopride tablets (2 mg/tablet, Jiangsu Hansoh Pharmaceutical Group Co., Ltd., Lot: 13320070,) were ground to powder, prepared into 0.018 mg/ml suspension with distilled water just before use. The daily dose of prucalopride in rats was 0.18 mg/kg, which was calculated based on its recommended dose 2 mg in human adults weighing 70 kg.

Loperamide hydrochloride (2 mg/capsule, Xian Janssen Pharmaceutical Ltd., Lot: JGJOM4J), was prepared with distilled water into 0.2 mg/ml suspension just before use.

Fu Zi (Guiyang Jirentang Traditional Chinese Medicine Tablet Factory, Lot: 20200801) and Rou Gui (Guiyang Jirentang Traditional Chinese Medicine Tablet Factory, Lot: 20200901) were purchased from the Second Affiliated Hospital of Guizhou University of Traditional Chinese Medicine. 15 g of Fu Zi, 15 g of Rou Gui and 10 g of Fu Zi/5 g of Rou Gui (2∶1) were respectively weighed, as their daily dose in human adults is 15 g. Their daily dose in rats was calculated based on body surface area (BSA) by the equation: Daily dose in rats = 6.25 × (15 g/70 kg) = 1.3 g/kg. Fu Zi decoction was prepared by boiling the herbal medicine in water twice, 60 min for the first time and 25 min for the second time. Rou Gui decoction was prepared by boiling the herbal medicine in water twice, 30 min for the first time and 25 min for the second time. Fu Zi-Rou Gui (2∶1) was prepared by the following steps: Fu Zi was boiled alone in purified water for 30 min, before Rou Gui was added, and boiled for another 30 min. After the liquid was filtered out, the remaining dregs were boiled in purified water for another 25 min. The two decoctions were put together to give the decoction of Fu Zi-Rou Gui (2∶1). All the decoctions prepared above were concentrated in boiling water bath to a concentration 0.13 g of raw herb/ml, and stored at 4 ℃.

Reagents The reagents used in the present study included rat VIP ELISA Kit (48 Tests, Shanghai Zhuocai Biotechnology Co., Ltd., Lot: ZC-37398), hematoxylin (500 ml/bottle, Wuhan Servicebio Technology Co., Ltd., Lot: ZH202509), eosin (500 ml/bottle, Wuhan Servicebio Technology Co., Ltd., Lot: CR2011064), Q5 High-Fidelity DNA Polymerase (NEB, Lot: M0491L), Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Lot: 75510-019), agarose (Invitrogen, Lot: 75510-019), Marker (Takara, Lot: DL2000) and TEA (Invitrogen, Lot: AM9870).

Instruments The instruments used in the present study included Spectra MAX Plus384 Microplate Reader (Molecular Devices Corporation), KZ-III-F high-speed tissue homogenizer (Wuhan Servicebio Technology Co., Ltd.), D3024 desktop microcentrifuge (Beijing Dragon Laboratory Instruments Limited), UPH-II-10T Ulupure laboratory water system (Chengdu Ultra-pure Technology Ltd.,), DZKW-4 electric-heated thermostatic water bath (Beijing Zhongxing Weiye Instrument Co., Ltd.), Leica-2016 rotary microtome (Germany), JT-12S rapid tissue processor (Wuhan Junjie Electronics Co., Ltd.), BMJ-A tissue embedder (Changzhou Zhongwei Electronic Instrument Factory), RS36 autostainer (Changzhou Paisijie Medical Equipment Co., Ltd.), PHY-III slide dryer (Changzhou Zhongwei Electronic Instrument Co., Ltd.), Pannoramic 250 digital scanner (3D Histech, Hungary), PCR instrument (2720, ABI), FLX800T microplate reader (BioTec), DYY-6C electrophoresis system (Beijing Liuyi), BG-gds Auto (130) gel imaging system (Beijing Baygene Biotech.) and NC2000 Nanodrop (Thermo Scientific), *etc.*

Methods

Modeling and grouping All rats were acclimatized for one week, 15 of them were randomly selected and used as control, and the remaining 55 rats were given 8 mg/kg loperamide hydrochloride suspension intragastrically for 45 days, while the rats of control group were given an equal volume of water. Then, five rats in each group were randomly selected to calculate their fecal water content and intestinal propulsion rate. Decreased fecal water content and intestinal propulsion rate proved the achievement of STC induction in rats. Finally, the STC rats were randomly divided into five groups: STC group, Fu Zi-Rou Gui (2∶1) group, Fu Zi group, Rou Gui group and prucalopride group, with 10 rats in each group.

Drug administration After STC modeling, rats were given the decoctions of Fu Zi, Rou Gui, Fu Zi-Rou Gui (2∶1), or 0.18 mg/kg prucalopride by gavage once every day for 14 days, while the rats of control group and STC group were given 10 ml/kg distilled water.

Measurement of the time to first black stool After STC modeling and drug administration, five rats were randomly selected from each group, and given Indian ink by gavage (2 ml/rat), and the duration from the gavage to the passage of first black stool was measured, defined as the time to first black stool.

