Original Paper

Magnolia Officinalis Bark Extract Induces Depolarization of Pacemaker Potentials Through M₂ and M₃ Muscarinic Receptors in Cultured Murine Small Intestine Interstitial Cells of Cajal

Hyun Jung Kim^{a,b} Taewon Han^c Yun Tai Kim^{c,d} Insuk So^e Byung Joo Kim^{a,b}

^aDivision of Longevity and Biofunctional Medicine, Pusan National University School of Korean Medicine, Yangsan, ^bHealthy Aging Korean Medical Research Center, Pusan National University School of Korean Medicine, Yangsan, ^cResearch group of Innovative Special Food, Korea Food Research Institute, Seongnam, ^dDepartment of Food Biotechnology, Korea University of Science & Technology, Daejeon, ^eDepartment of Physiology, Seoul National University College of Medicine, Seoul, Republic of Korea

Key Words

Magnolia officinalis Rehder and EH Wilson • M. officinalis • Interstitial Cells of Cajal • Gastric Emptying • Pacemaker Potentials • Intestinal Transit Rates • Gastrointestinal Motility

Abstract

Background: *Magnolia officinalis* Rehder and EH Wilson (*M. officinalis*) are traditional Chinese medicines widely used for gastrointestinal (GI) tract motility disorder in Asian countries. We investigated the effects of an ethanol extract of *M. officinalis* (MOE) on the pacemaker potentials of cultured interstitial cells of Cajal (ICCs) *in vitro* and its effects on GI motor functions *in vivo*. **Methods:** We isolated ICCs from small intestines, and the whole-cell patch-clamp configuration was used to record the pacemaker potentials in cultured ICCs *in vitro*. Both gastric emptying (GE) and intestinal transit rates (ITRs) were investigated in normal and GI motility dysfunction (GMD) mice models *in vivo*. **Results:** MOE depolarized ICC pacemaker potentials dose-dependently. Pretreatment with methoctramine (a muscarinic M₂ receptor antagonist) and 4-DAMP (a muscarinic M₃ receptor antagonist) inhibited the effects of MOE on the pacemaker potential relative to treatment with MOE alone. In addition, MOE depolarized pacemaker potentials after pretreatment with Y25130 (a 5-HT₃ receptor antagonist), GR113808 (a 5-HT₄ receptor antagonist) or SB269970 (a 5-HT₇ receptor antagonist). However, pretreatment with RS39604 (a 5-HT₄ receptor antagonist) blocked MOE-induced pacemaker potential depolarizations. Intracellular GDPβS inhibited MOE-induced pacemaker potential depolarization, as did pretreatment with Ca²⁺ free solution or thapsigargin. In normal mice, the GE and ITR values were significantly and dose-dependently increased by MOE. In loperamide-

Byung Joo Kim

Division of Longevity and Biofunctional Medicine, Pusan National University School of Korean Medicine 49 Busandaehakro, Mulgeum-eup, Yangsan, Gyeongsangnamdo, (Republic of Korea); Tel. 82-515108469, Fax 82-515108420, E-Mail vision@pusan.ac.kr

and cisplatin-induced GE delay models, MOE administration reversed the GE deficits. The ITRs of the GMD mice were significantly reduced relative to those of normal mice, which were significantly and dose-dependently reversed by MOE. **Conclusion:** These results suggest that MOE dose-dependently depolarizes ICCs pacemaker potentials through M_2 and M_3 receptors via internal and external Ca^{2+} regulation through G protein pathways *in vitro*. Moreover, MOE increased GE and ITRs *in vivo* in normal and GMD mouse models. Taken together, the results of this study show that MOE have the potential for development as a gastropromotric agent in GI motility function.

© 2017 The Author(s)
Published by S. Karger AG, Basel

Introduction

Magnolia officinalis Rehder and EH Wilson (*M. officinalis*) have been widely used in traditional Chinese medicine to treat various symptoms, including those of anxiety, stress, antibacterial diseases, allergic diseases and oxidative stress [1-6]. In addition, *M. officinalis* is an herb with a large variety of uses for the remedy of gastrointestinal (GI) disorders such as dyspepsia, abdominal distention and constipation [7-13]. *M. officinalis* has been shown to improve gastrointestinal peristaltic activity in mice [12], as well as to have anti-spasmodic effects in guinea pigs [8]. Moreover, *M. officinalis* has beneficial effects on atropine-induced GI tract dysmotility that are mediated by activation of the c-kit/stem cell factor (SCF) pathway in interstitial cells of Cajal (ICCs) [11]. In addition, Jeong et al. [14] suggested that the main component of *M. officinalis*, magnolol, showed the contractile activity in the rat GI tract. However, Zhang et al. [13] suggested that magnolol inhibited the contractions of distal colonic segments by inhibiting L-type Ca^{2+} channels in rat.

ICCs are the pacemaker cells in the GI tract that contribute to normal GI motility by generating electrical slow waves [15-17]. Damage to ICCs or reductions in ICC numbers have been described in many GI motility disorders [18, 19]. Therefore, it is important to identify the mechanisms underlying ICC pacemaker activity to understand GI function. However, no scientific studies conducted to date have presented evidence of any relationship between *M. officinalis* and ICCs.

In the present study, we investigated the effects of an ethanol extract of *M. officinalis* (MOE) on the pacemaker potentials of cultured ICCs *in vitro* and the effects of MOE on GI motor functions *in vivo* under the normal and abnormal conditions by determining the gastric emptying (GE) and the intestinal transit rates (ITR) in mice.

