Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
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ORIGINAL ARTICLE

The Traditional Medicine Bojungikki-Tang Increases Intestinal Motility

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Submitted: 25-Nov-2020 Revised: 04-Mar-2021 Accepted: 09-Mar-2021 Published: 10-Jun-2021

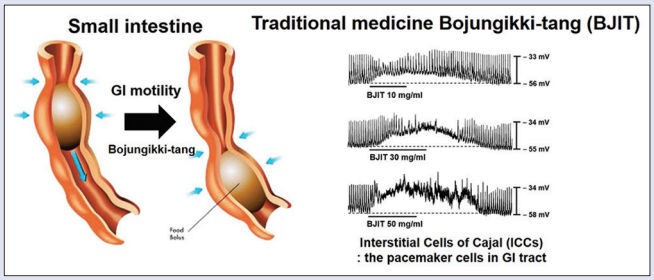
ABSTRACT

Background: Bojungikki-tang (BJIT) is a traditional formula used to treat Gastrointestinal (GI) diseases. **Objectives:** We investigated the GI motility functions *in vivo* and the pacemaker potential in interstitial cells of Cajal (ICCs) *in vitro* by BJIT. **Materials and Methods:** Intestinal transit rate (ITR) and serum levels of GI hormones were investigated in mice. ICC-induced pacemaker potential was evaluated using the electrophysiological method. **Results:** ITR values and the level of motilin significantly increased after treatment with BJIT. The BJIT-induced ITR increase was related to the increase in the expression of a c-kit. BJIT induced the pacemaker potential depolarizations and the frequency decrease of ICCs. Pretreatment with methoctramine resulted in the inhibition of BJIT-induced depolarization of the pacemaker potential. However, BJIT-induced effects were retained in the presence of 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide. Furthermore, thapsigargin pretreatment resulted in the inhibition of BJIT-induced effects. Moreover, BJIT blocked both transient receptor potential melastatin 7 and calcium-activated chloride (transmembrane protein 16A) channels. **Conclusion:** These results indicate that BJIT can be considered a good medicine for controlling GI motility.

Key words: Bojungikki-tang, interstitial cells of Cajal, intestinal transit rate, motilin, pacemaker potentials

SUMMARY

- The levels of hesperidin, naringin, decursin, nodakenin, glycyrrhizinic acid, liquiritigenin, and ginsenoside Rg1 in Bojungikki-tang (BJIT) were analyzed using ultra-performance liquid chromatography
- BJIT increased the intestinal transit rate in mice through an increase in motilin
- C-kit expression was higher after BJIT treatment
- BJIT depolarized the pacemaker potential of the interstitial cells of Cajal (ICCs)
- BJIT affected the ICC pacemaker potential through M_2 receptors via the regulation of internal Ca^{2+}
- BJIT suppressed both transient receptor potential melastatin 7 and transmembrane protein 16A channels .



Abbreviations used: BJIT: Bojungikki-tang; GI: Gastrointestinal; ICCs: Interstitial cells of Cajal; ITR: Intestinal transit rate; TRPM7: Transient receptor potential melastatin 7; TMEM16A: Transmembrane protein 16A; HEK: Human embryonic kidney.

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DOI: 10.4103/pm.pm_507_20

Access this article online
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INTRODUCTION

Bojungikki-tang (BJIT; Hochu-ekki-to in Japanese) is a popular traditional medicine that has been long used in East Asian countries in Gastrointestinal (GI) and respiratory diseases treatment.[1] It has been also used against general fatigue and lack of appetite and to treat stroke and cerebrovascular disease repellent due to qi deficiencies.[2,3] Among many roles, BJIT has been confirmed to be effective in recovering the functions of the digestive system.[4,5]

The GI tract is an important organ for food consumption, nutrient absorption, and waste discharge. GI motility disorders are known to affect the intestinal functions. There are many cells related to GI motility, and the typical cells are interstitial cells of Cajal (ICCs).[6] ICCs are special pacemaker cells,[7-9] and any damage to the number or structure of these ICCs may develop serious GI diseases.[10,11] Thus, ICCs play an essential role in normal GI motility control. However, BJIT's efficacy in ICCs and its role in GI movement have not been investigated. Therefore, we investigated the effects of GI motility functions and pacemaker potentials of ICCs by BJIT.

MATERIALS AND METHODS

Instrumentation and reagents

BJIT was provided by HANKOOKSHINYAK Pharmaceutical Co. Ltd., (Nonsan, Republic of Korea). The components and amounts of BJIT are

Cite this article as: Kwon HE, Kim JN, Kwon MJ, Lee JR, Kim SC, Nam JH, *et al.* The traditional medicine bojungikki-tang increases intestinal motility. Phcog Mag 2021;17:S1-8.

described in detail in Table 1. Hesperidin, naringin, decursin, nodakenin, glycyrrhizinic acid, liquiritigenin, and ginsenoside Rg1 were purchased from Sigma-Aldrich. A high-performance liquid chromatography method is the same as the previous research.[9]

Preparation of standard solutions

Appropriate amounts of compounds (hesperidin, naringin, decursin, nodakenin, glycyrrhizinic acid, and liquiritigenin) were measured and treated with methanol. In brief, ginsenoside Rg1 was accurately measured and dissolved in 60% methanol. An undiluted solution was prepared at 1 μg/mL and appropriately diluted with methanol to obtain 1, 5, and 10 ng/mL concentrations.

