Introduction

Diarrhea-predominant irritable bowel syndrome (D-IBS) is a chronic gastrointestinal (GI) disease characterized by abdominal pain, discomfort, and changes in bowel movement patterns without any evidence of underlying bowel damage, all of which impair life of patients who burden high healthcare cost [1-3]. The pathogenesis of is not yet fully understood. Because of the complex causes of the D-IBS, there is no accepted and effective standard therapy but treatment mostly follows a symptom-oriented trial and error method. Safe and effective treatment options are urgently needed. Some herbal medicines such as the BPYC pill from TCM is traditionally used to treat D-IBS and it can significantly ameliorate abdominal pain, diarrhea and improve patients’ quality of life.

It is generally perceived that altered GI motility is mentioned as one of the mechanisms of IBS [4].

Excessive contraction of the intestinal smooth muscle may lead to D-IBS symptoms [5], such as diarrhea and abdominal pain. However, there are no data of BPYC pill and its effect on colonic contractility. GI motility results from coordinated contractions of smooth muscle [6]. Our previous researches found some traditional Chinese medicine and their compounds took effect on regulation of GI motility via inhibiting GI smooth muscle contraction [7,8]. Therefore, the aim of the study at hand was to evaluate the impact of BPYC pill on the motoric activity of CLSMS and investigate the mechanism of its anti-spasmodic effect.

Materials and Methods

Animals

Forty male Sprague-Dawley rats (200 ± 20 g) were obtained from Vital River Laboratories Animal Technology Co. Ltd. (Beijing, China) and housed at the animal laboratory of Beijing Chinese Medicine Hospital affiliated to Capital Medical University under a 12 h light/dark cycle (lights on at 08:00 A.M.) with access to food and water ad libitum. After acclimatization for three days, rats were fasted for 24 h prior to the experimental treatment.

Drugs and reagents

BPYC pills (provided by Guangzhou Baiyun Mountain Chenliji Pharmaceutical Factory Company, Ltd, Guangzhou, China) are composed of Huangqi (Radix astragali), Dangshen (Radix codonopsis), Sharen (Fructus amomi), Baishao (Radix paeoniae alba), Danggui (Radix angelicae sinensis), Baizhu...
(Rhizoma atractylodis macrocephalae), Rougui (Cortex cinnamomi), Yanhusuo (Rhizoma corydalis), Lizhihe (Semem litchi), Ganjiang (Rhizoma zingiberis), Zhigancao (Radix glycyrrhizae praeparata), Fangfeng (Radix saposhnikoviae), Muxiang (Radix aucklandiae), Buguzhi (Fructus psoraleae), and Chishizhi (Halloysium rubrum). A decoction of the BPYC pills was prepared with water, concentrated, and freeze-dried into a powder that was stored in sealed bags at 4°C. Before each experiment, BPYC suspension was prepared by mixing the freeze-dried BPYC powder into Krebs solution (KS) to produce different concentrations of BPYC solution.

The following reagents were obtained from Sigma-Aldrich Co. LLC, USA: EGTA (batch number: E0396), Thapsigargin (batch number: T9033), Acetylcholine chloride, (batch number: A6625), Nifedipine (batch number: N7634), TMB-8 (batch number: T111), L-NAME (batch number: N5751), methylene blue (batch number: M9140), 4-AP (batch number: 275875), Apamin (batch number: A4950), Nifedipine (batch number: N7634), TMB-8 (batch number: T111), Krebs solution (mM): NaCl 120.6, KCl 5.9, NaH2PO4 1.2, MgCl2 1.2, NaHCO3 15.4, CaCl2 2.5 and glucose 11.5, pH: 7.35-7.45.

### Apparatus

A CH-1015 super thermostat bath (Shanghai Yueping Scientific Instrument Co. Ltd., China) was used for maintaining temperature of 37°C, ML110 Powerlab amplifier, ML740 four-channel recorder and MLT02021D tonotransducer were used for collecting data of tissue tension, and Power Lab/4sp analysis system Labchart7 was used for analysis of physiological data (AD Instruments Shanghai Trading Co. Ltd, Pudong New Area, Shanghai, China).

