

Modulation of Pacemaker Potentials by Pyungwi-San in Interstitial Cells of Cajal from Murine Small Intestine - Pyungwi-San and Interstitial Cells of Cajal -

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Key Words

interstitial cells of Cajal, pacemaker cell, Pyungwi-san, traditional herbal medicine, gastrointestinal tract

Abstract

Objective: Pyungwi-san (PWS) plays a role in a number of physiologic and pharmacologic functions in many organs. Interstitial cells of Cajal (ICCs) are pacemaker cells that generate slow waves in the gastrointestinal (GI) tract. We aimed to investigate the beneficial effects of PWS in mouse small-intestinal ICCs.

Methods: Enzymatic digestion was used to dissociate ICCs from the small intestine of a mouse. The whole-cell patch-clamp configuration was used to record membrane potentials from the cultured ICCs.

Results: ICCs generated pacemaker potentials in the GI tract. PWS produced membrane depolarization in the current clamp mode. Pretreatment with a Ca^{2+} -free solution and a thapsigargin, a Ca^{2+} -ATPase, inhibitor in the endoplasmic reticulum, eliminated the generation of pacemaker potentials. However, only when the thapsigargin was applied in a bath solution, the membrane depolarization was not produced by PWS. Furthermore, the membrane depolarizations due to

PWS were inhibited not by U-73122, an active phospholipase C inhibitor, but by chelerythrine and calphostin C, protein kinase C inhibitors.

Conclusions: These results suggest that PWS might affect GI motility by modulating the pacemaker activity in the ICCs.

1. Introduction

Pyungwi-san (PWS), a traditional herbal medicine, has been widely used for the treatment of gastrointestinal (GI) disorders such as inappetance, abdominal distension, borborygmus and diarrhea induced by gastric atony, gastric dilatation and GI catarrh [1, 2]. It is composed of *Atractylodes Rhizoma* (*Atractylodes japonica*), *Citri Pericarpium* (*Citrus unshiu*), *Magnoliae Cortex* (*Machilus thunbergii*), *Zingiberis Rhizoma Recens* (*Zingiber officinale*), *Zizyphi Fructus* (*Zizyphus jujube*), and *Glycyrrhizae Radix* (*Glycyrrhiza uralensis*) [3]. Previous studies have shown that PWS has antioxidant properties. PWS exhibited a concentration-dependent inhibition of 1,1-diphenyl-2-picryl-hydrazil (DPPH) radical adduct formation and it showed dose-dependent free radical scavenging activity onto superoxide anions [4]. Moreover, PWS has protective and pharmacological effects on the gastric mucosal membrane [5]. A

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previous study also demonstrated the safety of PWS and reported an acute toxicity by using a rat model [6]. However, little is known about the beneficial effects of PWS on gastrointestinal (GI) motility.

Interstitial cells of Cajal (ICCs) are the pacemaking cells in the GI muscles and generate the rhythmic oscillations in the membrane potential known as slow waves [7-9]. Slow waves propagate within ICC networks, are conducted into smooth muscle cells via gap junctions, and initiate phasic contractions by activating Ca^{2+} entry through L-type Ca^{2+} channels. The pacemaker activity in the murine small intestine is due mainly to periodic activation of nonselective cation channels (NSCC) [10, 11] or Cl^{-} channels [12, 13]. ICCs also mediate or transduce inputs from the enteric nervous system. However, the effects of PWS in mouse small intestinal ICCs have not yet been investigated. Therefore, we undertook an investigating of the characteristics of PWS in mouse small intestinal ICCs.