Measurement of the rate of intestinal propulsion After STC modeling and 30 min after the last administration of drugs, five rats were randomly selected from each group, given 10% carbon powder (suspended in 2% CMC-Na, 0.5 ml/rat) by gavage, executed *via* cervical dislocation20 min later. Then, their intestines were immediately removed, and the distance from the pylorus to ileocecal junction and the distance ink moved in the intestines (the distance from the pylorus to the front end of ink content) were measured using a ruler. The rate of intestinal propulsion = The distance ink moved/The distance from the pylorus to ileocecal junction × 100%".

Observation of pathological changes *via* hematoxylin-eosin (HE) staining After STC modeling and drug administration, the colonic tissues were collected, embedded in paraffin and

sectioned. In the next step, the sections were dewaxed, rehydrated, immersed in a hematoxylin solution, rinsed with tap water, followed by 5 – 10 s of acid alcohol differentiation. After a water rinse, the sections were immersed in warm water at 50 ℃ or in a weak base solution till the reddish stain of the Hematoxylin turned to blue. After that, the sections were rinsed under tap water, immersed in 85% alcohol, stained with eosin for 3 – 5 min, washed again with tap water for 3 – 5 s, dehydrated through ascending strengths of alcohol, cleared in xylene and finally fixed with neutral glue. Subsequently, the finished slides were observed and scanned under a Pannoramic 250 digital scanner (3D Histech, Hungary).

Measurement of colonic 5-HT, VIP and SP levels *via* ELISA

Part of colonic tissue was collected and accurately weighed, homogenized in nine volumes of PBS using tissue homogenizer. Then, the homogenate was centrifuged for 10 min at 5 000 × g, and the supernatant was subjected to a conventional ELISA assay to measure the levels of colonic 5-HT, VIP and SP.

Detection of changes in intestinal flora by 16S rRNA real-time PCR Some rat feces were sampled to extract total microbiome DNA, which was assayed and quantified *via* a real-time PCR targeting 16S-rRNA gene. Sequencing libraries were prepared using TruSeq Nano DNA LT Library Prep Kit (Illumina).

Statistical analysis The data of each group were expressed as mean ± standard de*via*tion. Spss. 21. 0 was used for data analysis, and Graphpad prism 8. 0 software for plotting. One-way ANOVA was used for significance analysis, least significant difference (LSD) test (equal variance assumed) and Tamhane's test (equal variance not assumed) were used for pairwise comparison. The results are considered to be statistically significant if $P < 0.05$.

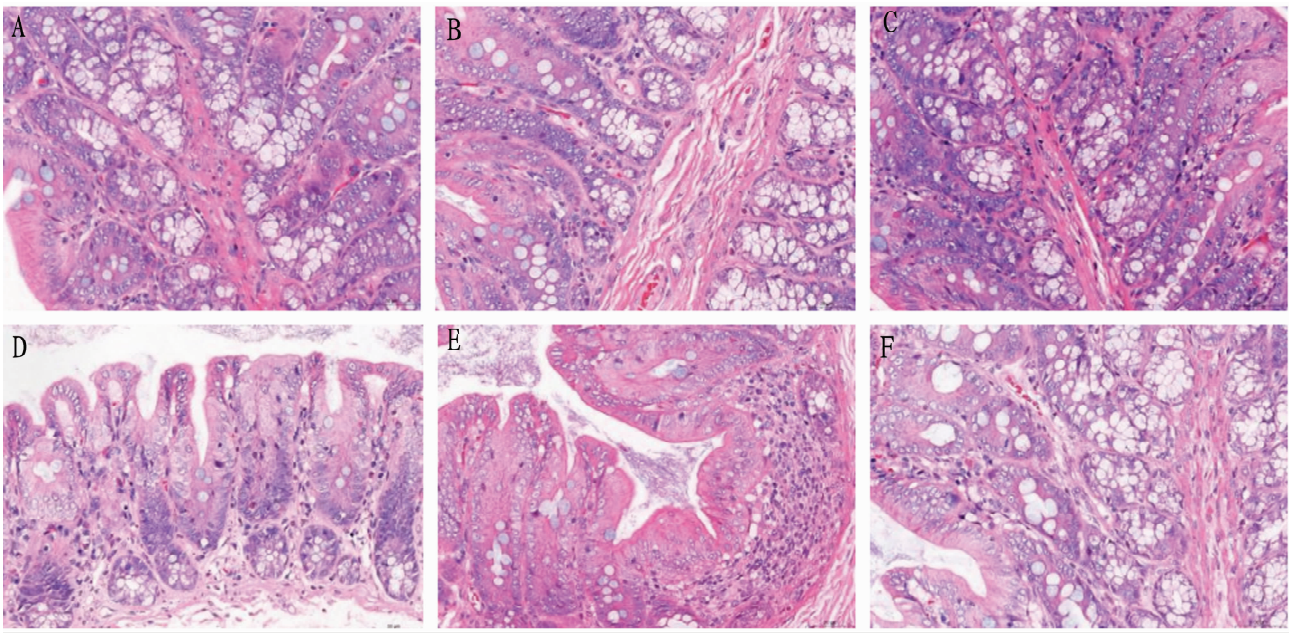
Results and Analysis

Comparison of general conditions of rats

During STC modeling, the healthy rats of the blank control moved freely, responded quickly, grasped and crawled vigorously, had shiny hair, thick whiskers, regular diet and bowel movement. However, the STC rats were less active and responsive, with bruised paw nails, bowed backs and thinner hair. These symptoms were improved after the rats were given herbal medicines.