### Tissue preparation

Rats were anesthetized by administering 7% chloral hydrate (35 mg/100 g body weight) via intraperitoneal injections. The distal colon (6–7 cm from the anus) was removed and quickly incised longitudinally along the mesenteric border. Two longitudinal smooth muscle strips (0.8 cm × 0.2 cm) were obtained with the layers of mucosa and sub-mucosa removed [8]. Each strip was suspended in a tissue chamber containing 15 ml Krebs solution that was constantly warmed by circulating water maintained at 37°C and oxygenated with 95% O2 and 5% CO2. Portions of CLSMS were ligatured with a medical thread at both ends. One end was fixed to the bottom of the bath (tissue chamber), while the other end was connected to a physiological recorder through the external isometric force transducer (MLT02021D). The initial load of CLSMS samples was 1 g (2 mV) to maintain basal tension. Krebs solution (15 mL, 37°C) was injected into the four water baths that bubbled continuously with 95% O2 and 5% CO2. The tissues were allowed to equilibrate for 60 min with rinsing every 15 min before starting the experiment.

### Experimental protocol

**Effect of the BPYC pill on CLSMS contraction induced by Ach:** After equilibration for 60 min, 0.1 mM Ach was added into the Kreb’s solution within the bath to induce CLSMS contraction. When regular and stable contractions were obtained, BPYC suspension was added continuously at intervals of 5 min, so that the cumulative concentration of BPYC (Krebs solution as a control) in each chamber reached 3 μg/ml, 10 μg/ml, 30 μg/ml, 100 μg/ml, 300 μg/ml, 1000 μg/ml, 3000 μg/ml, and 10000 μg/ml (crude drug dose). The effect of the BPYC powder on CLSMS contraction was measured as Tension (%) and a dose-dependent curve was obtained from the various BPYC concentrations used, Tension (%)=(tension after agent administration-basal tension)/(tension before agent administration-basal tension) × 100%

**Effect of the BPYC pill on CLSMS contraction induced by high K+:** After equilibration for 60 min, 0.1 M KCl was added. Using the same method described above, the effect of the BPYC pill on CLSMS contraction induced by high K+ was observed through Tension (%) and its associated dose-dependent curve was obtained.

**Effect of the BPYC pill on CLSMS contraction, induced by exhausting intracellular Ca2+, and internal flow of extracellular Ca2+:** After equilibration of CLSMS for 60 min, normal Krebs solution was replaced with Krebs solution without Ca2+, and 1 μM thapsigargin and 1 mM EGTA were added and incubated for 30 min. Krebs solution without Ca2+ was then replaced with Krebs solution containing 2.5 mM Ca2+ to induce CLSMS contraction. Using the same methods stated previously, the inhibitory effect of BPYC pill on CLSMS contraction in response to the exhaustion of intracellular Ca2+ and subsequent internal flow of extracellular calcium were observed.

**Study on the mechanism of effect of the BPYC pill on CLSMS contraction induced by Ach:** Using the methods stated above, a chart was prepared to visualize inhibitors’ influence on the dose-dependent effect of BPYC. The CLSMS were washed with Krebs solution and different inhibitors (EGTA 1 mM, thapsigargin 1 μM, nifedipine 10 μM, TMB-8 500μM, methylene blue 10 μM, 4-AP 50 μM, L-NAME 10 mM, apamin 0.1 μM) were added and incubated for 30 min. Following this, 0.1 mM Ach was added to induce CLSMS contraction, and the effect of inhibitors was determined by calculating Inhibition maximum (I50 %), half-inhibitory concentration (IC50), and PD value (log IC50). To ensure the experimental effect of individual inhibitor, each strip from eight rats was tested for 3.5–4 h, and only one inhibitor was added to each strip. Group with no incubation of inhibitor is control group. I50 (%)=1-(tension after 10000 μg/ ml agent - basal tension)/(maximum tension after Ach - basal tension) × 100%

At the end of each experiment CLSMS were flushed with fresh KS and re-exposed to the reference substance which induces muscle contraction, in order to verify their reactivity.

Animal care and experimental procedures were conducted according to the institutional ethical guidelines and conformed to the requirements of the Institutional Animal Care and Use Committee of Beijing University of Chinese Medicine and approval by Animal Ethics Committee of Beijing Institute of Traditional Chinese Medicine (No.20160601).
Statistical analysis

Data are presented as means ± standard deviation (SD) and n refers to the number of rats. Significance of differences between groups was analyzed using SPSS 20.0 software (SPSS, Chicago, IL, USA) with student t-test. P<0.05 was considered statistically significant.