2. Materials and methods

2.1. Preparation of cells and cell cultures

Balb/c mice (3-7 days old) of either sex were anesthetized with ether and sacrificed by cervical dislocation. The small intestines from 1 cm below the pyloric ring to the cecum were removed and opened along the mesenteric border. Luminal contents were removed by washing with Krebs-Ringer bicarbonate solution. The tissues were pinned to the base of a Sylgard dish, and the mucosa was removed by sharp dissection. Small tissue strips of the intestine muscle (consisting of both circular and longitudinal muscles) were equilibrated in Ca^{2+} -free Hanks solution (containing in mmol/L: KCl 5.36, NaCl 125, NaOH 0.34, Na_2HCO_3 0.44, glucose 10, sucrose 2.9, and HEPES 11) for 30 min. Then, the cells were dispersed using an enzyme solution containing collagenase (Worthington Biochemical Co., Lakewood, NJ, USA) at 1.3 mg/mL, bovine serum albumin (Sigma Chemical Co., St. Louis, MO, USA) at 2 mg/mL, trypsin inhibitor (Sigma) at 2 mg/mL and adenosine triphosphate (ATP) at 0.27 mg/mL. Cells were plated onto sterile glass coverslips coated with murine collagen (2.5 $\mu\text{g}/\text{mL}$, Falcon/BD, Franklin Lakes, NJ, USA) in a 35-mm culture dish and were then cultured at 37°C in a 95% O_2 , 50-mL/L CO_2 incubator in a smooth muscle growth medium (Clonetics Corp., San Diego, CA, USA) supplemented with 2% antibiotics/antimycotics (Gibco, Grand Island, NY, USA) and murine stem cell factor (SCF, 5 ng/mL, Sigma). ICCs were identified immunologically by using an anti-c-kit antibody (phycoerythrin-conjugated rat anti-mouse c-kit monoclonal antibody; eBioscience, San Diego, CA, USA) at

a dilution of 1:50 for 20 min [14]. ICCs were morphologically distinct from other cell types in the culture, thus, identifying the cells by using phase contrast microscopy once they had been verified with the anti c-kit antibody was possible.

2.2 Patch-clamp experiments

The whole-cell patch-clamp configuration was used to record membrane potentials (current clamp) for the cultured ICCs. An axopatch ID (Axon Instruments, Foster, CA, USA) was used to amplify the membrane currents and potentials. The command pulse was applied using an IBM-compatible personal computer and pClamp software (version 6.1, Axon Instruments). Data obtained were filtered at 5 kHz and displayed on an oscilloscope, a computer monitor, and a pen recorder (Gould 2200, Gould, Valley View, OH, USA). Results were analyzed using pClamp and Origin (version 6.0) software. All experiments were performed at 30-32°C.

2.3 Solutions and drugs

The physiological salt solution used to bathe the cells (Na⁺-Tyrode) contained (mmol/L): KCl 5, NaCl 135, CaCl_2 2, glucose 10, MgCl_2 1.2 and HEPES 10, adjusted to pH 7.4 with NaOH. The pipette solution contained (mmol/L): KCl 140, MgCl_2 5, K_2ATP 2.7, NaGTP 0.1, creatine phosphate disodium 2.5, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 5 and ethylene glycol tetraacetic acid (EGTA) 0.1, adjusted to pH 7.2 with KOH.

PWS was purchased from I-WORLD Pharmaceuticals (South Korea). PWS is composed of *Atractylodes Rhizoma* (*Atractylodes japonica*), *Citri Pericarpium* (*Citrus unshiu*), *Magnoliae Cortex* (*Machilus thunbergii*), *Zingiberis Rhizoma Recens* (*Zingiber officinale*), *Zizyphi Fructus* (*Zizyphus jujube*), and *Gycyrrhizae Radix* (*Glycyrrhiza uralensis*) (Table 1). The dosage for adult is 10-15 g (crude material) per day. More information about PWS can be found at I-WORLD Pharmaceuticals homepage (<http://i-pharm.koreasme.com>). The PWS was dissolved in distilled water at a concentration of 0.5 g (crude drug)/ml and stored in a refrigerator. All other drugs were obtained from Sigma (Sigma Chemical Co., USA). Drugs were dissolved in distilled water and added to the bath solution just prior to use to make the desired concentrations. Addition of these chemicals to the bath solution did not alter the pH of the solution. Thapsigargin, U-73122, and U-73343 were dissolved in dimethyl sulfoxide (DMSO) to obtain a 50- or 100-mmol/L stock solution that was added (1000 times dilution) to the bath solution on the day of the experiment. The final concentration of DMSO in the bath solution was always < 0.1%, and we confirmed that this concentration

of DMSO did not affect the results that were recorded.