Pathological changes in rat colon

HE staining showed that at the end of STC modeling, the healthy rats had intact mucosal layer, submucosal layer, muscle layer and plasma layer in the colon, and the mucosal surface was covered with a single layer of columnar epithelial cells that had normal morphology, and no obvious pathological changes were observed. In contrast, pathological changes such as mucosal epithelial cells peeling off, necrosis of mucosal lamina propria, infiltration of the lamina propria by inflammatory cells, and reduced number of cup-shaped cells, were observed in only a few STC rats, no obvious pathological changes were seen in most STC rats. In the majority of the rats treated with drugs, the colon tissues had normal morphology and structure, showing no obvious histopathological damage, except that decreased number of cupped cells and inflammatory cell infiltration were observed in very few of the rats treated with drugs, suggesting that STC modeling and drug interventions did not induce obvious histopathological changes in the colon tissues of rats (Fig. 1).



A. Control, showing no obvious histopathological damages; B. STC model rats, showing slight inflammatory cell infiltration; C. Fu Zi-Rou Gui (2∶1) treated rats, showing slight reduction in the number of cupped cells; D. Fu Zi-treated rats, showing no obvious histopathological damages; E. Rou Gui-treated rats, showing slight inflammatory cell infiltration; F. prucalopride-treated rats, showing no obvious histopathological damages.

Fig. 1 Histomorphological changes of colon in rats of the six groups (HE ×400)

Changes in the time to first black stool and the rate of intestinal propulsion

Compared with the healthy rats of control group, the time to first black stool and the rate of intestinal propulsion in STC rats obviously decreased, and the difference was statistically significant ($P<0.01$), indicating that STC model was achieved, as shown in Table 1. Compared with the STC rats, the time to first black stool and the rate of intestinal propulsion in rats treated with drugs both increased, and the increases in rats treated with Fu Zi-Rou Gui (2∶1) and prucalopride were statistically significant ($P<0.01$), as shown in Table 2.

Table 1 Comparison of the time to first black stool and the rate of intestinal propulsion between healthy and STC rats ($n=5$, $\bar{x}\pm s$) after STC modeling

Groups	The time to first black stool//min	The rate of intestinal propulsion//%
Control group	327.00±21.38	66.25±5.01
STC rats	604.80±22.04**	38.88±2.29**

**, Difference was statistically significant at $P<0.01$.

Table 2 Comparison of the time to first black stool and the rate of intestinal propulsion in rats after drug administration

Groups	Dose	The time to first black stool//min	The rate of intestinal propulsion//%
Control group	–	346.00±18.21	64.11±6.00
STC rats	–	598.25±21.02**	40.08± 2.31**
Fu Zi-Rou Gui (2∶1)-treated rats	1.3 g/kg	398.55±16.26##	58.12±0.98##
Fu Zi-treated rats	1.3 g/kg	453.21±15.32**	50.08±1.62
Rou Gui-treated rats	1.3 g/kg	469.13±4.32#	52.44±1.04#
Prucalopride-treated rats	0.18 mg/kg	403.15±11.82##	56.02±0.55##

Compared with the blank control, *$P<0.05$, **$P<0.01$; compared with STC rats, #$P<0.05$, ##$P<0.01$.

Changes in colonic 5-HT, VIP and SP in rats

Compared with the healthy rats of control group, colonic 5-HT and SP levels in STC rats significantly decreased, while VIP level significantly increased. The differences between the two groups were statistically significant ($P<0.01$). Compared with the STC rats, colonic 5-HT and SP levels increased and VIP level decreased in rats treated with Fu Zi-Rou Gui (2∶1), Fu Zi and prucalopride, and the differences were statistically significant ($P<0.01$), as shown in Table 3.

Table 3 Changes in colonic 5-HT, VIP and SP levels in rats after drug administration ($\bar{x}\pm s$, $n=10$)

Group	Dosage	5-HT//ng/mL	VIP//pg/mL	SP//ng/mL
Control group	–	4.03±0.20	6.45±1.01	8.23±1.17
STC rats	–	1.85±0.40**	12.38±1.69**	5.08±0.47**
Fu Zi-Rou Gui (2∶1)-treated rats	1.3 g/kg	3.82±0.45##	7.55±0.09##	7.09±1.02##
Fu Zi-treated rats	1.3 g/kg	3.08±0.28##	8.72±0.32#	6.65±1.12#
Rou Gui-treated rats	1.3 g/kg	2.99±0.29	8.32±1.01	6.41±0.43
Prucalopride-treated rats	0.18 mg/kg	3.90±0.21##	7.00±0.58##	7.52±0.43##

Compared with the blank control, *$P<0.05$, **$P<0.01$; compared with STC rats, #$P<0.05$, ##$P<0.01$.