Quantitation of Bojungikki-tang

The Waters ACQUITY™ Ultra Performance LC (UPLC) system (USA) equipped with Waters ACQUITY™ photodiode array detector (PDA) was used along with Waters ACQUITY™ BEH C_{18} column (1.7 μm, 2.1 mm × 100 mm) and Empower software. With respect to the PDA wavelength, hesperidin and liquiritigenin were analyzed at 245 nm and decursin, at 360 nm. In addition, naringin and nodakenin were analyzed at 280 nm and glycyrrhizinic acid was analyzed at 285 nm [Table 2]. In addition, ginsenoside Rg1 was analyzed at 203 nm [Table 3].

Intestinal transit rate measurement

With Evans blue (5%, w/v), intestinal transit rate (ITR) was measured. Thirty minutes after Evans administration, the ITR was measured. According to the regulations of Pusan National University's Institutional Animal Care and Use Committee, animal care and experiments were conducted (PNU-2019-2462).

Measurement of gut hormones in the serum

Serum levels of gut hormones were checked using commercial kits (Abbkine Scientific Co., Ltd., Wuhan, China).

Western blotting

Anti-transmembrane protein 16A (TMEM16A; Abcam, Cambridge, UK), anti-c-kit (Cell Signaling Technology, Danvers, MA, USA.), anti-transient receptor potential melastatin 7 (TRPM7; Abcam, Cambridge, UK), and anti-β-actin (Santa Cruz Biotechnology, Dallas, TX, USA) antibodies were used. All other procedures were carried out as previously described.[12]

Preparation of interstitial cell of Cajal clusters and patch-clamp experiments

The ICC isolation method and solution are the same as the existing research method.[12,13] We used whole-cell electrophysiological experiment. Experimental apparatus and solutions are identical to the previous study.[13,14]

Transient receptor potential melastatin 7 channel expression in human embryonic kidney 293 cells

TRPM7 (LTRPC7/pCDNA4-TO) constructs were transiently transfected in human embryonic kidney (HEK) 293 cells. The composition and testing methods of the solution used are the same as those of the past.[13,14]

Table 1: Composition of Bojungikki-tang used in the study. BJIT extract was obtained from HANKOOKSHINYAK Corp. (Nonsan, Chungcheongnam-do, Republic of Korea) in a pack of 2.06 g. BJIT: Bojungikki-tang

Latin name	Scientific name	Amount (g)
Astragali Radix	Astragalus membranaceus	0.41
Ginseng radix	Panax ginseng C. A. Meyer	0.30
Atractylodis Rhizome	Atractylodes macrocephala Koidzumi	0.46
Glycyrrhizae Radix	Glycyrrhiza uralensis Fischer	0.34
Angelicae gigantis Radix	Angelica gigas Nakai	0.23
Citri unshii Pericarpium	Citrus unshiu Markovich	0.20
Cimicifugae Rhizoma	Cimicifuga heracleifolia Komarov	0.06
Bupleuri Radix	Bupleurum falcatum Linne	0.06
Total		2.06

Table 2: The analysis condition of hesperidin, liquiritigenin, decursin, naringin, nodakenin and glycyrrhizinic acid

Time (min)	0.1% FA/ Water(%)	0.1% FA/ Acetonitrile(%)	Flow rate (ml/ min)
0	98	2	0.40
1.0	98	2	0.40
2.0	90	10	0.40
4.0	70	30	0.40
7.0	50	50	0.40
9.0	30	70	0.40
10.0	10	90	0.40
12.0	0	100	0.40
14.0	98	2	
16.0	98	2	0.40

Table 3: The analysis condition of Ginsenoside Rg1

Time (min)	Water (%)	Acetonitrile(%)	Flow rate (ml/min)
0	85	15	0.40
1.0	85	15	0.40
14.0	70	30	0.40
15.0	68	32	0.40
16.0	60	40	0.40
17.0	45	55	0.40
19.0	45	55	0.40
21.0	10	90	0.40
22.0	10	90	0.40
23.0	85	15	0.40

Calcium-activated chloride (transmembrane protein 16A) channel expression in human embryonic kidney 293 cells

TMEM16A (pEGFP-N1-mANO1) constructs were transiently transfected in HEK 293 cells. The composition and testing methods of the solution used are the same as those of the past.[13,14]

Statistical analysis

Results are expressed as means ± standard error of mean. For multiple comparison, one-way analysis of variance with Bonferroni's *post*

hoc comparison was used. Analyses were performed using Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA). $P < 0.05$ was considered significant.