Table 1 Amount and composition of PWS

Herb	Scientific name	Amount (g)
Chang Chul	Atractylodis Rhizoma	2.01 g
Jin Pi	Citri Pericarpium	1.28 g
Hu Pak	Magnoliae Cortex	0.22 g
Kam Cho	Glycyrrhizae Radix	0.43 g
Saeng Kang	Zingiberis Rhizoma Recens	0.26 g
Dae Chu	Jujubae Fuctus	1.65 g
	Total amount	5.85 g

2.4. Statistics

All data are expressed as means \pm standard error (SE). The Student's *t*-test for unpaired data was used to compare the control and the experimental groups. A *P* value of less than 0.05 was considered statistically significant.

3. Results

3.1. Effect of PWS on pacemaker potentials in cultured ICCs

The patch-clamp technique was tested on ICCs that formed network-like structures in the culture (2-4 days). Spontaneous rhythms were routinely recorded from cultured ICCs under current- and voltage-clamp conditions, and the ICCs within networks displayed more robust electrical rhythms. Tissue-like spontaneous slow waves have been recorded from these cells [15]. To understand the relationship between PWS and the modulation of pacemaker activity in ICCs, we examined the effects of PWS on the pacemaker potentials. Recording from cultured ICCs under the current-clamp mode ($I = 0$) showed spontaneous pacemaker potentials. The resting membrane potential was -51.4 ± 1.2 mV, and the amplitude was 21 ± 3 mV. In the presence of PWS (5 - 50 mg/ml), the membrane potentials were depolarized to 1.0 ± 0.1 mV at 5 mg/ml, 8.4 ± 1.3 mV at 10 mg/ml, 18.3 ± 1.4 mV at 30 mg/ml, and 25.4 ± 1.1 mV at 50 mg/ml (Figs. 1A-D). A summary of the values and a bar graph for the effects of PWS on the pacemaker potentials are presented in Fig. 1E ($n = 4$).