Materials and Methods

Preparation of the sample and HPLC Analysis

The dried bark of *Magnolia officinalis* was purchased from Kapdang Co. (Seoul, Korea). Honokiol and magnolol were purchased from Sigma-Aldrich (St. Louis, MO, USA). The plant samples were confirmed by Dr. Yun Tai Kim and a voucher specimen (#NP-1601) was deposited with the research group of innovative special food, Korea Food Research Institute. Dried bark of *M. officinalis* (600 g) was extracted with 70% ethanol (6000 mL) for 1 hour at 20°C and the process was repeated once. The extracts were combined, then passed through a membrane filter (0.45 µm; Millipore, Billerica, MA, USA), after which the solvents were removed by rotary evaporation (N-21NS, EYELA, Tokyo, Japan). The remaining extracts were then freeze-dried, yielding about 1.47% of the dried bark weight (w/w). The freeze-dried extract powder (100 mg) was subsequently dissolved in 10 mL of methanol for one hour, then filtered through a 0.45 µm RC-membrane filter (Sartorius Stedim, Goettingen, Germany) and diluted prior to injection (20 µL) into the HPLC system. Analytical HPLC was conducted using a Jasco HPLC system (Jasco, Hachioji, Tokyo, Japan) comprising a PU-980 pump, an AS-950-10 autosampler, and a MD-2010 Plus multi-wavelength detector. Chromatic separation was conducted at 30°C on a Waters Symmetry® C18 (4.6 mm × 250 mm, particle size 5 µm) column, after which samples were eluted using a mobile phase composed of 0.4% formic acid and acetonitrile (35:65 v/v) that was applied for 15 min at 1.0 mL/min and the samples were detected at 294 nm. Quantitative analysis

was replicated three times. The regression equation and correlation coefficient (r^2) of each standard curve were automatically determined using a Jasco HPLC system. The regression equations of honokiol and magnolol were $y=4228.1899x + 18613.9165$ with an R^2 of 0.99959 and $y=5766.4127x - 13822.8914$ with an R^2 of 1, respectively, indicating that a high linear correlation was achieved for all standard curves. The concentrations of honokiol and magnolol were determined to be 29.417 ± 0.303 mg/g and 56.609 ± 1.447 mg/g based on the peak area in the chromatogram and the regression equation (Fig. 1).

Ethics

Animal care and experiments were conducted in accordance with the guidelines issued by the Institutional Animal Care and Use Committee (IACUC) at Pusan National University (Busan, Republic of Korea; Approval no. PNU-2015-1036) and those issued by the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Preparation of ICC and ICC clusters

ICR mice (Samtako Bio Korea Co., Ltd., Osan, Republic of Korea; 3–7 days old; weighing 1.9–2.2 g) of either sex were used to examine the effects of MOE on ICC pacemaker potentials. Animals were maintained under controlled conditions ($21 \pm 3^\circ\text{C}$, relative humidity (RH) $50 \pm 6\%$, lights on 6 a.m.–6 p.m.). Small intestines were removed and opened along the mesenteric border. Luminal contents were removed using Krebs-Ringer bicarbonate solution. Mucosae were then removed by sharp dissection, and small tissue strips of intestine muscle were then equilibrated for 30 min in Ca^{2+} -free Hank's solution (containing (in mM); KCl 5.36, NaCl 125, NaOH 0.34, Na_2HCO_3 0.44, glucose 10, sucrose 2.9 and HEPES 11; pH 7.4). Cells were then dispersed in an enzyme solution containing 1.7 mg ml^{-1} collagenase (Worthington Biochemical, Lakewood, NJ, USA), 2.5 mg ml^{-1} bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA), 3.0 mg ml^{-1} trypsin inhibitor (Sigma-Aldrich, St. Louis, MO, USA), and 0.60 mg ml^{-1} ATP (Sigma-Aldrich, St. Louis, MO, USA). Next, cells were cultured at 37°C in a 95% O_2 -5% CO_2 incubator in smooth muscle growth medium (SMGM) (Clonetics, San Diego, CA, USA) supplemented with 2% antibiotics/antimycotics (Gibco, Grand Island, NY, USA) and 5 ng ml^{-1} of murine stem cell factor (SCF) (Sigma-Aldrich, St. Louis, MO, USA). At first, ICCs were cultured only for $<12 \text{ h}$ and we checked using anti-c-kit antibody immunologically (eBioscience, San Diego, CA, USA) at a dilution of 1:50 for 20 min [20]. However, in this case, we could not experiment with patch-clamp methods because of bad state of ICCs. Therefore, because ICCs differed morphologically from other cell types in cultures, they were identified by phase contrast microscopy. The morphology of ICCs was spindle-shaped, with several branches emanating from a central soma, and connected with neighboring cells, showing networks. The ICCs in the cultures were morphologically distinct from the other cells. Therefore, the patch-clamp technique was tested from ICCs that showed the network-like structures in cultures and after that, we identified c-Kit protein by immunostaining with anti c-kit antibody [21]. In this case, almost all cells are c-kit positive and at this time, spontaneous rhythmicity was routinely recorded from cultured ICC under current-clamp conditions and ICC within networks had a more robust electrical rhythmicity, and tissue-like spontaneous slow waves were recorded from these cells [22].

Patch-clamp experiments

We used whole-cell patch clamp methods to record the effects of MOE on ICC pacemaker potentials. The physiological salt solution used to bathe cultured ICC clusters contained (in mM): 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 used to examine pacemaker potentials contained (in mM): KCl 140, MgCl_2 5, K_2ATP 2.7, NaGTP 0.1, creatine phosphate disodium 2.5, HEPES 5, and EGTA 0.1 (adjusted to pH 7.2 with KOH). Patch-clamp techniques were conducted using Axopatch I-D and Axopatch 200B amplifiers (Axon Instruments, Foster, CA, USA). Command pulses were applied using an IBM-compatible personal computer and pClamp software (version 6.1 and version 10.0; Axon Instruments, Foster, CA, USA). Data were filtered at 5 kHz and displayed on an oscilloscope, a computer monitor, and/or on a pen recorder (Gould 2200) (Gould, Valley View, OH, USA). Results were analyzed using pClamp and Origin software (version 6.0) (Microcal, Northampton, MA, USA). All experiments were performed at 30°C – 32°C .

Evaluation of gastric emptying (GE)

GE was performed by administering 0.05% (w/v) phenol red solution 30 min after administering MOE. Mice were sacrificed 20 min later, after which their stomachs were immediately removed, cut into several

pieces in 5 mL of 0.01 N NaOH, homogenized, and treated with 0.2 mL of 20% trichloroacetic acid per mL of homogenate. Mixtures were then centrifuged for 10 min at 1050 *g*, after which the supernatants (0.05 mL) were added to 0.5 N NaOH (0.2 mL). GE (%) was calculated using $100 - (A/B) \times 100$, where A is the test stomach absorbance (560 nm) and B is the control stomach absorbance (560 nm) immediately after phenol red administration.