RESULTS

Analysis of Bojungikki-tang

The levels of hesperidin, naringin, decursin, nodakenin, glycyrrhizinic acid, liquiritigenin, and ginsenoside Rg1 in BJIT were analyzed using UPLC. The concentrations of the seven compounds are shown in Table 4 and Figure 1.

Effects of Bojungikki-tang on intestinal transit rate in mice

[Figure 2A]. Poncirus trifoliata was used to compare the efficacy of BJIT. *Poncirus trifoliata* (1 g/kg), which is known to exhibit the GI motility increase activity,[15] increased the ITR (56.0% ± 3.1%; $P < 0.01$). BJIT increased the ITR in a BJIT dose-dependent manner (42.4% ± 3.6% at 0.01 g/kg, 48.2% ± 4.4% [$P < 0.01$] at 0.1 g/kg, and 50.3% ± 4.6% [$P < 0.01$] at 1 g/kg) [Figure 2A]. These results suggest that BJIT increased the ITR in mice.

Variation in intestinal hormones following Bojungikki-tang treatment

GI hormone levels in the mouse serum were evaluated by radioimmunoassay. The level of motilin (MTL) in the GI was significantly elevated [Figure 2Ba], but the levels of substance *P* (SP) [Figure 2Bb], somatostatin (SS) [Figure 2Bc], and vasoactive intestinal peptide (VIP) [Figure 2Bd] showed no significant changes after BJIT administration. These results suggest that the BJIT-induced increase in ITR was represented by an increase in MTL.

Variation in the protein expression of transmembrane protein 16A, c-kit, and transient receptor potential melastatin 7 after Bojungikki-tang treatment

TMEM16A channel[16,17] or TRPM7 channel[8] is involved in the ICC activity. Further, c-kit is associated with the population of ICCs.[18] Therefore, TMEM16A, TRPM7, or c-kit may be a biomarker of GI motility. After treatment with BJIT, the expression of TMEM16A, TRPM7, and c-kit was evaluated by Western blotting. The c-kit expression was higher after BJIT treatment [Figure 3a]. C-kit expression significantly increased by 25.1% ($P < 0.01$) after the treatment of mice with BJIT [Figure 3b]. The expression of TMEM16A and TRPM7 in mice was almost unchanged after treatment with BJIT [Figure 3c and d].

Effects of Bojungikki-tang on the pacemaker potential of the interstitial cells of Cajal

The ICCs induced the pacemaker potential generation with –56.7 ± 2.3 mV resting membrane potential and 23.1 ± 1.8 mV amplitude (current-clamp mode) [Figure 4a]. The BJIT-induced depolarization was 7.5 ± 0.5 mV ($P < 0.01$), 11.3 ± 0.7 mV ($P < 0.01$), and 18.4 ± 0.8 mV ($P < 0.01$), and the frequencies were 18.6 ± 1.0 cycles/min, 7.6 ± 1.1 cycles/min ($P < 0.01$), and 3.7 ± 1.1 cycles/min ($P < 0.01$) [Figure 4, $n = 19$] at 10, 30, and 50 mg/mL BJIT, respectively. These results show that BJIT depolarized the pacemaker potential.

Confirmation of Bojungikki-tang receptor types in interstitial cells of Cajal

Muscarinic receptors are expressed in the GI tract and are related to the altered motility of the GI smooth muscle.[19,20] The murine small intestinal ICCs only express muscarinic M_2 and M_3 receptors.[21] Therefore, the antagonist was used to identify receptors involved in BJIT-induced pacemaker potential depolarization. ICC was administered muscarinic M_2 receptor antagonist methoctramine or M_3 receptor antagonist 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide (4-DAMP). Pretreatment with methoctramine resulted in inhibition of the BJIT-induced effects [Figure 5Aa]. Further, 4-DAMP failed to interrupt the effects induced by BJIT [Figure 5Ab]. Depolarization and frequency were 2.4 ± 1.1 mV ($P < 0.01$) and 18.4 ± 1.2 cycles/min ($P < 0.01$) by methoctramine [Figure 5B and C; $n = 6$) and 11.2 ± 0.9 mV and 8.8 ± 0.8 cycles/min by 4-DAMP [Figure 5B and C; $n = 7$], respectively. These results suggest that BJIT affected the pacemaker potential by M_2 receptors.