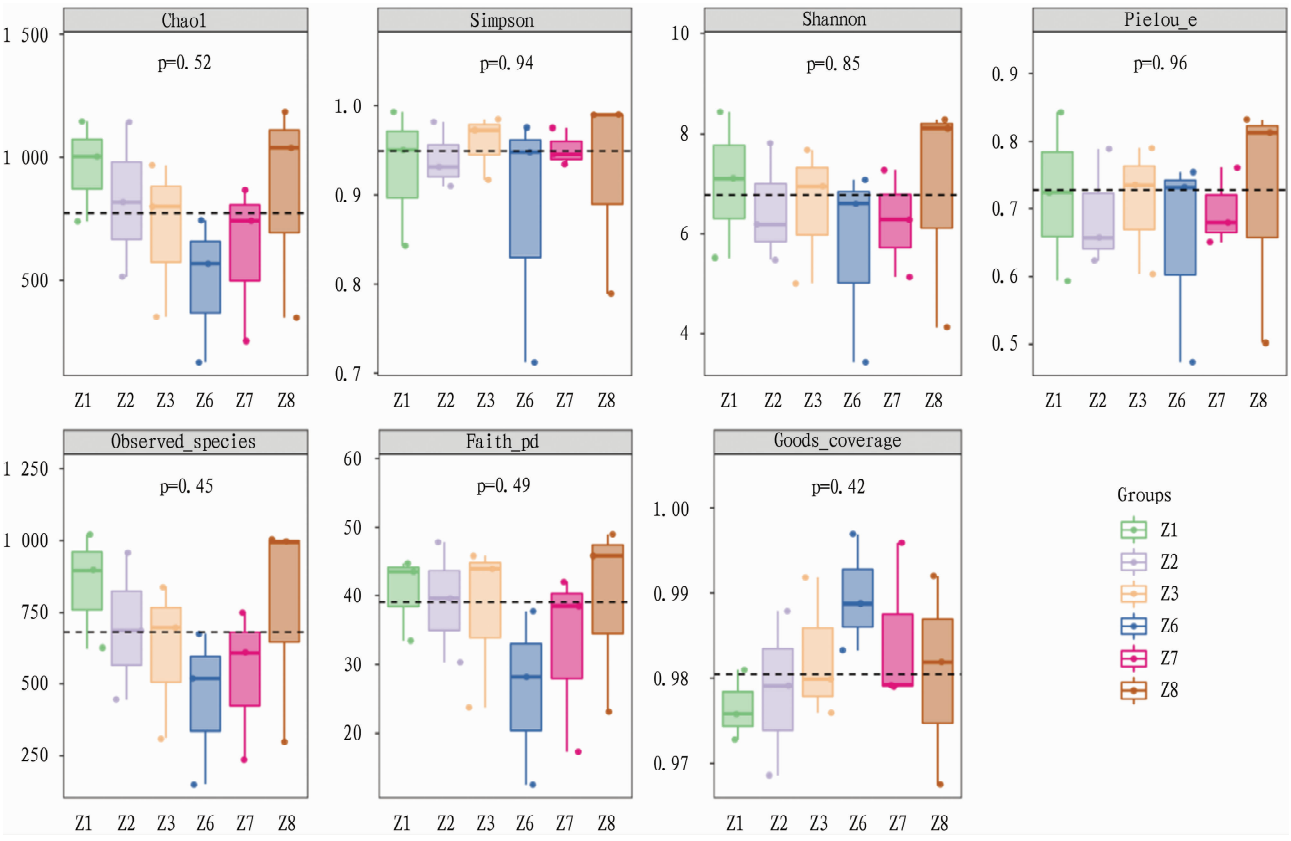
Changes in intestinal flora in rats treated with different drugs

Effects of Fu Zi-Rou Gui (2∶1) on alpha diversity of intestinal flora in rats To assess the alpha diversity of intestinal flora in rats treated with drugs more precisely, the richness was characterized by Chao1 and Observed species indices, the diversity by Shannon and Simpson indices, the evolutionary-based diversity by Faith's PD index, the evenness by the Pielou's evenness index, and the coverage by Good's coverage index. The results showed that there were no significant differences in these six indices between the six groups, i.e., their alpha diversity was similar, suggesting that there were no significant differences in species richness of the intestinal flora between drug-treated rats and STC rats (Fig. 2).