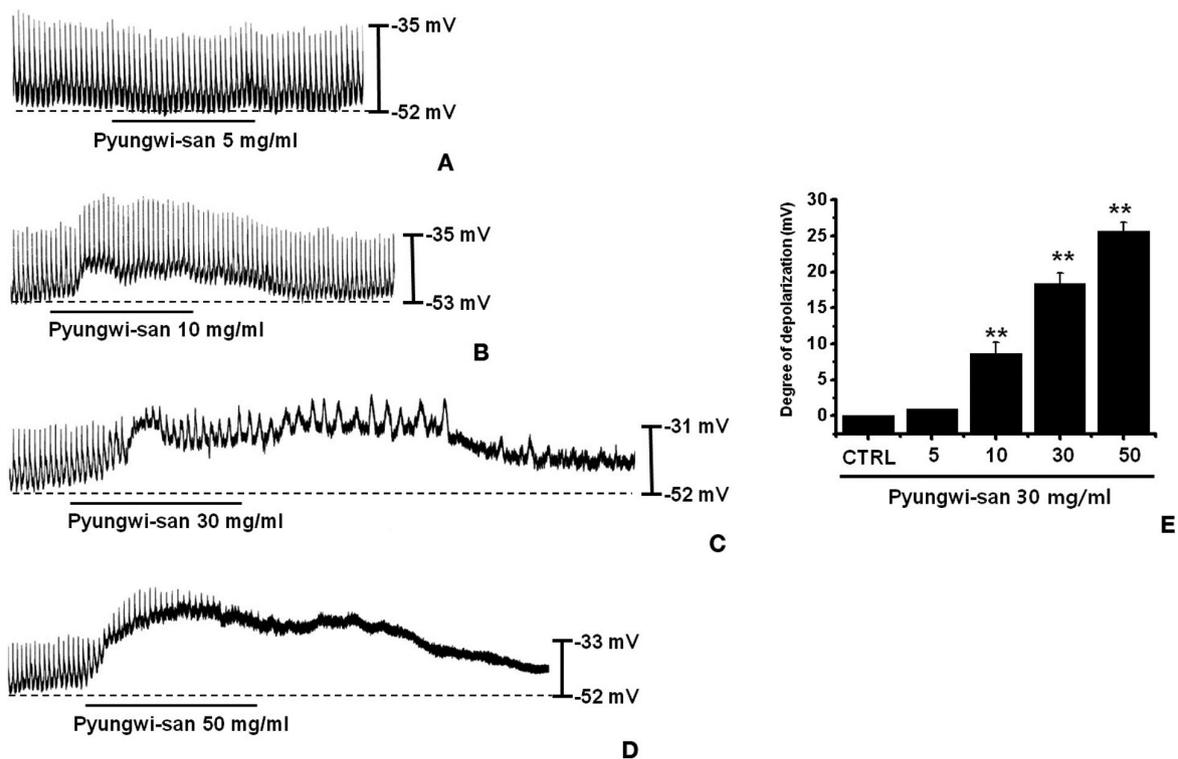


Figure 1 Effects of PWS on pacemaker potentials in cultured ICCs from murine small intestine. A-D: ICCs were isolated from the Balb/c small intestine after primary cell culture for various doses of PSW (5 to 50 mg/mL) and the clamping mode ($I = 0$). Summary of the responses to PWS in E. The error bars represent mean values \pm standard error (SE). **($P < 0.01$) Significantly different from the untreated control. CTRL: Control.

3.2 Effects of pretreatment with an external Ca^{2+} -free solution and a Ca^{2+} -ATPase inhibitor in the endoplasmic reticulum on PWS-induced pacemaker potentials in cultured ICCs

An external Ca^{2+} influx is necessary for GI smooth muscle contractions and is essential for generating pacemaker potentials in the ICCs. The generation of pacemaker currents depended upon intracellular Ca^{2+} oscillation [16]. To investigate the role of external Ca^{2+} vs internal Ca^{2+} , we tested PWS under external Ca^{2+} -free conditions and in the presence of thapsigargin, a Ca^{2+} -ATPase inhibitor of the endoplasmic reticulum [10, 17]. The pacemaker potentials were completely eliminated with an external Ca^{2+} -free solution was used. In this condition, PWS induced membrane depolarizations (n = 4; Fig. 2A). Under external Ca^{2+} -free conditions, the membrane depolarizations with PWS (30 mg/ml) were not significantly different when compared to those with PWS (30 mg/ml) in normal Ca^{2+} solution (n = 4, Fig. 2C). In addition, PWS-induced membrane depolarizations were inhibited by pretreatment with thapsigargin (Fig. 3B). In the presence of thapsigargin (5 μm), the membrane depolarizations with PWS were significantly different from those with PWS in the absence of thapsigargin (n = 4, Fig. 3C).

3.3. Effects of phospholipase C inhibitor on PWS-induced pacemaker potentials in cultured ICCs

Since the membrane depolarizations caused by PWS

were related to intracellular Ca^{2+} mobilization, we examined whether the effects on the pacemaker potentials required phospholipase C (PLC) activation. To test this possibility, we measured the PWS-induced membrane depolarizations in the absence and the presence of U-73122, an active PLC inhibitor [18]. The pacemaker membrane depolarizations were completely eliminated when U-73122 (5 μm) was applied; however, under these conditions, PWS-induced (30 mg/ml) membrane depolarizations were produced (n = 4; Fig. 3A). In the presence of U-73122, the membrane depolarization produced by using PWS were 18.1 ± 1.5 mV. The membrane depolarizations caused by PWS with U-73122 were not different from those caused by PWS in the absence of U-73122 (n = 4, Fig. 3C). The treatment of U-73343 (5 μm), an inactive analog of U-73122, had no influence on the PWS-induced pacemaker potentials and under these conditions, PWS-induced (30 mg/ml) membrane depolarizations were not suppressed by using U-73343 (Fig. 3B).