Effects of an external or internal Ca^{2+} on Bojungikki-tang-induced pacemaker potential depolarization in interstitial cells of Cajal

Ca^{2+} regulation is thought to serve as a novel therapeutic strategy for controlling GI motility.[22] External Ca^{2+}-free solution suppressed the pacemaker potential, and then, BJIT induced pacemaker potential

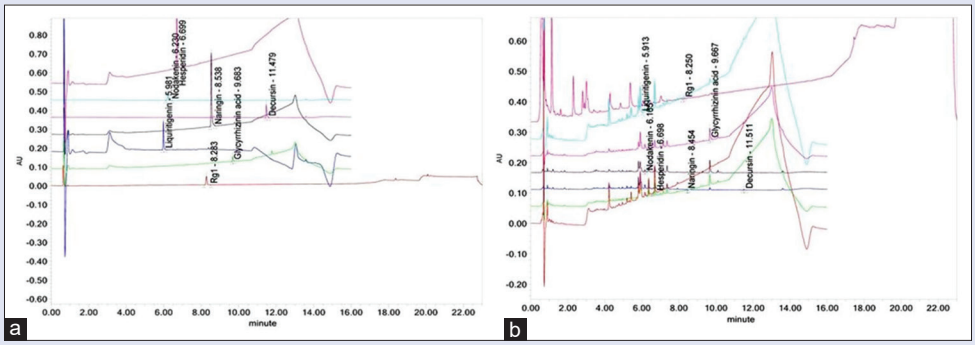

Figure 1: UPLC profiles of seven major compounds identified in BJIT. (a) UPLC profile of commercial standard compounds. (b) UPLC profile of seven major compounds in BJIT. 245 nmol/L (hesperidin and liquiritigenin), 360 nmol/L (decursin), 280 nmol/L (naringin and nodakenin), 285 nmol/L (glycyrrhizinic acid), and 203 nmol/L (ginsenoside Rg1). UPLC: Ultra-performance liquid chromatography; BJIT: Bojungikki-tang

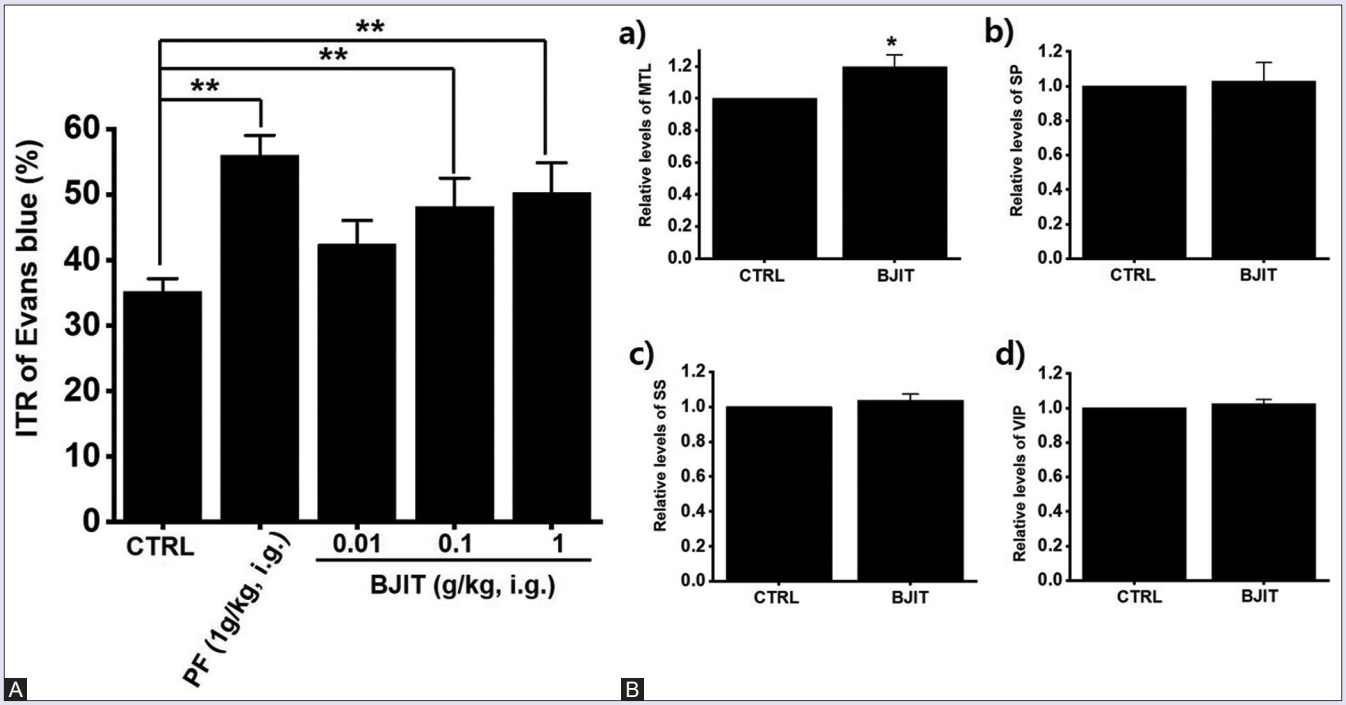

Figure 2: Effects of BJIT on ITR and intestinal hormones in mice. (a) BJIT increased ITR. (b) The levels of GI hormones such as a: MTL, b: SP, c: SS, and d: VIP. *$P < 0.05$. **$P < 0.01$. CTRL, control; BJIT, Bojungikki-tang; PF: *Poncirus trifoliata* Raf.; ITR: Intestinal transit rate; VIP: Vasoactive intestinal peptide