Measurement of intestinal transit rates (ITR%) using Evans blue

We used Evans blue solution (5%, w/v) to determine the ITR% of MOE *in vivo*. Evans blue solution was administered (0.1 mL/kg of body weight; i.g.) through an orogastric tube 30 min after administering MOE i.g. to normal ICR mice. Animals were sacrificed 30 min after Evans blue administration, and the intestinal transit distances were determined by measuring the distance the Evans blue had migrated in the intestine (from the pylorus to its most distal point).

Induction of GI motility dysfunction (GMD) in mice

Two experimental GMD models, an acetic acid (AA)-induced peritoneal irritation (PI) and a streptozotocin (STZ)-induced diabetic mouse model, were used. For the AA model, PI was induced by administering AA to ICR mice 30 min after the i.g. administration of MOE by injecting AA (0.5 %, w/v, in saline) intraperitoneally (i.p.) at 10 mL/kg. After injecting AA, mice were allowed to recover for 30 min. For the STZ-induced diabetic mouse model, male ICR mice weighing 20–25 g aged 4–5 weeks were used. Mice were randomly allocated to two groups, a control group or a diabetic group. To produce diabetes, mice were fasted overnight, then administered STZ (Sigma-Aldrich, St. Louis, MO) solution i.p. on the following day. Fresh STZ solution was prepared in 0.1M/L ice-cold citrate buffer (pH=4.0) and administered at 200 mg/kg body weight [23]. Control mice were administered the same volume of 0.1 M/L citrate buffer i.p. Two months after STZ injection, blood was withdrawn from the tail vein after an 8 h fast and blood glucose concentrations were measured using a ONE-TOUCH Select Simple kit (Johnson & Johnson Medical Company). Diabetes was defined as a blood glucose level >16 mM. No mortality occurred during the study period and no mice recovered from STZ-induced diabetes.

Drugs

Y25130, RS39604, SB269970, GR113808, loperamide and cisplatin were purchased from Tocris Bioscience (Bristol, United Kingdom), while methocramine, 4-DAMP, GDP-β-S, thapsigargin, mosapride and domperidone were obtained from Sigma-Aldrich (St. Louis, MO, USA). For stock solutions, all drugs were dissolved in distilled water (DW) or dimethylsulfoxide (DMSO) and stored at -20°C. The final concentration of DMSO in the bath solution was always <0.1%, and we confirmed that it did not affect the results at this concentration. Furthermore, the additions of these chemicals did not alter bath solution pH.

Statistical analysis

Results are expressed as the means ± SEMs. N values refer to the numbers of cells used in the experiments. For multiple comparison analysis, we used one-way ANOVA with Bonferroni's *post hoc* comparison. For statistical analyses, we used Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA) and Origin version 8.0 (OriginLab Corporation, Northampton, MA, USA). *P* values of <0.05 were considered statistically significant.

Results

Functional components of MOE

The presence of magnolol and honokiol in MOE was established by HPLC, and their levels were quantified using calibration curves obtained from purchased standards (Fig. 1). Validation of the method used confirmed its reliability and stability.

Effects of MOE on the pacemaker potentials in ICCs

Using the whole cell patch clamp technique, we investigated the characters of cell clusters. Cell clusters formed network-like structures after culture for <12 h and were viable as demonstrated by the generation of spontaneous rhythmic contractions. Under

Fig. 1. HPLC Chromatogram of the composition of an ethanol extract of *Magnolia officinalis* (MOE). H: Honokiol; M: Magnolol.

Fig. 2. Effects of MOE on the pacemaker potentials of cultured ICCs of the murine small intestine. (A–C) The change of pacemaker potentials of ICCs by MOE (1–10 mg/ml) in current-clamp mode ($I = 0$). MOE concentration-dependently depolarized pacemaker potentials and suppressed pacemaker potential amplitudes. Responses to MOE are summarized in (D) and (D). Bars represent means $\pm$ SEMs. *** $P < 0.001$: significantly different from the control. CTRL: Control. MOE: an ethanol extract of *Magnolia officinalis*.

current clamp mode ($I=0$), ICCs generated pacemaker potentials (Fig. 2) with a mean resting membrane potential of -58.2 ± 1.3 mV and a mean amplitude of 23.6 ± 0.6 mV. We initially examined the effects of MOE on the pacemaker potentials of ICCs. MOE (1–10 mg/ml) depolarized pacemaker potentials and decreased their amplitudes in a concentration-dependent manner (Fig. 2A–2C). In the presence of MOE, the mean degrees of depolarization were 5.5 ± 0.4 mV at 1 mg/ml, 11.2 ± 0.8 mV at 5 mg/ml, and 25.1 ± 0.9 mV at 10 mg/ml (Fig. 2D, $n = 15$), while the mean amplitudes were 17.4 ± 0.8 mV at 1 mg/ml, 4.8 ± 1.2 mV at 5 mg/ml, and 1.5 ± 0.4 mV at 10 mg/ml (Fig. 2E, $n = 15$). Summarized values and a bar graph

showing the effects of MOE on pacemaker potentials are provided in Figs. 2D and 2E. Overall, the results show that MOE depolarizes ICC pacemaker potentials dose-dependently.

Identification of MOE-target receptor subtypes in ICCs.