3.4. Effect of protein kinase C inhibitor on PWS-induced pacemaker potentials in cultured ICCs

We tested the effects of chelerythrine or calphostin C, an inhibitor of protein kinase C [17, 19], to investigate whether the PWS-induced responses to the pacemaker potentials were mediated by the activation of protein kinase C. Chelerythrine (1 μm) or calphostin C (10 μm), had no effect

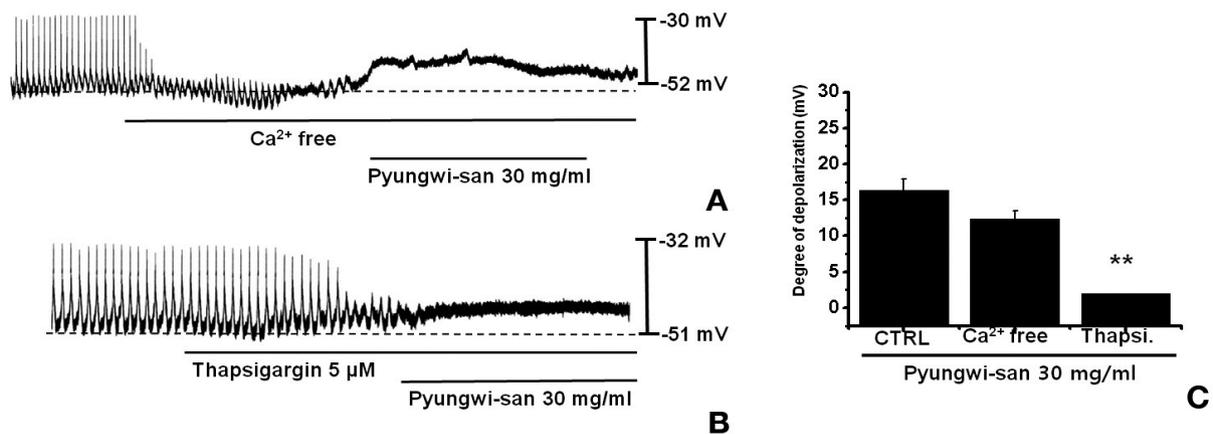


Figure 2 Effects of pretreatment with an external Ca^{2+} -free solution or thapsigargin, a Ca^{2+} -ATPase inhibitor, in the endoplasmic reticulum and of U-73122, an active phospholipase C inhibitor, on PWS-induced pacemaker potentials in cultured ICCs. A: ICCs were isolated from the Balb/c small intestine after primary cell culture for various doses of PSW (5 to 50 mg/mL) and the clamping mode ($I = 0$). The external Ca^{2+} -free solution eliminated the generation of pacemaker potentials. Under these conditions, PWS-induced (30 mg/ml) membrane depolarizations were produced. B: Thapsigargin (5 μm) eliminated the generation of pacemaker potentials. Thapsigargin blocked the PWS-induced (30 mg/ml) membrane depolarizations. C: Summary of the responses to PWS in the presence of an external Ca^{2+} -free solution and thapsigargin. Bars represent the mean values \pm SE. **($P < 0.01$) Significantly different from the untreated control. CTRL: Control.

on the membrane depolarizations caused by PWS (30 mg/ml; Fig. 4) and the values of the membrane depolarizations were significantly different from those

caused by PWS in the absence of chelerythrine or calphostin C (n = 5; Fig. 4C).