Effect of Fu Zi-Rou Gui (2∶1) on beta diversity of intestinal flora in rats Beta diversity was analyzed using principal coordinate analysis (PCoA). As shown in Fig. 3, each point represented a sample, and the closer the samples were, the more similar composition they shared. It could be seen that the samples of healthy rats and STC rats were distantly located in different quadrants, indicating that gavage with loperamide hydrochloride changed the composition of intestinal microflora. In addition, most samples of Fu Zi-Rou Gui (2∶1)-treated rats, Fu Zi-treated rats, Rou Gui-treated rats and prucalopride-treated rats were not located in the quadrant where the samples of STC rats were, and only one sample of Rou Gui-treat rats, one of Fu Zi-treated rats and one of prucalopride-treated rats were located in the same quadrant where the samples of STC rats were, but at far distances, suggesting that these drugs affected similar species and changed the composition of intestinal flora.

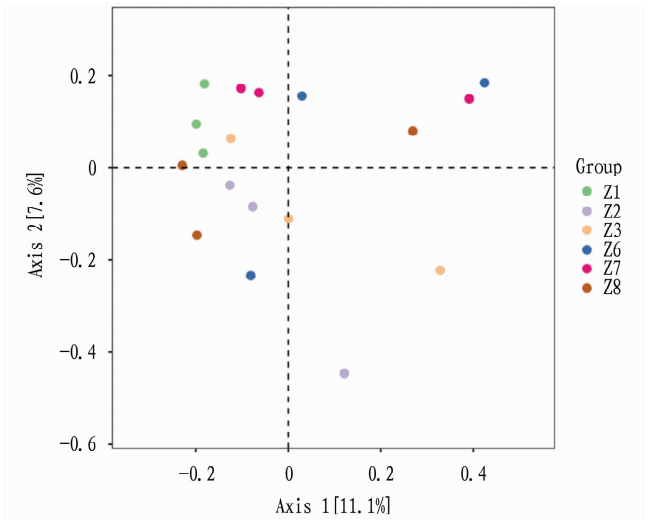
Effect of Fu Zi -Rou Gui (2∶1) on abundance of intestinal flora in rats

(1) The number of intestinal flora species shared by the six groups. The petal diagram in Fig. 4 showed the number of the same amplicon sequence variants (ASVs) shared by the six groups. In this diagram, each ellipse represents a group. The overlapping area in the middle of all ellipses indicates the number of ASVs shared by all groups, and the number at the other end of each ellipse is the number of ASVs of that group. The diagram shows that a total of 119 ASVs were shared by the six groups.



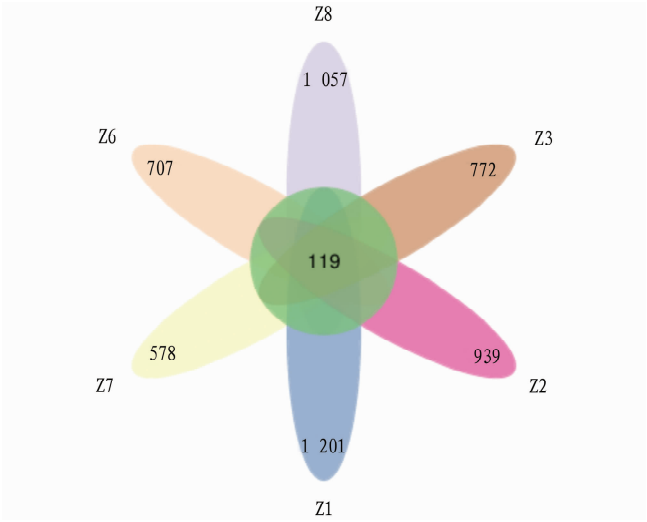
Z1, Control group; Z2, STC rats; Z3, Fu Zi-Rou Gui (2∶1)-treated rats; Z6, Fu Zi-treated rats; Z7, Rou Gui-treated rats; Z8, prucalopride treated rats.

Fig. 2 Boxplots of alpha diversity of the six groups



Z1, Control group; Z2, STC rats; Z3, Fu Zi-Rou Gui (2∶1)-treated rats; Z6, Fu Zi-treated rats; Z7, Rou Gui-treated rats; Z8, prucalopride treated rats.

Fig. 3 Analysis of beta diversity (PCoA)



Z1, Control group; Z2, STC rats; Z3, Fu Zi-Rou Gui (2∶1)-treated rats; Z6, Fu Zi-treated rats; Z7, Rou Gui-treated rats; Z8, prucalopride treated rats.

Fig. 4 Petal diagram based on ASV showing the number of microbial species shared by the six groups

(2) Analysis of composition of intestinal flora in rats. At the phylum level, all the six groups were dominated by Firmicutes and Bacteroidetes. The intestinal microflora of the control group was dominated by Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria and Verrucomicrobia. Compared with the control group, the relative abundance of Firmicutes, Proteobacteria and Verrucomicrobia in STC rats significantly increased, while the relative abundance of Bacteroidetes decreased. Compared with STC rats, the relative abundance of Proteobacteria in Fu Zi-Rou Gui (2∶1)-treated rats decreased, while the relative abundance of

Bacteroidetes, Verrucomicrobia and Firmicutes increased; the relative abundance of Bacteroidetes and Proteobacteria in Fu Zi-treated rats decreased, while that of Verrucomicrobia and Firmicutes increased; the relative abundance of Proteobacteria and Verrucomicrobia in Rou Gui-treated rats decreased, while that of Bacteroidetes increased; the relative abundance of Firmicutes, Proteobacteria and Verrucomicrobia in prucalopride-treated rats decreased, while that of Bacteroidetes increased (Fig. 5).