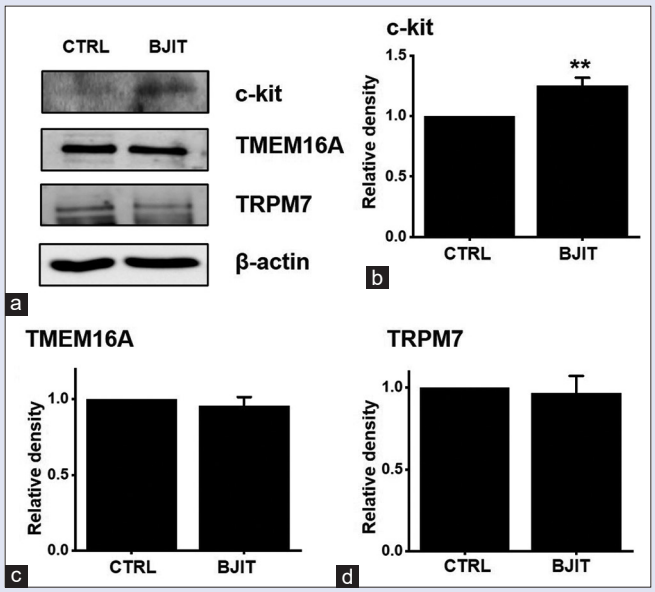

Figure 3: Effects of BJIT on the expression of c-kit, TMEM16A, and TRPM7 in mice. (a) Western blotting showed that the c-kit expression was higher but that of TMEM16A and TRPM7 was almost unchanged. (b-d) The expression of c-kit, TMEM16A, and TRPM7 is presented as band density relative to CTRL. **$P < 0.01$. CTRL: Control; BJIT: Bojungikki-tang; TMEM16A: Transmembrane protein 16A; TRPM7: Transient receptor potential melastatin 7

Table 4: Contents of the seven marker compounds of BJIT measured by UPLC

Compound	Content (ppm)
Hesperidin	25.57±0.88
Naringin	0.16±0.07
Decursin	1.68±0.24
Nodakenin	8.35±1.05
Glycyrrhizinic acid	80.38±3.02
Liquiritigenin	20.25±1.80
Ginsenoside Rg1	17.14±0.89

BJIT: Bojungikki-tang. UPLC: ultra-performance liquid chromatography

depolarization [$n = 6$; Figure 5Da and E]. Thapsigargin, an inhibitor of Ca^{2+}-ATPase of the endoplasmic reticulum, suppressed the pacemaker potential, and BJIT failed to induce pacemaker potential depolarization under this condition [$P < 0.01$; $n = 6$; Figure 5Db and E]. These results suggest that the BJIT-induced depolarization was dependent on the internal Ca^{2+}.

No involvement of TRPM7 and TMEM16A channels was checked during BJIT-induced pacemaker potential depolarization in ICCs.

The change in TRPM7 or TMEM16A channel activity is related to ICC activity.[8,16,17] BJIT (50 mg/ml) decreased the TRPM7 inward and outward currents [Figure 6a and b]. Relative densities were 24.8% ± 3.4% ($P < 0.01$) at + 100 mV [Figure 6c]. TMEM16A currents were inhibited by BJIT [Figure 6d and e]. Relative densities after BJIT treatment were 33.9% ± 4.8% ($P < 0.01$) at + 100 mV [Figure 6f]. These results showed that BJIT suppressed both TRPM7 and TMEM16A channels.

DISCUSSION

We found that the ITR values significantly increased in response to BJIT treatment [Figure 2A]. The level of MTL in the GI significantly increased [Figure 2Ba], but the levels of SP [Figure 2Bb], SS [Figure 2Bc], and VIP [Figure 2Bd] were unaffected by BJIT. The expression of c-kit in the murine small intestine was considerably