Muscarinic receptors mediate GI smooth muscle cell membrane depolarizations [24, 25], and isolated ICCs express muscarinic M_2 and M_3 receptor subtypes in the murine small intestine [26]. Moreover, 5-HT receptors are involved in the GI tract motility [27], and previous studies have shown seven 5-HT receptor subtypes are expressed in the GI tract, but only murine small intestine ICCs express three of these receptors (5-HT₃, 5-HT₄, and 5-HT₇) [28, 29]. Therefore, we used the muscarinic or serotonergic receptor subtype antagonists to determine which receptor subtypes are involved in MOE-induced pacemaker potential depolarizations. First, ICCs were exposed to the muscarinic M_2 receptor antagonist, methoctramine (10 μ M for 5 min), or the muscarinic M_3 receptor antagonist, 4-DAMP (10 μ M for 5 min), respectively. Methoctramine or 4-DAMP alone did not affect the pacemaker potentials, and pretreatment with methoctramine or 4-DAMP did not completely inhibit the MOE-induced effects (Fig. 3A and 3B). MOE-induced pacemaker potential depolarization was 6.5 ± 0.4 mV in the presence of methoctramine (Fig. 3A; $n = 9$) and 3.3 ± 0.6 mV in 4-DAMP (Fig. 3B; $n = 7$). However, when we applied these antagonists together, MOE-induced effects were completely blocked (Fig. 3C and 3D). In addition, various 5-HT receptor antagonists were also pretreated in ICCs, respectively. Y25130 (a 5-HT₃ receptor antagonist), RS39604 (a 5-HT₄ receptor antagonist), GR113808 (a 5-HT₄ receptor antagonist), or SB269970 (a 5-HT₇ receptor antagonist) were pretreated in ICCs at 10 μ M for 5 min, respectively, and after that, MOE was added. In case of pretreatment with RS39604 in ICCs, the MOE-induced effects were blocked, and MOE-induced pacemaker potential depolarization was 5.1 ± 0.9 mV in the presence of RS39604 (Fig. 4B; $n = 6$). However, in case of pretreatment with Y25130, SB269970 or GR113808, MOE depolarized ICCs resting membrane pacemaker potential (Fig. 4A, 4C and 4D). The MOE-induced pacemaker potential depolarization was 11.6 ± 0.6 mV in the presence of Y25130 (Fig. 4A; $n = 6$), 10.7 ± 0.5 mV in GR113808 (Fig. 4C; $n = 7$) and 10.2 ± 0.3 mV in SB269970 (Fig. 4D; $n = 5$). These results suggest that MOE affects ICC pacemaker potentials through the M_2 and M_3 receptors.

Fig. 3. Effects of muscarinic receptor subtype antagonists on MOE-induced pacemaker potential depolarizations in the cultured ICCs of murine small intestines. (A) In the presence of methoctramine muscarinic M_2 receptor antagonist, pacemaker potential depolarization was inhibited by MOE (5 mg/ml). (B) In the presence of 4-DAMP muscarinic M_3 receptor antagonist, pacemaker potential depolarization was inhibited by MOE. (C) In the presence of both methoctramine and 4-DAMP, pacemaker potential depolarization was completely inhibited by MOE. (D) Responses to MOE in the presence of different receptor antagonists. Bars represent means $\pm$ SEMs. *** $P < 0.001$: significantly different from non-treated controls. CTRL: Control. Methoc.: methoctramine. MOE: an ethanol extract of *Magnolia officinalis*.

Fig. 4. Effects of 5-HT receptor subtype antagonists on MOE-induced pacemaker potential depolarizations in the cultured ICCs of murine small intestine. (A) Y25130 (a 5-HT₃ receptor antagonist) did not block MOE-induced responses. (B) RS39604 (a 5-HT₄ receptor antagonist) blocked MOE-induced responses. (C) GR113808 (a 5-HT₄ receptor antagonist) did not block MOE-induced responses. (D) SB269970 (a 5-HT₇ receptor antagonist) did not block MOE-induced responses. (E) Responses to 5-HT receptor antagonists are summarized. Bars represent the means $\pm$ SEMs. ***P<0.001: significantly different from non-treated controls. CTRL: Control. MOE: an ethanol extract of *Magnolia officinalis*.

Fig. 5. Effects of GDP- β -S on MOE-induced pacemaker potential depolarizations in cultured ICCs of murine small intestine. (A) When GDP- β -S (1 mM) was in the pipette, MOE did not depolarize the pacemaker potentials. (B) MOE-induced responses are summarized. Bars represent the means $\pm$ SEMs. **P<0.01. Significantly different from non-treated controls. CTRL: Control. MOE: an ethanol extract of *Magnolia officinalis*.

Involvement of G protein in MOE-induced pacemaker potential depolarization in ICCs

To investigate if G-proteins are required for MOE-induced pacemaker potential depolarization, we used GDP- β -S, which permanently inactivates G-protein binding proteins [30, 31]. When GDP- β -S (1 mM) was applied, MOE (5 mg/ml) only caused slight pacemaker potential depolarization (2.5 ± 0.5 mV, $n = 6$; Fig. 5). These results indicate that G-protein is involved in MOE-induced ICC pacemaker potential depolarization.

Effects of an external Ca²⁺-free solution and endoplasmic reticulum Ca²⁺-ATPase inhibitor on MOE-induced pacemaker potential depolarization in ICCs

Internal and external Ca²⁺ have important roles in GI motility regulations [32]. To investigate the roles of internal and external Ca²⁺ in MOE-induced pacemaker potential

Fig. 6. Effects of external and internal Ca^{2+} regulations on MOE-induced pacemaker potential depolarizations in cultured ICCs of murine small intestine. (A) MOE did not induce depolarization under external Ca^{2+} -free solution conditions. (B) Thapsigargin abolished the generation of pacemaker potentials, and MOE did not induce depolarization in the presence of thapsigargin. (C) MOE-induced pacemaker potential depolarizations are summarized. Bars represent the means $\pm$ SEMs. *** P <0.001. Significantly different from untreated controls. CTRL: Control. MOE: an ethanol extract of *Magnolia officinalis*.

Fig. 7. Increasing effect of MOE on normal GE and on loperamide- or cisplatin- induced delayed GE. (A) After a 24 h fast, animals ($n = 7$ /group) were orally administered MOE at the indicated dosages. (B) Loperamide-induced GE delay was prevented by MOE. (C) MOE treatment prevented cisplatin-induced GE delay. GE percentages were calculated as described in the Materials and Methods. 5-HT₄ receptor agonist (mosapride, 5 mg/kg) or dopamine receptor antagonist (domperidone, 5 mg/kg) were administered in distilled water. Bars represent the means $\pm$ SEMs. *** P <0.001. Significantly different from normal controls. CTRL: Control. Mosa.: Mosapride. Dom.: Domperidone. p.o. –per os. i.p. –intraperitoneally. MOE: an ethanol extract of *Magnolia officinalis*.

depolarization, MOE was applied under external Ca^{2+} -free conditions or in the presence of thapsigargin, an inhibitor of Ca^{2+} -ATPase in the endoplasmic reticulum. Pretreatment with an external Ca^{2+} -free solution (Fig. 6A) or thapsigargin (Fig. 6B) abolished pacemaker potentials, and MOE did not induce pacemaker potential depolarizations under these conditions ($n=7$; respectively, Fig. 6). These results indicate that MOE-induced pacemaker potential depolarization is dependent on internal and external Ca^{2+} regulation.