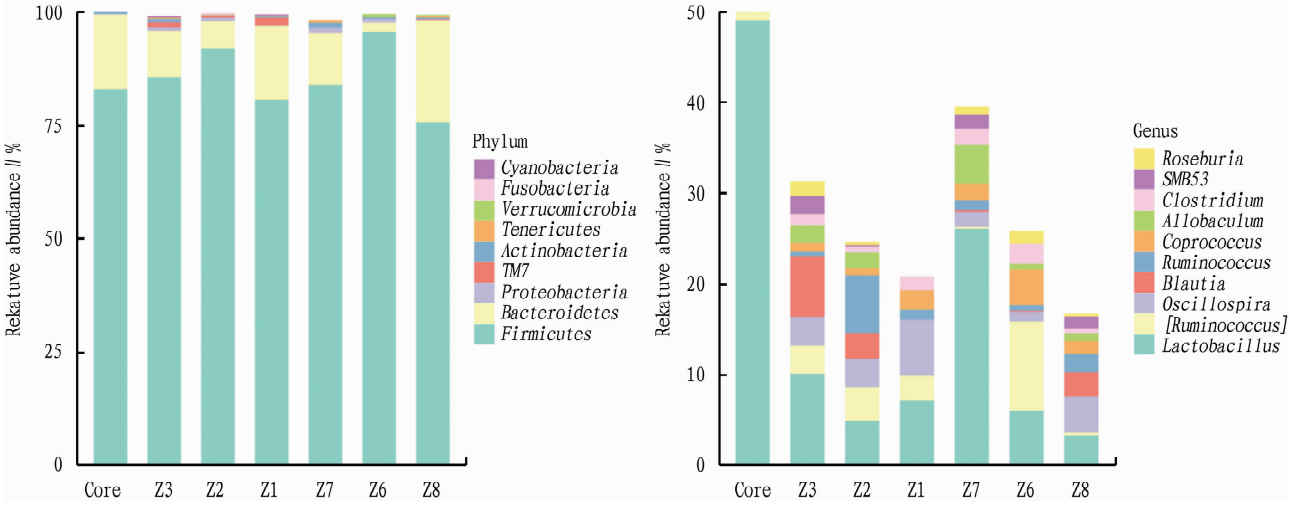


Fig. 5 Relative abundance of intestinal flora at the phylum and genus levels

At the genus level, the intestinal microflora of all groups was dominated by *Lactobacillus*. In detail, *Lactobacillus*, *Ruminococcus*, *Oscillospira*, *Clostridium* and *Coprococcus* were dominant genera in the control group, *Lactobacillus*, *Allobaculum*, *Oscillospira*, *Ruminococcus* and *Blautia* were dominant genera in STC rats. Compared with the control group, the relative abundance of *Lactobacillus*, *Oscillospira*, *Coprococcus* and *Clostridium* in STC rats decreased, while that of *Blautia*, *Rumenococcus* and *Allobaculum* increased. Compared with STC rats, the relative abundance of *Lactobacillus*, *Blautia*, *Clostridium*, SMB53 and *Roseburia* in Fu Zi-Rou Gui (2∶1) treated rats increased, while that of *Ruminococcus* decreased; the relative abundance of *Lactobacillus*, *Coprococcus*, *Clostridium* and *Roseburia* in Fu Zi-treated rats increased, while that of *Oscillospira*, *Ruminococcus*, *Allobaculum* and *Blautia* decreased; the relative abundance of *Lactobacillus*, *Coprococcus*, *Allobaculum*, *Clostridium*, *SMB53* and *Roseburia* in Rou Gui-treated rats increased, while that of *Oscillospira* and *Ruminococcus* decreased; the relative abundance of *Oscillospira*, *Coprococcus* and SMB53 in prucalopride-treated rats increased, while that of *Lactobacillus*, *Ruminococcus* and *Allobaculum* decreased (Fig. 5).