Results

Inhibitory effect of the BPYC pill on CLSMS contraction induced by Ach

The addition of 0.1 mM Ach caused muscle tension to increase rapidly to a peak and then decline to plateau at a relatively stable level. The tension reduced in all BPYC and control groups. When BPYC concentration reached 100 μg/ml, there was a significant difference in muscle tension compared to the control group (*P<0.01). With increasing concentration, more significant differences were found (**P<0.001). At a BPYC concentration of 10,000 μg/ml, tension initially stimulated by Ach decreased by 93% (Figure 1).

(A) Original trace of spontaneous contraction of CLSMS induced by Ach in response to BPYC. Ach: 0.1 mM Ach was added to induce CLSMS contraction.

(B) The concentration-dependently inhibitory effect of the BPYC pill on the tension of CLSMS induced by Ach. Tension (%)=(tension after agent administration-basal tension)/(tension before agent administration-basal tension) × 100%. The solid line represents the tension (%) of CLSMS in BPYC solution. The dotted line represents the tension (%) of CLSMS in KS. Data were analyzed by student t-test and represented as mean ± SD (n=6, *P=0.0005, p<0.01, **P<0.001).

Inhibitory effect of the BPYC pill on CLSMS contraction induced by high K+

The addition of 0.1 M KCl increased tension to a relatively stable level. After BPYC was added to this solution, a dose-dependent decrease in tension was observed. No change was observed in the control group. When BPYC reached concentrations above 100 μg/ml, a significant difference in muscle tension was measured (**P<0.01). At a BPYC concentration of 10,000 μg/ml, tension originally stimulated by high K⁺ decreased to 7% of that before the BPYC was added (Figure 2).

(A) Original trace of spontaneous contraction of CLSMS induced by high K⁺ in response to BPYC. KCl: 0.1 M KCl was added to induce CLSMS contraction.

(B) The concentration-dependently inhibitory effect of the BPYC pill on the tension of CLSMS induced by high K⁺. Tension (%)=(tension after agent administration-basal tension)/(tension before agent administration-basal tension) × 100%The solid line represents the tension (%) of CLSMS in BPYC solution. The dotted line represents the tension (%) of CLSMS in Krebs solution. Data were analyzed by student t-test and represented as mean ± SD (n=4, **P<0.001).

Figure 1. The inhibitory effect of the BPYC pill on CLSMS contraction induced by Ach.
Inhibitory effect of the BPYC pill on CLSMS contraction induced by exhausting intracellular Ca$^{2+}$ and internal flow of extracellular Ca$^{2+}$

After BPYC was added to CLSMS, muscle tension decreased rapidly while this decrease was much slower in the control group. When the BPYC concentration was more than 10 μg/ml, there was a significant difference compared with the control group (**$P<0.001$). At a BPYC concentration of 10,000 μg/ml, tension stimulated by exhausting intracellular Ca$^{2+}$ and internal flow of extracellular calcium decreased to 9% of that before BPYC was added (Figure 3).

Study on the mechanism of inhibitory effect of the BPYC pill on CLSMS contraction induced by Ach

The participation of various ion channels and intercellular pathways were verified by application of several pharmacological inhibitors. After incubation of CLSMS with different inhibitors, $I_{\text{max}}$, IC50, and PD values were assessed. Compared to the control group, there were significant differences between the nifedipine group, the TMB-8 group, and the thapsigargin group ($P<0.05$). However, the EGTA group, L-NAME group, Methylene blue group, 4-AP group, Apamin group showed no significant difference from the control ($P>0.05$) (Table 1).

Notes: $I_{\text{max}}$: Inhibition maximum, $I_{\text{max}}$ (%)=$1- (tension after agent administration-basal tension)/(maximum tension after Ach - basal tension) × 100\%$, IC50: half-inhibitory concentration, PD value= log (IC50). Data were analyzed by student $t$-test and represented as mean ± SD, n=8; *$P=0.046$, **$P=0.012$, ***$P=0.025$, ****$P=0.042$, *****$P=0.014$, ******$P=0.028$, *******$P=0.031$, *******$P=0.037$, *******$P=0.026$, vs control group.