Effects of MOE on GE in normal mice

In normal mice, MOE (1 g/kg)-treated groups showed significantly higher GE (%) values than non-treated controls [GE values for MOE at 0.01, 0.1 or 1 g/kg were $51.4 \pm 1.2\%$, $51.8 \pm 1.7\%$ and $62.5 \pm 1.3\%$ ($p < 0.001$), respectively; Fig. 7A]. Additionally, MOE at 1 g/kg had effects similar to those of mosapride (5 mg/kg) [$61.8 \pm 1.9\%$ ($p < 0.001$)] and domperidone

Fig. 8. Effects of MOE on intestinal transit rates (ITR%) in normal mice and in two GI motility disorder (GMD) mouse models. (A) ITR% values of normal mice treated with MOE prior to Evans blue administration ($n = 12-14$ for each bar). (B) ITR% values of acetic acid (AA)-induced mice induced 30 min before the i.g. administration of Evans blue ($n = 7-11$ for each bar). (C) ITR% values of streptozotocin (STZ)-induced mice induced 2 months before the i.g. administration of Evans blue ($n = 9-11$ for each bar). Bars represent the mean values $\pm$ SEs. *** $P < 0.001$. Significantly different from normal controls. CTRL: Control. PF: Poncirus trifoliata Raf. Lope.: Loperamide. MOE: an ethanol extract of *Magnolia officinalis*.

(5 mg/kg) [$61.3 \pm 1.4\%$ ($p < 0.001$)] (Fig. 2A). We next examined loperamide-induced and cisplatin-induced GE delay models to determine if MOE could increase GE in these abnormally depressed GE models. In the loperamide-induced GE delay model, the mean GE was lower than normal [$46.8 \pm 4.1\%$ ($p < 0.001$); Fig. 7B], and this decrease was inhibited by MOE at 1 g/kg [GE values for MOE at 0.01, 0.1 and 1 g/kg were $44.5 \pm 1.5\%$, $47.6 \pm 1.9\%$ and $53.5 \pm 0.9\%$ ($p < 0.001$), respectively; Fig. 7B]. The effects of MOE at 1 g/kg were comparable to those of mosapride (5 mg/kg) [$53.5 \pm 1.1\%$ ($p < 0.001$)] or domperidone (5 mg/kg) [$54.6 \pm 0.9\%$ ($p < 0.001$)] (Fig. 7B). In addition, decreased GE was also inhibited by MOE (0.1 or 1 g/kg) in the cisplatin-induced GE delay model, with GE values at MOE 0.01, 0.1 and 1 g/kg of $47.2 \pm 1.5\%$, $51.6 \pm 1.6\%$ ($p < 0.001$) and $53.2 \pm 1.5\%$ ($p < 0.001$), respectively, being observed (Fig. 7C). The effects of MOE at 1 g/kg were comparable to those of mosapride (5 mg/kg) [$54.6 \pm 1.1\%$ ($p < 0.001$)] or domperidone (5 mg/kg) [$55.1 \pm 1.4\%$ ($p < 0.001$)] (Fig. 7C).

Effects of MOE on ITR% in normal and GMD mice

The mean ITR% for Evans blue after 30 min in normal mice was $46.5 \pm 0.9\%$ (Fig. 8A). PF (1 g/kg), which has been demonstrated to have prokinetic activity in the GI tract [33], significantly increased ITR% [$67.8 \pm 1.1\%$ ($p < 0.001$)]. The MOE increased ITR% dose-dependently [ITR% values at 0.01, 0.1 and 1 g/kg were $54.6 \pm 0.6\%$ ($p < 0.001$), $60.5 \pm 1.2\%$ ($p < 0.001$) and $68.5 \pm 1.1\%$ ($p < 0.001$), respectively; Fig. 8A]. Loperamide decreased

ITR%, which is consistent with the results of previous studies [34], and MOE inhibited this loperamide-induced ITR% decrease [ITR% for loperamide was $34.4 \pm 1.5\%$, while it was $46.1 \pm 0.8\%$ for loperamide with MOE ($p < 0.001$); Fig. 8A]. To examine the effects of MOE on GMD models, we used AA and STZ-induced diabetic mouse models of experimental GMD. The AA mouse model showed a significant ITR% decrease [to $32.6 \pm 1.2\%$ vs $44.4 \pm 0.7\%$ for normal controls; $p < 0.001$; Fig. 8B]. However, intragastric treatment with MOE at 0.01, 0.1, or 1 g/kg significantly inhibited this reduction [to $33.3 \pm 1.1\%$, $40.0 \pm 2.0\%$ ($p < 0.001$) and $44.1 \pm 0.9\%$ ($p < 0.001$), respectively; Fig. 8B]. In addition, loperamide decreased ITR% in AA mice and MOE reduced this decrease (Fig. 8B). STZ-induced diabetic mice also showed significant ITR% reduction (to $28.5 \pm 1.3\%$; Fig. 8C), which was also significantly inhibited by MOE at 0.01, 0.1 or 1 g/kg [to $31.9 \pm 1.3\%$ ($p < 0.001$), $37.0 \pm 1.4\%$ ($p < 0.001$) and $43.1 \pm 0.7\%$ ($p < 0.001$), respectively; Fig. 8C]. In addition, loperamide decreased ITR% in STZ-induced diabetic mice, while MOE reduced this decrease (Fig. 8C). Taken together, these results indicate that MOE increased ITR% in mice with GMD.

Discussion

The present study was conducted to investigate the effects of ethanol extracts of *M. officinalis* on GI motor function. *M. officinalis* has been widely used for the remedy of a variety of digestive dysfunctions, and of that, a few studies have reported their effects on GI function [7-13].