Conclusion and Discussion

In Chinese traditional medicine, it is believed that STC is a type of constipation, which occurs in the large intestine, and is associated with the lung, spleen and kidney[5]. Modern clinical research indicates that STC is mostly caused by overwork, exhaustion of qi, consuming too much cold food, overuse of cold drugs, especially abuse of antibiotics, bitter, cold and purgative drugs such as Da Huang (rhubarb) and Mang Xiao (Natrii Sulfas), resulting in yang deficiency of the spleen and kidney, inability to mobilize and evaporate body fluids and intestinal atony[6-7]. The clinical manifestations of STC patients include difficult defecation, symptoms caused by yang deficiency, such as intolerance of cold and cold limbs, fatigue, soreness and coldness of loins and knees[8]. In this study, loperamide hydrochloride was used to induce STC in rats, and then the rats became less responsive, with bruised paw nails and bowed backs and other symptoms. In addition, their intestinal propulsion rate and fecal water content decreased. All the symptoms were consistent with previous reports on STC model[9], indicating that the STC rat model in our study was successfully induced with loperamide hydrochloride.

The combination of Fu Zi and Rou Gui effectively improves intestinal motility in STC rats

The combination of Fu Zi-Rou Gui was commonly used in warming prescriptions for the treatment of constipation from the ancient pre-Qin dynasty to the Ming and Qing dynasties[10]. As one of the commonly used herbal medicines in clinical practice, Fu Zi is pungent, sweet, hot, has the action of restoring yang from collapse, reinforcing fire and strengthening yang, dispelling cold to alleviating pain[11]. Rou Gui is pungent, sweet and hot, has the action of reinforcing fire and strengthening yang, dispelling cold and relieving pain, warming meridians and collaterals. The combination of the two herbs may perform better, as Fu Zi tends to enter qi, warming yang and dispersing cold, while Rou Gui tends to enter blood, warming the meridians and opening the veins. It has been reported that the contents of total base and ester base in Fu Zi both decrease slightly after its combination with Rou Gui, so that the active ingredients of Fu Zi are better retained[12]. In the present study, STC rats were treated with Fu zi alone, Rou Gui alone or both together, then we found that the rate of intestinal propulsion and fecal water content of rats were all increased after drug administration, and the symptoms of STC such as bruised paw nails and bowed back all changed for the better, especially in the rats treated with the combination of Fu Zi and Rou Gui (2∶1). Yang deficiency will slow down bowel movement and decrease

the rate of intestinal propulsion. The present study showed that the combination of Fu Zi and Rou Gui can improve intestinal motility and propulsion in STC rats, and performed better than Fu Zi alone or Rou Gui alone due to the synergistic effects between them.

The combination of Fu Zi and Rou Gui can regulate colonic 5-HT, SP and VIP levels in STC rats

Reduced intestinal transport capacity, prolonged colonic transport time, and hampered colonic contents are the main pathological features of STC[13]. Studies have shown that STC patients also have colonic motility disorder and colonic inertia[14]. STC intestinal dysmotility is closely related to intestinal neurotransmitters. Substance P (SP), an excitatory neurotransmitter in the gastrointestinal tract, can promote smooth muscle contraction and intestinal peristalsis. Decreased SP level in the intestine is one of the important reasons resulting in the occurrence of STC[15]. 5-Hydroxytryptamine (5-HT) as an intestinal neurotransmitter can regulate gastrointestinal motility and intestinal cell secretion. Studies have shown that colonic 5-HT expression is reduced in STC patients[16]. Vasoactive intestinal peptide (VIP) is a peptidergic neurotransmitter that can slow down colonic motility by relaxing smooth muscle[17]. And as an inhibitory neurotransmitter, the level of VIP is negatively correlated with colonic motility. It has been reported that low VIP level can lead to excessive segmental peristalsis and reduce effective propulsion in the colon, while increased VIP level can relax smooth muscle of the gastrointestinal tract, inhibit colonic and rectal tension, impede peristaltic contraction, leading to the occurrence of STC[18]. The present study showed that compared with the healthy rats, the SP and 5-HT levels decreased and VIP level increased in STC rats, which is consistent with previous findings. Compared with the ST rats, colonic 5-HT and SP level increased and VIP level decreased in the rats treated with Fu Zi-Rou Gui (2∶1), Fu Zi alone, Rou Gui alone and prucalopride, suggesting that Fu Zi-Rou Gui (2∶1) improves intestinal motility in STC rats by regulating 5-HT, SP, and VIP levels.

The combination of Fu Zi and Rou Gui can regulate intestinal microflora in STC rats

The mechanism for changing intestinal motility based on intestinal flora has received much attention in recent years. It is believed that intestinal microbiota plays a key role in intestinal health. So maintaining the homeostasis of intestinal flora and a beneficial equilibrium between it and human body are important to ensure normal intestinal function[19]. Intestinal flora and its metabolites affect intestinal function by influencing the immune system, metabolic processes, neurotransmitters, *etc.* For example, intestinal flora act on enterochromaffin cells (ECs) using short-chain fatty acids (SCFAs) as mediators to regulate the synthesis and release of 5-HT. In addition, bacteriophage-producing anaerobic bacteria are able to react with colonic ECs, and 5-HT produced during the reaction increases the frequency of colonic peristalsis, thus affecting intestinal motility[20-21]. Clinical studies have found evident changes in the composition of intestinal flora in STC patients, including significant decreases in probiotic bacteria and significant increases in potentially pathogenic bacteria or fungi, and these changes positively correlate with the severity of constipation[22].