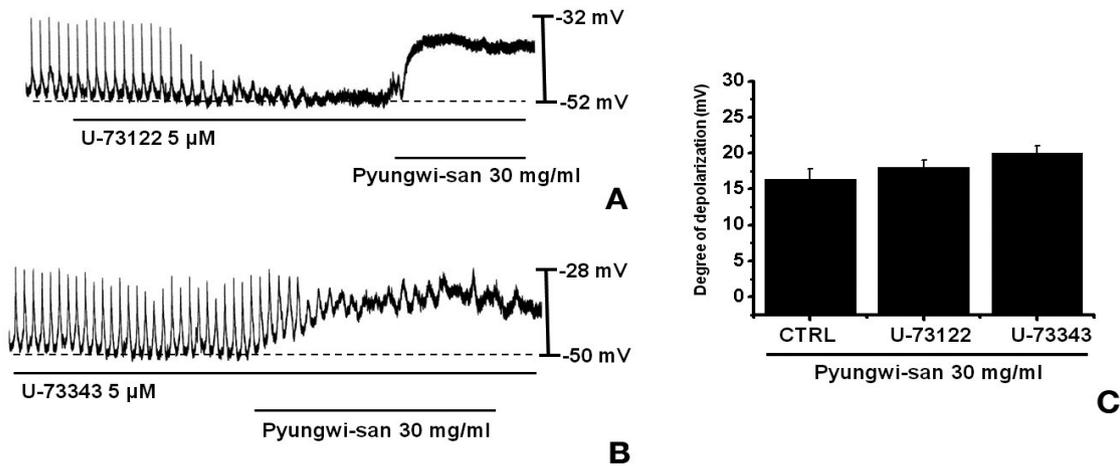


Figure 3 Effects of PWS on phospholipase C inhibitors in cultured ICCs. A: ICCs were isolated from the Balb/c small intestine after primary cell culture for various doses of PSW (5 to 50 mg/mL) and the clamping mode ($I = 0$). U-73122 ($5 \mu\text{m}$), a phospholipase C inhibitor, eliminated the generation of pacemaker potentials. U-73122 did not block the PWS-induced (30 mg/ml) membrane depolarizations. B: The application of U-73343 ($5 \mu\text{m}$) did not influence the generation of pacemaker currents. Also, U-73343 did not block the PWS-induced (30 mg/ml) membrane depolarizations. C: Summary of the responses to PWS in phospholipase C inhibitors. Bars represent the mean values \pm SE. CTRL: Control.