All reactions caused by BPYC were reversible after flushing with fresh KS.

Discussion

The BPYC pill is an effective Zheng-based drug for D-IBS

Zheng is a key concept of TCM, an ancient medical practice system which emphasizes regulating the integrity of the human body and its interrelationship with natural environments [9]. Zheng (meaning syndrome or pattern) is the overall physiological and/or pathological pattern of the human body in response to a given internal and external condition, which usually is
an abstraction of internal disharmony [10]. D-IBS belongs to disease of “Futong (abdominal pain)” and “Xiexie (diarrhea)” with a TCM Zheng of Pi-deficiency. Bu Pi Yi Chang in Chinese means invigorating Pi and regulating the bowel which is the strategy to treat Zheng. As an important role in BPYC formula, Baizhu were found to have an analgesic effect on visceral pain and inhibitory effect on bowel movement [10]. Further investigations are needed not only to find effective compounds in BPYC pill, but also to systematically understand how this recipe can adjust gastrointestinal digestive functions.

**BPYC pill could regulate the motility of colonic smooth muscle and its antispasmodic effect is partially mediated by calcium channels**

Smooth muscle contraction is controlled by Ca2+ signaling [11]. An increase in intracellular Ca2+ is the primary trigger of GI smooth muscle contraction [12]. It is confirmed that Ca2+ plays a core role in mediating colon smooth muscle contraction [13]. Intracellular free Ca2+ levels determine the contraction of smooth muscle cells, and the two main sources of free Ca2+ are: (1) the internal flow of extracellular Ca2+ and (2) Ca2+ release from intracellular storage compartments within the muscle. Among the two, the internal flow of extracellular Ca2+ plays the major role [5]. There are three main calcium channels, voltage-operated channels (VOC), receptor-operated channels (ROC), and store-operated channels (SOC). However, in GI smooth muscle, the influx of extracellular Ca2+ is achieved mainly through L-type VOC [14,15].

Ach, the most classic and primary excitatory neurotransmitter in the gastrointestinal tract, acts on cell-membrane receptor M to increase intracellular Ca2+ concentration, excites gastrointestinal smooth muscle and generates spontaneous contractions, and therefore plays an important role in gastrointestinal movements. Intestinal smooth muscle excitation-contraction coupling caused by Ach leads to an increase in the number of defecations that may lead to diarrhea and other associated symptoms. Smooth muscle contraction, induced by Ach, is involved in both extracellular calcium influx and intracellular calcium storage release. The former is related mainly to VOC and ROC [16]. The latter is involved mainly in G-protein coupling, first by activating protein kinase C (PKC), producing inositol triphosphate (IP3) that docks to the IP3 receptor in sarcoplasmic reticulum, opening calcium pools thereby releasing intracellular Ca2+ and increasing its concentration. These experimental

**Figure 3.** The inhibitory effect of the BPYC pill on CLSMS contraction induced by exhausting intracellular Ca2+ and resuming the internal flow of extracellular Ca2+.

| Table 1: Influence of different inhibitors on the inhibitory effect of the BPYC pill on CLSMS contraction induced by Ach. |
|---|---|---|---|
| **Group** | **I_{max}(\%)** | **IC50(\mu g/ml)** | **PD-value** |
| Control | 99.93 ± 0.19 | 730.63 ± 98.05 | 2.65 ± 1.89 |
| EGTA | 98.99 ± 0.05 | 674.55 ± 40.09 | 2.98 ± 1.78 |
| L-NAME | 99.87 ± 0.20 | 654.32 ± 20.67 | 2.98 ± 1.46 |
| Methylene blue | 99.82 ± 0.06 | 865.76 ± 41.87 | 2.96 ± 1.42 |
| 4-AP | 98.60 ± 2.00 | 699.08 ± 40.69 | 2.76 ± 1.43 |
| Apamin | 98.77 ± 0.12 | 712.55 ± 47.39 | 2.98 ± 2.27 |
| Nifedipine | 72.66 ± 2.87 | 6220.12 ± 59.89 | 3.20 ± 1.15 |
| TMB-8 | 68.21 ± 14.33 | 3701.00 ± 95.03 | 3.98 ± 1.99 |
| Thapsigargin | 79.00 ± 3.86 | 6587.01 ± 69.98 | 3.99 ± 1.98 |