The present study indicated that alpha diversity was similar between rats with loperamide hydrochloride-induced STC and healthy rats, suggesting that there was no significant difference in the species abundance and evenness of intestinal flora between them. However, after intervention with drugs, there was still no significant difference in alpha diversity between STC rats and drug-treated rats, proving that Fu Zi-Rou Gui combination, Fu Zi, Rou Gui and prucalopride cannot change the alpha diversity of intestinal microflora in rats. Our results also showed that there were obvious differences in beta diversity between STC rats and healthy rats, suggesting that administration of loperamide hydrochloride to rats changed the composition of intestinal microflora. The analysis on beta diversity revealed that there was certain difference in the composition of intestinal microflora species among the six groups. In overall, beta diversity in rats treated with Fu Zi-Rou Gui (2∶1) and Fu Zi, especially that in rats treated with Rou Gui was similar to beta diversity of control group, proving that these herbal medicines changed the composition of intestinal microflora species, and the effect of Rou Gui alone was most significant.

At the phylum level, the intestinal microflora in healthy rats of the control group was dominated by Firmicutes, Bacteroidetes and Proteobacteria, which was consistent with existing studies[23]. Compared with the control group, the relative abundance of Firmicutes and Proteobacteria in STC rats significantly increased, while that of Bacteroidetes decreased, and this is consistent with previous study, which shows that the proportion of Bacteroidetes decreases while that of Firmicutes, Actinobacteria and Proteobacteria increases in patients with constipation[24]. The abundance of genera in the phylum Bacteroidetes is positively associated with constipation symptoms, but just with intestinal water absorption, and not with colonic motility, whereas the abundance of genera in Firmicutes and Proteobacteria is associated with intestinal motility[25-26]. The present study also revealed that compared with STC rats, the relative abundance of Proteobacteria decreased and that of Bacteroidetes increased in rats treated with Rou Gui alone or Fu Zi-Rou Gui (2∶1), indicating that Fu Zi-Rou Gui combination and Rou Gui alone can relieve constipation by changing the proportion of Proteobacteria, which is related with intestinal motility, and the proportion of Bacteroidetes, which is related with intestinal water reabsorption. Compared with STC rats, the relative abundance of Bacteroidetes and Proteobacteria in Fu Zi treated rats decreased while that of Firmicutes increased, suggesting that Fu Zi can change the proportion of Proteobacteria, which is related with intestinal motility. In addition, the relative abundance of Firmicutes and Proteobacteria in prucalopride treated rats decreased while that of Bacteroidetes increased, suggesting that prucalopride can relieve constipation by effectively regulating the flora related to intestinal motility and intestinal water reabsorption.

At the genus level, *Lactobacillus* was changed most significantly by drug administration in the present study. Numerous studies[27-29] have shown that the abundance of *Lactobacillus* spp. decreases in patients with constipation and in rats with STC. *Lactobacillus* spp. are the dominant flora and also the probiotic flora in

humans. *Lactobacillus reuteri* can effectively improve the bowel movements in constipated patients. Anaerobic bacteria are dominant flora in human colon[30]. The results of this study showed that *Lactobacillus*, *Ruminococcus*, *Oscillospira*, *Clostridium* and *Coprococcus*, all of which are anaerobic, were dominant intestinal flora in healthy rats of the control group. Compared with the control group, the relative abundance of *Lactobacillus*, *Oscillospira* (gram-negative), *Coprococcus* (gram-positive) and *Clostridium* (gram-positive) in STC rats decreased, while that of *Blautia* (gram-negative), *Ruminococcus* (Gram-positive) and *Allobaculum* increased, suggesting that the relative abundance of *Lactobacillus* spp. and anaerobic bacteria decreased in STC rats, which is consistent with previous studies. Compared with STC rats, the relative abundance of *Lactobacillus* spp. in all drug-treated rats increased, while the relative abundance of *Roseburia* increased in Fu Zi-Rou Gui (2∶1) -treated rats. *Roseburia* produces butyric acid, which is a SCFA that can affect the secretion and release of 5-HT, suggesting that Fu Zi-Rou Gui (2∶1) combination can regulate not only the abundance of *Lactobacillus* spp., but also 5-HT level to improve intestinal motility.

In conclusion, yang deficiency, reduced warming and propulsive ability, and concretion of yin and coldness lead to intestinal contents unable to transport properly. The combination of Fu Zi and Rou Gui can improve intestinal dysfunction in STC rats, probably by upregulating colonic 5-HT, VIP and SP level and the composition of intestinal flora. The present study only explains part of the intervention mechanism. However, intestinal flora regulates intestinal motility through multiple pathways. For example, *Bacteroides* spp., *Bifidobacterium* spp., *Lactobacillus* spp., *Clostridium* spp., *Prevotella* spp. among intestinal flora can produce SCFAs, thus regulating the secretion and release of 5-HT. Their effects on the metabolites of intestinal flora will be explored in future studies.

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