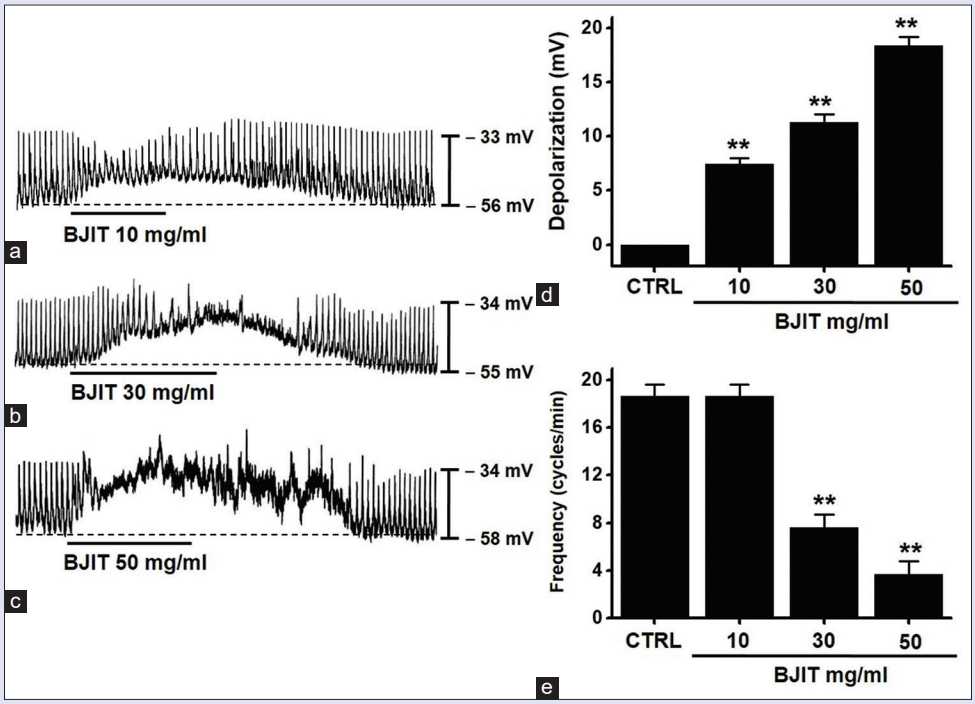

Figure 4: Effects of BJIT on the pacemaker potential of ICCs. (a-c) The pacemaker activity of ICCs stimulated by BJIT (10–50 mg/mL) in the current-clamp mode (I = 0). (d and e) Responses are summarized. ***P* < 0.01. CTRL: Control; BJIT: Bojungikki-tang; ICCs: Interstitial cells of Cajal

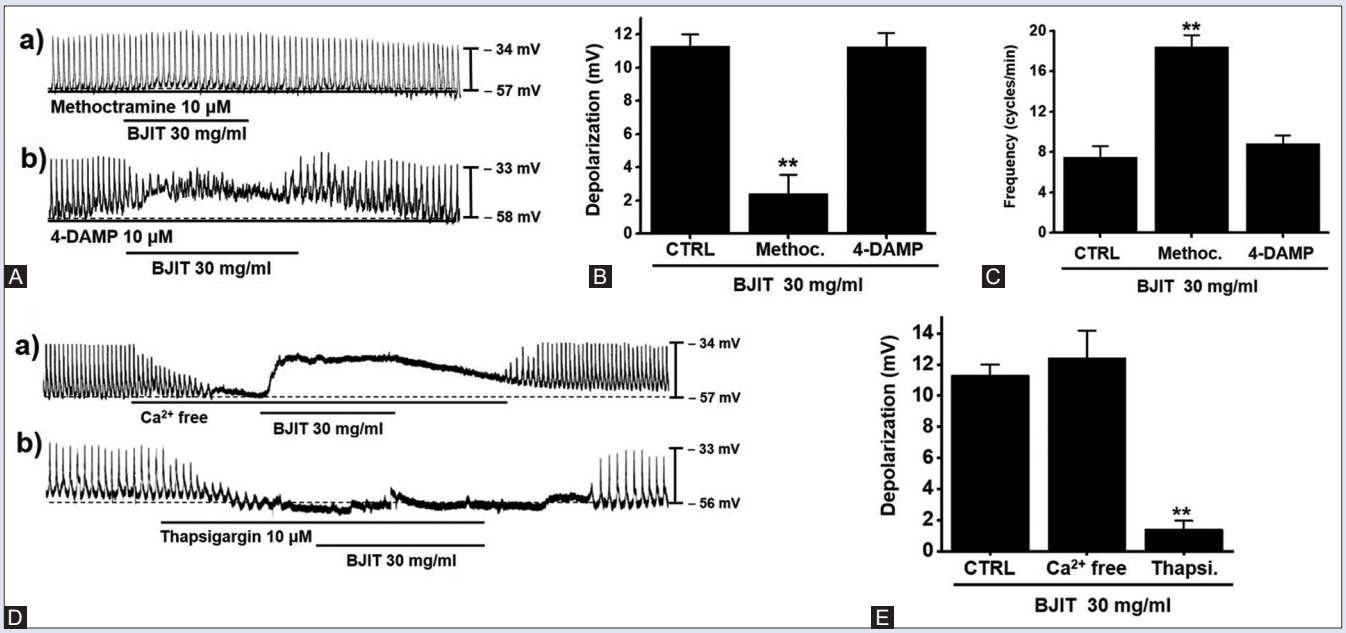

Figure 5: Effects of muscarinic receptor antagonists and external and internal Ca^{2+} on BJIT-induced pacemaker potential depolarization of ICCs. (Aa) Methoctramine inhibited BJIT-induced responses. (Ab) 4-DAMP had no effect on BJIT-induced responses. (Da) In case of external Ca^{2+}-free solution, BJIT induced the pacemaker potential depolarization. (Db) Thapsigargin prevented the BJIT-induced pacemaker potential depolarization. (B, C and E) Responses are summarized. ***P* < 0.01. CTRL: Control; BJIT: Bojungikki-tang; ICCs: Interstitial cells of Cajal; 4-DAMP: 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide

higher after BJIT treatment [Figure 3a and b]. However, TMEM16A and TRPM7 expression was almost unchanged [Figure 3c and d]. Furthermore, BJIT depolarized the pacemaker potential of ICCs [Figure 4]. While methoctramine resulted in the inhibition of BJIT-induced pacemaker potential depolarization [Figure 5Aa], 4-DAMP failed to interfere with the effect of BJIT [Figure 5Ab]. Pretreatment of cells with a Ca^{2+}-free solution failed to inhibit BJIT-mediated effects [Figure 5Da], while the pretreatment with thapsigargin resulted in inhibition of BJIT-induced effects [Figure 5Db]. Furthermore, BJIT blocked both TRPM7 and

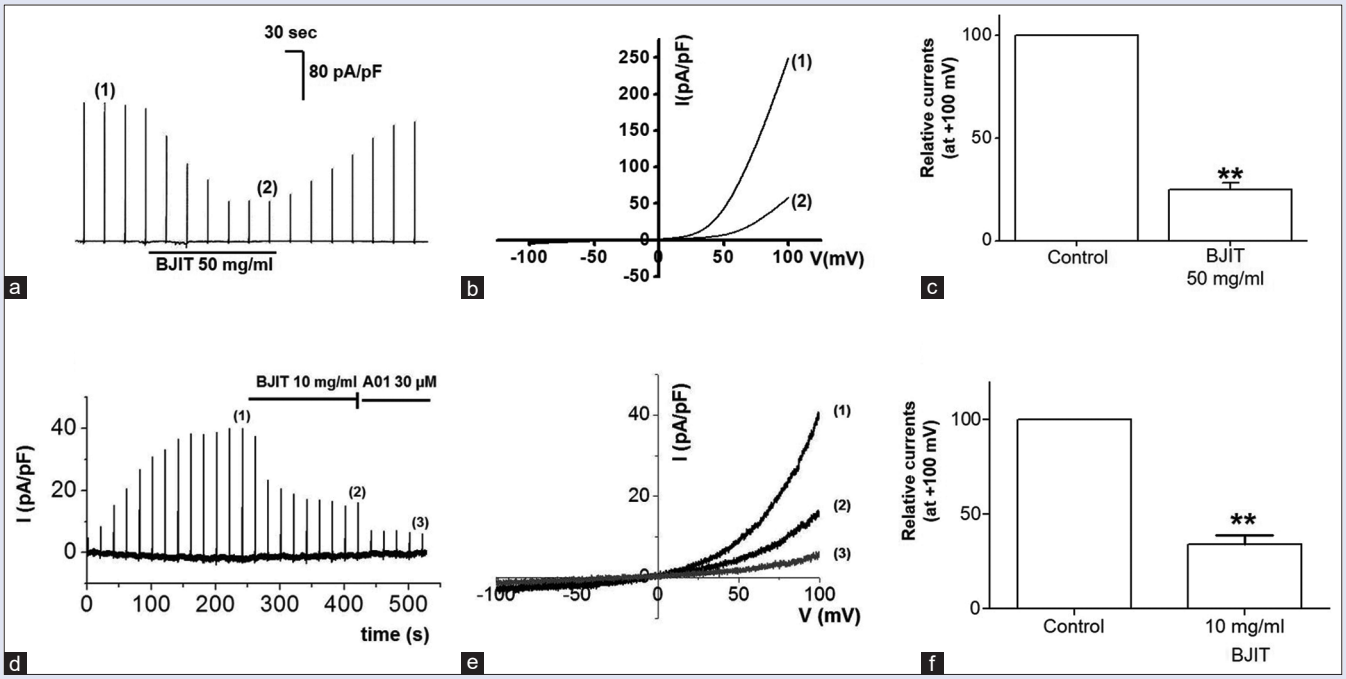

Figure 6: Effects of BJIT on overexpressed TRPM7 or TMEM16A in HEK 293 cells. (a) BJIT blocked overexpressed TRPM7 currents. (b) Current-voltage relationships were measured before (1) and during (2) treatment. (d) BJIT blocked the overexpressed TMEM16A currents. (e) Current-voltage relationships were measured before (1) and during (2) treatment. Responses to BJIT during overexpressed (c) TRPM7 or (f) TMEM16A are summarized. $^{**}P < 0.01$. A01 (a selective TMEM16A inhibitor): A positive control. BJIT: Bojungikki-tang; TRPM7: Transient receptor potential melastatin 7; HEK: Human embryonic kidney; A01, T16Ainh-A01; TMEM16A: Transmembrane protein 16A

TMEM16A channels [Figure 6]. Therefore, BJIT served as an effective prokinetic agent and induced GI motility function.