In the present study, we found that MOE dose-dependently depolarized ICC pacemaker potentials through M_2 and M_3 receptors via internal and external Ca^{2+} regulation through G protein pathways *in vitro* and that MOE increased GE and ITRs in normal and GMD mouse models *in vivo*. Methoctramine or 4-DAMP inhibited the effects of MOE on pacemaker potential when compared with MOE treatment alone (Fig. 3). In addition, after pretreatment with Y25130, GR113803 or SB269970, MOE depolarized ICCs pacemaker potential. However, the pretreatment with RS39604 blocked the MOE-induced effects (Fig. 4). Both Intracellular GDP β S and in case of pretreatment with Ca^{2+} free solution or thapsigargin inhibited MOE-induced effects (Fig. 5 and 6). In normal mice, the GE and ITR values increased significantly and dose-dependently in response to MOE (Fig. 7 and 8). In loperamide- and cisplatin-induced GE delay models, MOE administration reversed the GE deficits (Fig. 7). The ITRs of the GMD mice were significantly reduced relative to those of normal mice, which were significantly and dose-dependently reversed by MOE (Fig. 8). Therefore, MOE showed a potent gastropromotory activity in both normal and abnormal mice.

The main components of *M. officinalis* are magnolol and honokiol, show a variety of biological functions. The biological activity of these compounds appears to be due to the presence of hydroxyl and allylic groups on a biphenolic moiety [4, 35, 36]. Several previous studies have reported the effects of *M. officinalis* on the GI tract. *M. officinalis* inhibited the contractile activity of gastric fundus strips in rats [12] and it inhibited acetylcholine-induced contraction in guinea pig isolated ileum [8]. Also, Zhang et al. [13] suggested that magnolol inhibited the contractions of distal colonic segments by inhibiting L-type Ca^{2+} channels. However, another studies suggested that *M. officinalis* improved atropine and L-Arg induced GI motility dysfunction and enhanced the intestinal propulsion rate by recording the time of excreting carbo medicinalis [10]. In addition, Tian et al. [11] suggested that atropine impaired the SCF/c-kit signaling pathways in ICCs and *M. officinalis* recovered the atropine-induced gastric dysmotility in rats. They investigated the *M. officinalis* effects in atropine-related GI dysmotility in rats *in vivo* [10, 11] and they checked the molecular (c-kit and SCF) and enzyme (gastrin, motilin, somatostatin, and substance P) levels in GI whole segments tissue [10, 11]. Also, Jeong et al. [14] also suggested that magnolol showed contractile activity in the rat GI tract. GI motility patterns are highly integrated behaviours requiring coordination between smooth muscle cells and utilizing regulatory inputs from ICCs, neurons, and endocrine and immune cells [37]. In this study, we investigated the effects *M. officinalis* on only ICCs. MOE-induced effects were not completely blocked by either methoctramine (a muscarinic M_2

antagonist) or by 4-DAMP (a muscarinic M_3 antagonist) (Fig. 3A and 3B). However, when we applied these antagonists together, MOE-induced effects were completely blocked (Fig. 3C). In addition, in case of pretreatment with Y25130 (a 5-HT₃ antagonist), GR113808 (a 5-HT₄ antagonist) or SB269970 (a 5-HT₇ antagonist), MOE-induced effects have no changed (Fig. 4A, 4C and 4D). However, in case of pretreatment with another type 5-HT₄ antagonist, RS39604, the MOE-induced effects were blocked (Fig. 4B). Therefore, we think that the MOE-induced effects on 5-HT₄ receptors by RS39604 may be non-specific effects and these results suggest that MOE affects ICC pacemaker potentials through the M_2 and M_3 receptors. Smooth muscle cells of the GI tract express muscarinic receptors and binding of ACh activates non-selective cation channels in these cells [38-40]. These channels are encoded by canonical transient receptor potential (TRPC) channels, types 4 and 6 (i.e. TRPC4 and TRPC6) [41, 42]. The small intestine ICCs have also been shown to express both M_2 and M_3 receptors [23, 43]. Carbachol (CCh) induced the pacemaker potential depolarizations of ICCs [44] and we think that MOE have similar effects with CCh on ICCs. CCh induced effects on ICCs were regulated by G-protein via internal and external Ca^{2+} modulation [44] like MOE-induced effects. Therefore, we think that in ICCs, TRPC4 and TRPC6 channels may be involved in MOE-induced muscarinic receptor activation via G protein. These mechanisms may be responsible for the MOE-induced ICCs depolarization, and thereby accelerate intestinal motility *in vivo*. Therefore, although the results have been inconsistent, inhibitory effect or enhancing effect, in our hands, *M. officinalis* exerted an enhancing effect on GI motilities as references 10, 11, and 14.

ICCs act as pacemaker cells in GI motility function [15-17, 37, 45]. Therefore, they play a role in the generation and propagation of electrical properties in smooth muscles [15, 46]. ICCs have also been shown to be involved in neurotransmission [32, 37, 47-49]. Therefore, we believe that *M. officinalis* act on smooth muscle cells or enteric neurons through ICCs in the GI tract. Because many GI motility disorders have been associated with ICC abnormalities, it is extremely important to understand the mechanisms underlying pacemaker activity and ICC excitability [50].

In summary, the results of the present study show that (1) MOE concentration-dependently depolarizes the pacemaker potentials in ICCs; (2) MOE affects ICC pacemaker potentials through the M_2 and M_3 receptors; (3) G-protein is involved in MOE-induced ICC pacemaker potential depolarizations; (4) MOE-induced pacemaker potential depolarization is dependent on internal and external Ca^{2+} regulation; and (5) MOE shows potent gastropromotory activity in both normal and abnormal mice. Taken together, these results indicate that *M. officinalis* has the potential for development of a gastropromotory agent for use in GI motility function.

Acknowledgements

This study was supported by a Korean National Research Foundation (NRF) grant funded by the Korean government (MSIP) (Grant no. 2014R1A5A2009936).

Disclosure Statement

The authors have no potential conflict of interest to declare.

References

- 1 Hon-Yen Hsu CSH: Commonly Used Chinese Herbs Formulas with Illustrations. Oriental Healing Arts Institute, Long Beach CA, rev. edition. 1980.
- 2 Kanzuo T: In: Satyajit D. Sarkar, Yuji M. (Eds.). *Magnolia: The Genus Magnolia*. first ed., Boca raton CRC press, London, 2002.
- 3 Nilausen TD, Johansen JD, Thyssen JP: Allergic contact dermatitis of the face caused by *Magnolia officinalis* bark extract. *Contact Dermatitis* 2016;75:385-387.