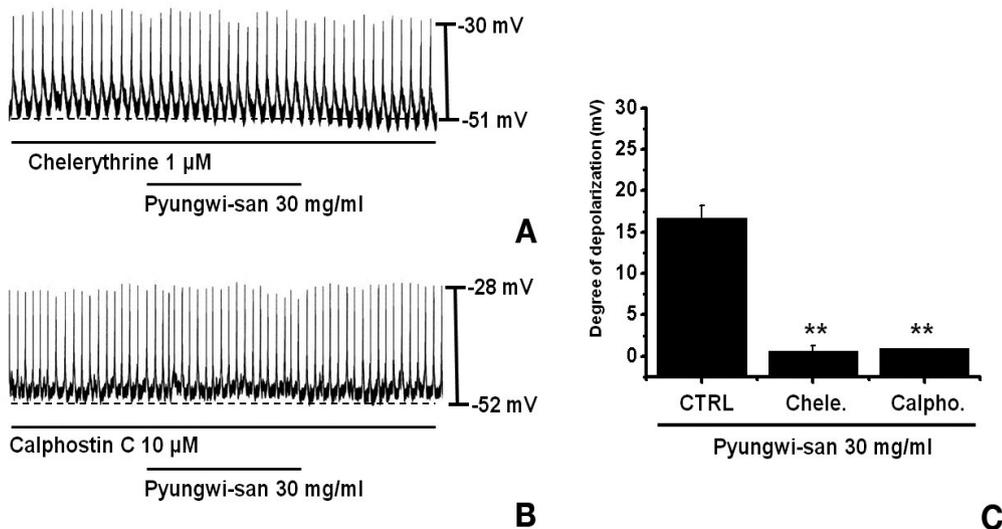


Figure 4 Effects of chelerythrine or calphostin C, an inhibitor of protein kinase C, on PWS-induced pacemaker potentials in cultured ICCs of the murine small intestine. A, B: ICCs were isolated from the Balb/c small intestine after primary cell culture for various doses of PSW (5 to 50 mg/mL) and the clamping mode ($I = 0$). Pacemaker potentials of ICCs exposed to PWS (30 mg/ml) in the presence of chelerythrine ($1 \mu\text{m}$) or calphostin C ($10 \mu\text{m}$) are shown. Under these conditions, PWS did not cause membrane depolarizations. C: Summary of the responses to PWS in the presence of chelerythrine or calphostin C. Bars represent the mean values \pm SE **($P < 0.01$) Significantly different from the untreated control. CTRL: Control.

4. Discussion

ICCs are the pacemaking cells in the GI muscles and generate the rhythmic oscillations in the membrane potential known as slow waves [8-10]. Slow waves propagate within ICC networks, conduct into smooth muscle cells via gap junctions, and initiate phasic contractions by activating Ca^{2+} entry through L-type Ca^{2+} channels. The pacemaker activity in the murine small intestine is due mainly to periodic activation of nonselective cation channels (NSCCs) [11] or Cl^- channels [12, 13]. ICCs also mediate or transduce inputs from the enteric nervous system. Because of the central role of ICCs in GI motility, loss of these cells would be extremely detrimental. Research on the biological function of ICCs provides exciting new opportunities for understanding the etiology of diseases that have long eluded comprehension [9, 11].

PWS is a mixture of six herbs and is traditionally used in northeast Asia (especially Korea and Japan) for the treatment of gastrointestinal disorders, such as dyspepsia and inappetence induced by gastric dilatation and gastrointestinal catarrh. A composite traditional Korean medicine, PWS is a basic prescription consisting of six herbs and contains hesperidin, 6-gingerol, honokiol, glycyrrhizin and magnolol [20]. Although, the beneficial effects of PWS have been elucidated for antioxidant and clinical uses, the effects of PWS on GI motility and on the ICCs have not been investigated yet.

In this study, PWS produced membrane depolarization in the current-clamp mode. Pretreatment with a Ca^{2+} -free solution and thapsigargin, a Ca^{2+} -ATPase inhibitor in the endoplasmic reticulum, eliminated the generation of pacemaker potentials. Under Ca^{2+} -free solution conditions, PWS was found to cause membrane depolarizations. Also, the pacemaker membrane depolarizations were not inhibited by the use of U-73122, an active phospholipase C inhibitor. Furthermore, the protein kinase C inhibitors, chelerythrine and calphostin C, did block the PWS-induced pacemaker potentials. These results suggest that PWS might affect GI motility through a modulation of the pacemaker activity in the ICCs, and that the activation is associated with protein kinase C activation, and with Ca^{2+} release from internal storage in an external- Ca^{2+} -independent and phospholipase C-independent manner.

Taken together, our data suggest that the gastroprokinetic effects of PWS might be mediated by the induction of pacemaker potentials in the ICCs. Considering the effects of this drug on the ICCs, further research, including finding active compound(s) and examining their action mechanisms, is clearly needed.