Traditional medicine offers the advantage of exploiting the healing instinct inherent in nature and is an attractive alternative to compensate the limitations or shortcomings of modern medicine.[23] BJIT (also called Hochu-ekki-to in Japanese) is a traditional herbal formula in Asian countries.[4] BJIT comprises eight herbal components [Table 1]. In general, BJIT has been traditionally used to ameliorate severe weakness in Asian countries.[24] Recent studies have shown that it exhibits immunosuppressive properties against allergic rhinitis[25] and reduces IgE levels in atopic dermatitis.[26] It exerts antibacterial effects against *Helicobacter* infections[27] and suppresses arthritis.[28] In addition, BJIT protects the GI tract from radiation damage[29] and enhances the quality of life and food intake after surgery or cancer treatment-related chemotherapy in addition to reducing the side effects associated with chemotherapy.[30,31] BJIT is also known to enhance the digestive functions[4,5] and increase the defense mechanisms against various infections.[4]

In the present study, pretreatment with methoctramine, but not 4-DAMP, resulted in the blockade of BJIT-induced effects [Figure 5A]. These results suggest that BJIT affected ICC activity through the M_2 receptors. The M_2 and M_3 receptors are in the GI tract and are involved in contraction.[32,33] ICCs express M_2 and M_3 receptors and regulate the slow wave in the GI tract.[21,34] M_2 receptors play an essential role in controlling rhythmic activity, whereas M_3 receptors exhibit regulatory functions.[35] However, a recent study reported that only M_5 and nicotine receptors are expressed in mouse ICCs.[36] Liu *et al.*[37] suggested that muscarinic receptors mediate the inhibitory effect of acetylcholine (ACh) on the ileal pacemaker potential in mice. Further, electrophysiological experiments revealed the ACh- and carbachol (CCh)-mediated increase in the pacemaker frequency and amplitude of ICCs derived from the mouse stomach[38] and the CCh-mediated decrease in the pacemaker frequency and amplitude of ICCs.[39] Thus, further in-depth research on the relevance and mechanism underlying ICCs and muscarinic receptors is warranted. Nevertheless, these results suggest that the effect of BJIT is similar to that of CCh and is mediated through M_2 receptors.

ICCs are specialized gut pacemaker cells. GI motor disorders are related to various chronic diseases and affect the quality of life of patients.[40] As ICC dysfunction or loss has been involved in GI motor disease, ICCs can serve as valuable treatment targets.[10,11] Studies on GI motility are limited by the difficulty involved in obtaining human GI tissue samples.[6] Therefore, various studies on the control of GI motility under normal and diseased conditions have been conducted using ICCs. Furthermore, the pacemaker activity was known to be mainly associated with the activation of TMEM16A or TRPM7.[8,16,17] TRPM7 is expressed in cultured ICCs,[8] consistent with the expression of TMEM16A.[16,17] Therefore, TMEM16A and TRPM7 play key roles in the treatment of GI motility diseases. Further, ICCs express the proto-oncogene c-kit, which is essential for their functions and morphology.[18,41,42] In this study, BJIT inhibited the TRPM7 and ANO1 channels [Figure 6]. In addition, Western blotting revealed the higher expression of c-kit in the murine small intestine after BJIT treatment [Figure 3a and b]. However, the expression of TMEM16A and TRPM7 was almost unchanged following exposure to BJIT [Figure 3c and d]. Therefore, we believe that the BJIT-induced increase in ITR may be associated with the upregulation of c-kit in ICCs.

GI motility is also regulated by various GI hormones such as VIP, MTL, SP, and SS.[43,44] These hormones play key roles in controlling GI motility.[45,46] Therefore, changes in hormone levels are involved in controlling GI

motility. In this study, MTL level considerably increased [Figure 2Ba], but the levels of SP [Figure 2Bb], SS [Figure 2Bc], and VIP [Figure 2Bd] remained unchanged after the administration of BJIT. Therefore, we believe that the increase in the secretion of the GI hormone MTL could be one of the key mechanisms involved in the BJIT-mediated control of intestinal motility.

CONCLUSION

This study shows that (1) the BJIT-induced increase in ITR values was related to the increase in the expression of c-kit; (2) BJIT promoted ITR and increased the level of MTL without affecting the expression of SP, SS, and VIP in mice; (3) BJIT depolarized the ICC pacemaker potential through M_2 receptors via internal Ca^{2+}-dependent pathways; (4) BHSST inhibited TRPM7 and TMEM16A channels. Taken together, BJIT could serve as an effective agent specific for GI motility.

Financial support and sponsorship

This research was supported by the National Research Foundation of Korea (NRF) Grant funded by the Korea Government (MSIP) (No. NRF-2017R1A2B2003764).

Conflicts of interest

There are no conflicts of interest.

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