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**Control** | **EGTA** | **L-NAME** | **Methylene blue** | **4-AP** | **Apamin** | **Nifedipine** | **TMB-8** | **Thapsigargin**
---|---|---|---|---|---|---|---|---
99.93 ± 0.19 | 98.99 ± 0.05 | 99.87 ± 0.20 | 99.82 ± 0.06 | 98.60 ± 2.00 | 98.77 ± 0.12 | 72.66 ± 2.87 | 68.21 ± 14.33 | 79.00 ± 3.86
730.63 ± 98.05 | 674.55 ± 40.09 | 654.32 ± 20.67 | 865.76 ± 41.87 | 699.08 ± 40.69 | 712.55 ± 47.39 | 6220.12 ± 59.89 | 3701.00 ± 95.03 | 6587.01 ± 69.98
2.65 ± 1.89 | 2.98 ± 1.78 | 2.98 ± 1.46 | 2.96 ± 1.42 | 2.76 ± 1.43 | 2.98 ± 2.27 | 3.20 ± 1.15 | 3.98 ± 1.99 | 3.99 ± 1.98
results show that the BPYC pill had a concentration-dependently inhibitory effect on CLSMS contraction induced by Ach, which restricts both the internal flow of extracellular calcium and the release of intracellular calcium storage. The possible inhibitory mechanism of internal flow of extracellular calcium may rely on blocking VOC and ROC simultaneously.

It is well known that extracellular high K+ depolarizes the smooth muscle cell membrane and opens VOC, resulting in an influx of extracellular Ca2+ and an activation of contractile machinery [17]. This experiment found that BPYC pills inhibit the CLSMS contraction caused by KCl, and its mechanism may be via the inhibition of the internal flow of extracellular Ca2+ by blocking VOC.

The antispasmodic effect of BPYC pill was partially blocked by TMB-8, thapsigargin, and nifedipine (P<0.05). Nifedipine has the function of blocking L-VOC [18] and inhibiting the influx of extracellular calcium. In this experiment, after CLSMS incubation with the inhibitor nifedipine for 20 min, Ach was added to stimulate contraction, which was significantly lower than the control group. After administration of different doses of BPYC pill to CLSMS, there was a concentration-dependent inhibitory effect on muscle contraction, reducing muscle tension, and leading to muscle relaxation. Therefore, the BPYC pill may inhibit muscle contraction by blocking VOC and reducing intracellular Ca2+ concentration. TMB-8 is capable of inhibiting intracellular calcium release from the sarcoplasmic reticulum, leading to a decline in intracellular calcium concentration. In this experiment, after incubation of CLSMS with the inhibitor TMB-8 for 20 min, Ach was added to stimulate contraction. Contractions of CLSMS in TMB-8 were significantly lower than those of the control group. After administration of different doses of the BPYC pill, there was a concentration-dependent inhibitory effect on muscle contraction leading to reduced muscle tension. Therefore, the BPYC pill may inhibit muscle contraction by promoting reuptake of calcium and inhibiting intracellular calcium release from the sarcoplasmic reticulum. Thapsigargin, as the specific inhibitor of the calcium pump and its antispasmodic effect is partially mediated by calcium channels, possibly by blocking the internal flow of extracellular Ca2+, inhibiting the release of intracellular Ca2+, and reducing free intracellular Ca2+ concentration via VOC, ROC, and SOC, respectively.

Conclusion

BPYC pills could inhibit colonic contraction of rats in vitro and its antispasmodic effect is partially mediated by calcium channels, possibly by blocking the internal flow of extracellular Ca2+, inhibiting the release of intracellular Ca2+, and reducing free intracellular Ca2+ concentration via VOC, ROC, and SOC, respectively.

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Conflict of Interest

All authors declare no conflict of interest in association with this work.

References


*Correspondence to:*
Professor Zheng-Fang Wang and Sheng-sheng Zhang
Digestive Disease Center of Beijing Chinese Medicine Hospital Affiliated to Capital Medical University
23 Meishuguanhou Street, Dongcheng District
Beijing, 100010, China
E-mail: bjwzf0442@163.com; zhss2000@163.com