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References

1. Heo J. [Dongeuibogam]. Seoul:Namsandang;1994. 435 p. Korean.
2. Riedlinger JE, Tan PW, Lu W. Ping wei san, a Chinese medicine for gastrointestinal disorders. *Ann Pharmacother.* 2001;35(2):228-35.
3. Shin IS, Seo CS, Ha HK, Lee MY, Huang DS, Huh JI, et al. Genotoxicity assessment of Pyungwi-san (PWS), a traditional herbal prescription. *J Ethnopharmacol.* 2011;133(2):696-703.
4. Lee HS, Moon JY. [Scavenging property of Pyungwi-san herbal-acupuncture solution on ROS and RNS]. *Korean J Oriental Physiology & Pathology.* 2007;21(1):165-70. Korean.
5. Ji HC, Baek TH. [A comparative study of Pyeongwi-san, Ijin-tang and Pyeongjintang extracts on indomethacin-induced gastric mucosal lesions in mice.] *J Korean Oriental Med.* 2011;32(2):102-17. Korean.
6. Shin IS, Kim JH, Ha HK, Huang DS, Huh JI, Shin HK. [Study on safety of Pyungwi-san in Sprague-Dawley rats]. *Korean J Oriental Physiology & Pathology.* 2010;24(3):426-9. Korean.
7. Ward SM, Burns AJ, Torihashi S, Sanders KM. Mutation of the proto-oncogene c-kit blocks development of interstitial cells and electrical rhythmicity in murine intestine. *J Physiol.* 1994;480(Pt 1):91-7.
8. Huizinga JD, Thuneberg L, Kluppel M, Malysz J, Mikkelsen HB, Bernstein A. W/kit gene required for interstitial cells of Cajal and for intestinal pacemaker activity. *Nature.* 1995;373(6512):347-9.
9. Sanders KM. A case for interstitial cells of Cajal as pacemakers and mediators of neurotransmission in the gastrointestinal tract. *Gastroenterology.* 1996;111(2):492-515.
10. Koh SD, Jun JY, Kim TW, Sanders KM. A Ca^{2+} -inhibited non-selective cation conductance contributes to pacemaker currents in mouse interstitial cell of Cajal. *J Physiol.* 2002;540(Pt 3):803-14.
11. Kim BJ, Lim HH, Yang DK, Jun JY, Chang IY, Park CS, et al. Melastatin-type transient receptor potential channel 7 is required for intestinal pacemaking activity. *Gastroenterology.* 2005;129(5):1504-17.
12. Huizinga JD, Zhu J, Molleman A. High-conductance chloride channels generate pacemaker currents in

- interstitial cells of Cajal. *Gastroenterology*. 2002;123(5):1627-36.
13. Zhu MH, Kim TW, Ro S, Yan W, Ward SM, Koh SD, et al. A Ca^{2+} -activated Cl^- conductance in interstitial cells of Cajal linked to slow wave currents and pacemaker activity. *J Physiol*. 2009;587(Pt 20):4905-18.
 14. Goto K, Matsuoka S, Noma A. Two types of spontaneous depolarizations in the interstitial cells freshly prepared from the murine small intestine. *J Physiol*. 2004;559(Pt 2):411-22.
 15. Koh SD, Sanders KM, Ward SM. Spontaneous electrical rhythmicity in cultured interstitial cells of Cajal from the murine small intestine. *J Physiol*. 1998;513(Pt 1):203-13.
 16. Ward SM, Ordog T, Koh SD, Baker SA, Jun JY, Amberg G, et al. Pacemaking in interstitial cells of Cajal depends upon calcium handling by endoplasmic reticulum and mitochondria. *J Physiol*. 2000;525 Pt 2:355-61.
 17. Choi S, Choi JJ, Jun JY, Koh JW, Kim SH, Kim DH, et al. Induction of pacemaker currents by DA-9701, a prokinetic agent, in interstitial cells of Cajal from murine small intestine. *Mol Cells*. 2009;27(3):307-12.
 18. Sakamoto T, Unno T, Matsuyama H, Uchiyama M, Hattori M, Nishimura M, et al. Characterization of muscarinic receptor-mediated cationic currents in longitudinal smooth muscle cells of mouse small intestine. *J Pharmacol Sci*. 2006;100(3):215-26.
 19. Aiello EA, Clement-Chomienne O, Sontag DP, Walsh MP, Cole WC. Protein kinase C inhibits delayed rectifier K^+ current in rabbit vascular smooth muscle cells. *Am J Physiol*. 1996;271(1 Pt 2):H109-19.
 20. Yamauchi Y, Ueda J, Ohsawa K. [A simultaneous determination of various main components in oriental pharmaceutical decoction "heii-san" by ion-pair high-performance liquid chromatography]. *Yakugaku Zasshi*. 1996;116(10):776-82. Japanese.