- 4 Rajgopal A, Missler SR, Scholten JD: *Magnolia officinalis* (Hou Po) bark extract stimulates the Nrf2-pathway in hepatocytes and protects against oxidative stress. *J Ethnopharmacol* 2016;193:657-662.
- 5 Stoger DBSCE: *Chinese Herbal Medicine: Materia Medica* third ed. Eastland Press, Seattle, 2004.
- 6 Wu JN: *An Illustrated Chinese Materia Medica*. Oxford University Press, Oxford, 2002.
- 7 Bensoussan A, Kellow JE, Bouchier SJ, Fahey P, Shim L, Malcolm A, Boyce P: Efficacy of a Chinese Herbal Medicine in Providing Adequate Relief of Constipation-predominant Irritable Bowel Syndrome: A Randomized Controlled Trial. *Clin Gastroenterol Hepatol* 2015;13:1946-1954.
- 8 Chan SS, Zhao M, Lao L, Fong HH, Che CT: Magnolol and honokiol account for the anti-spasmodic effect of *Magnolia officinalis* in isolated guinea pig ileum. *Planta Med* 2008;74:381-384.
- 9 Han X, Pang Y, Liu S, Tan Z, Tang S, Zhou C, Wang M, Xiao W: Antidiarrhea and antioxidant activities of honokiol extract from *magnoliae officinalis* cortex in mice. *Trop J Pharm Res* 2014;13:1643-1651.
- 10 Peng B, He R, Yang B, Lu Y, Gao J, Li J: Comparative study of effect of *Magnolia officinalis* and *M. officinalis* var. *biloba* on gastrointestinal motility dysfunction. *Zhongguo Zhong Yao Za Zhi*. 2010;35:2624-2627.
- 11 Tian H, Huang D, Li T, Huang L, Zheng X, Tang D, Zhang L, Wang J: The protective effects of total phenols in *magnolia officinalis* rehderi on gastrointestinal tract dysmotility is mainly based on its influence on interstitial cells of Cajal. *Int J Clin Exp Med* 2015;8:20279-20286.
- 12 Zhang WW, Li Y, Wang XQ, Tian F, Cao H, Wang MW, Sun QS: Effects of magnolol and honokiol derived from traditional Chinese herbal remedies on gastrointestinal movement. *World J Gastroenterol* 2005;11:4414-4418.
- 13 Zhang M, Zang KH, Luo JL, Leung FP, Huang Y, Lin CY, Yang ZJ, Lu AP, Tang XD, Xu HX, Sung JJ, Bian ZX: Magnolol inhibits colonic motility through down-regulation of voltage-sensitive L-type Ca²⁺ channels of colonic smooth muscle cells in rats. *Phytomedicine* 2013;20:1272-1279.
- 14 Jeong SI, Kim YS, Lee MY, Kang JK, Lee S, Choi BK, Jung KY: Regulation of contractile activity by magnolol in the rat isolated gastrointestinal tracts. *Pharmacol Res* 2009;59:183-188.
- 15 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;373:347-349.
- 16 Kim BJ, Lim HH, Yang DK, Jun JY, Chang IY, Park CS, So I, Stanfield PR, Kim KW: Melastatin-type transient receptor potential channel 7 is required for intestinal pacemaking activity. *Gastroenterology* 2005;129:1504-1517.
- 17 Eisenman ST, Gibbons SJ, Verhulst PJ, Cipriani G, Saur D, Farrugia G: Tumor necrosis factor alpha derived from classically activated "M1" macrophages reduces interstitial cell of Cajal numbers. *Neurogastroenterol Motil* 2017;29:e12984.
- 18 He CL, Soffer EE, Ferris CD, Walsh RM, Szurszewski JH, Farrugia G: Loss of interstitial cells of Cajal and inhibitory innervation in insulin-dependent diabetes. *Gastroenterology* 2001;121:427-434.
- 19 Zarate N, Mearin F, Wang XY, Hewlett B, Huizinga JD, Malagelada JR: Severe idiopathic gastroparesis due to neuronal and interstitial cells of Cajal degeneration: pathological findings and management. *Gut* 2003;52:966-970.
- 20 Hong NR, Park HS, Ahn TS, Kim HJ, Ha KT, Kim BJ: Ginsenoside Re inhibits pacemaker potentials via adenosine triphosphate-sensitive potassium channels and the cyclic guanosine monophosphate/nitric oxide-dependent pathway in cultured interstitial cells of Cajal from mouse small intestine. *J Ginseng Res* 2015;39:314-321.
- 21 Jun JY, Choi S, Chang IY, Yoon CK, Jeong HG, Kong ID, So I, Kim KW, You HJ: Deoxycholic acid inhibits pacemaker currents by activating ATP-dependent K⁺ channels through prostaglandin E2 in interstitial cells of Cajal from the murine small intestine. *Br J Pharmacol* 2005;144:242-251.
- 22 Koh SD, Sanders KM, Ward SM: Spontaneous electrical rhythmicity in cultured interstitial cells of Cajal from the murine small intestine. *J Physiol* 1998;513:203-213.
- 23 Wu YS, Lu HL, Huang X, Liu DH, Meng XM, Guo X, Kim YC, Xu WX: Diabetes-induced loss of gastric ICC accompanied by up-regulation of natriuretic peptide signaling pathways in STZ-induced diabetic mice. *Peptides* 2013;40:104-111.
- 24 Huizinga JD, Chang G, Diamant NE, El-Sharkawy TY: Electrophysiological basis of excitation of canine colonic circular muscle by cholinergic agents and substance P. *J Pharmacol Exp Ther* 1984;231:692-699.
- 25 Inoue R, Chen S: Physiology of muscarinic receptor operated nonselective cation channels in guinea-pig ileal smooth muscle. *Exs* 1993;66:261-268.
- 26 Epperson A, Hatton WJ, Callaghan B, Doherty P, Walker RL, Sanders KM, Ward SM, Horowitz B: Molecular markers expressed in cultured and freshly isolated interstitial cells of Cajal. *Am J Physiol Cell Physiol* 2000;279: C529-C539.

- 27 Gershon MD, Tack J: The serotonin signaling system: from basic understanding to drug development for functional GI disorders. *Gastroenterology* 2007;132:397–414.
- 28 Liu HN, Ohya S, Nishizawa Y, Sawamura K, Iino S, Syed MM, Goto K, Imaizumi Y, Nakayama S: Serotonin augments gut pacemaker activity via 5-HT₃ receptors. *PLoS One* 2011;6:e24928.
- 29 Shahi PK, Choi S, Zuo DC, Yeum CH, Yoon PJ, Lee J, Kim YD, Park CG, Kim MY, Shin HR, Oh HJ, Jun JY: 5-hydroxytryptamine generates tonic inward currents on pacemaker activity of interstitial cells of cajal from mouse small intestine. *Korean J Physiol Pharmacol* 2011;15:129–135.
- 30 Komori S, Kawai M, Takewaki T, Ohashi H: GTP-binding protein involvement in membrane currents evoked by carbachol and histamine in guinea-pig ileal muscle. *J Physiol* 1992;450:105–126.
- 31 Ogata R, Inoue Y, Nakano H, Ito Y, Kitamura K: Oestradiol-induced relaxation of rabbit basilar artery by inhibition of voltage-dependent Ca channels through GTP-binding protein. *Br J Pharmacol* 1996;117:351–359.
- 32 Ward SM: Interstitial cells of Cajal in enteric neurotransmission. *Gut* 2000;47:40–43.
- 33 Kim BJ, Lee GS, Kim HW: Involvement of transient receptor potential melastatin type 7 channels on Poncirus fructus-induced depolarizations of pacemaking activity in interstitial cells of Cajal from murine small intestine. *Integr Med Res* 2013;2:62–69.
- 34 Tan-No K, Nijima F, Nakagawasai O, Sato T, Satoh S, Tadano T: Development of tolerance to the inhibitory effect of loperamide on gastrointestinal transit in mice. *Eur J Pharm Sci* 2003;20:357–363.
- 35 Atanasov AG, Wang JN, Gu SP, Bu J, Kramer MP, Baumgartner L, Fakhrudin N, Ladurner A, Malainer C, Vuorinen A, Noha SM, Schwaiger S, Rollinger JM, Schuster D, Stuppner H, Dirsch VM, Heiss EH: Honokiol: a nonadipogenic PPAR γ agonist from nature. *Biochim Biophys Acta* 2013;1830:4813–4819.
- 36 Wei W, Dejie L, Xiaojing S, Tiancheng W, Yongguo C, Zhengtao Y, Naisheng Z: Magnolol inhibits the inflammatory response in mouse mammary epithelial cells and a mouse mastitis model. *Inflammation* 2015;38:16–26.
- 37 Rumessen JJ, Vanderwinden JM: Interstitial cells in the musculature of the gastrointestinal tract: Cajal and beyond. *Int Rev Cytol* 2003;229:115–208.
- 38 Benham CD, Bolton TB, Lang RJ: Acetylcholine activates an inward current in single mammalian smooth muscle cells. *Nature* 1985;316:345–347.
- 39 Inoue R, Isenberg G: Acetylcholine activates nonselective cation channels in guinea pig ileum through a G protein. *Am J Physiol Cell Physiol* 1990;258:C1173–C1178.
- 40 Lee YM, Kim BJ, Kim HJ, Yang DK, Zhu MH, Lee KP, So I, Kim KW: TRPC5 as a candidate for the nonselective cation channel activated by muscarinic stimulation in murine stomach. *Am J Physiol Gastrointest Liver Physiol* 2003;284:G604–616.
- 41 Tsvilovskyy VV, Zholos AV, Aberle T, Philipp SE, Dietrich A, Zhu MX, Birnbaumer L, Freichel M, Flockerzi V: Deletion of TRPC4 and TRPC6 in mice impairs smooth muscle contraction and intestinal motility *in vivo*. *Gastroenterology* 2009;137:1415–1424.
- 42 Lee KP, Jun JY, Chang IY, Suh SH, So I, Kim KW: TRPC4 is an essential component of the nonselective cation channel activated by muscarinic stimulation in mouse visceral smooth muscle cells. *Mol Cells* 2005;20:435–441.
- 43 Chen H, Redelman D, Ro S, Ward SM, Ordög T, Sanders KM: Selective labeling and isolation of functional classes of interstitial cells of Cajal of human and murine small intestine. *Am J Physiol Cell Physiol* 2007;292:C497–C507.
- 44 So KY, Kim SH, Sohn HM, Choi SJ, Parajuli SP, Choi S, Yeum CH, Yoon PJ, Jun JY: Carbachol regulates pacemaker activities in cultured interstitial cells of Cajal from the mouse small intestine. *Mol Cells* 2009;27:525–531.
- 45 Kim HJ, Park SY, Kim DG, Park SH, Lee H, Hwang DY, Jung MH, Ha KT, Kim BJ: Effects of the roots of *Liriope platyphylla* Wang Et tang on gastrointestinal motility function. *J Ethnopharmacol* 2016;184:144–153.
- 46 Sanders KM, Koh SD, Ro S, Ward SM: Regulation of gastrointestinal motility--insights from smooth muscle biology. *Nat Rev Gastroenterol Hepatol* 2012;9:633–645.
- 47 Sanders KM, Ward SM: Kit mutants and gastrointestinal physiology. *J Physiol* 2007;578:33–42.
- 48 Kim HJ, Wie J, So I, Jung MH, Ha KT, Kim BJ: Menthol modulates pacemaker potentials through TRPA1 channels in cultured interstitial cells of Cajal from murine small intestine. *Cell Physiol Biochem* 2016;38:1869–1882.
- 49 Gim H, Nam JH, Lee S, Shim JH, Kim HJ, Ha KT, Kim BJ: Quercetin inhibits pacemaker potentials via nitric oxide/cGMP-dependent activation and TRPM7/ANO1 channels in cultured interstitial cells of Cajal from mouse small intestine. *Cell Physiol Biochem* 2015;35:2422–2436.
- 50 Vanderwinden JM, Rumessen JJ: Interstitial cells of Cajal in human gut and gastrointestinal disease. *Microsc Res Tech* 1999;47:344–360.