Effects of Chaihu-Shugan-San on Small Intestinal Interstitial Cells of Cajal in Mice

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Received December 4, 2019; accepted January 14, 2020

Chaihu-Shugan-San (CSS) has been widely used as an alternative treatment for gastrointestinal (GI) diseases in East Asia. Interstitial cells of Cajal (ICCs) are pacemakers in the GI tract. In the present study, we examined the action of CSS on pacemaker potentials in cultured ICCs from the mouse small intestine in vitro and on GI motility in vivo. We used the electrophysiological methods to measure the pacemaker potentials in ICCs. GI motility was investigated by measuring intestinal transit rates (ITRs). CSS inhibited the pacemaker potentials in a dose-dependent manner. The capsazepine did not block the effect of CSS. However, the effects of CSS were blocked by glibenclamide. In addition, N^G-nitro-L-arginine methyl ester (L-NAME) also blocked the CSS-induced effects. Pretreatment with SQ-22536 or with KT-5720 did not suppress the effects of CSS; however, pretreatment with ODQ or KT-5823 did. Furthermore, CSS significantly suppressed murine ITR enhancement by neostigmine in vivo. These results suggest that CSS exerts inhibitory effects on the pacemaker potentials of ICCs via nitric oxide (NO)/cGMP and ATP-sensitive K^+ channel dependent and transient receptor potential vanilloid 1 (TRPV1) channel independent pathways. Accordingly, CSS could provide the basis for the development of new treatments for GI motility dysfunction.

Key words Chaihu-Shugan-San; interstitial cells of Cajal; gastrointestinal tract; ATP-sensitive K^+ channel; intestinal transit rate

INTRODUCTION

Traditional medicines are widely prescribed in many countries and improves the healing power of the body and helps the body recover to its natural state. Many recent studies have indicated that herbal preparations and their extracts have favorable effects on the treatment of various diseases. Among various diseases, an estimated 51% of patients have gastrointestinal (GI) disorders and 10% of alternative medicines are being used for digestive symptoms. These GI conditions have been reduced the quality of human life and increased the cost of maintaining health. However, these various available drugs treatment for these diseases are chemical drugs and these chemical drugs have a large number of adverse effects. Traditional medicine may be an alternative medicine with a naturalistic approach and be known to have fewer side effects on GI diseases. Therefore, currently, many people are interested in treating GI tract diseases by natural medicine.

Chaihu-Shugan-San (CSS; 柴胡疏肝散) is a traditional medicine consisting of seven herbs, Chuanxiong Rhizoma, Citri Reticulatae Pericarpium Viride, Bupleuri Radix, Cyperti Rhizoma, Aurantii Fructus Immaturus, Paeoniae Radix Alba and Glycyrrhizae Radix. CSS has been widely used as an herbal prescription to treat various symptoms. CSS is commonly efficient in treating neurologic impairment and depression. Moreover, CSS controls the inflammatory reaction in the liver by controlling the insulin response and regulates the phospholipids to liver damage caused by chronic stress. In addition, CSS has an effect on nonalcoholic fatty liver disease with insulin resistance in rats. Furthermore, CSS has been used for centuries to improve some GI disorders that are similar to functional dyspepsia, gastric ulcers, diarrhea, and gastritis.

Interstitial cells of Cajal (ICCs) are pacemaker cells in the GI tract and generate pacemaker potentials. The damage and decrease in numbers of these are associated with many GI diseases. Therefore, this study is of considerable importance to the study of GI function. Many neurotransmitters and hormones affect these ICCs and thus regulate GI motility. To date, however, whether CSS has effects on ICCs and GI motility has not been clarified. Therefore, in this study, we examined the efficacy of CSS on the pacemaker potentials of ICCs in small intestine in vitro and the function of GI motility by measuring intestinal transit rates (ITRs) in vivo in mice.

MATERIALS AND METHODS

Chemical Profiling of CSS by Ultra Performance Liquid Chromatography (UPLC) UPLC was conducted using a Waters ACQUITY™ ultra performance LC system (Milford, MA, U.S.A.). Waters ACQUITY™ photodiode array detector (PDA; Milford) and HPLC Column were used for Waters ACQUITY™ BEH C18 column (1.7 µm, 2.1×100; Milford). The software was used to operate the system Empower (Milford). In addition, samples were extracted using an ultrasonic cleaner 8210R-DTH (Branson Company; San Jose, CA, U.S.A.). The reagents used for this experiment were methanol (Junsei, Tokyo, Japan), acetonitrile (JT-BAKER; Radnor, PA, USA), methanoic acid (Waters ACQUITY™), acetonitrile (JT-BAKER; Radnor, PA, USA).
Cassia twig (C. officinalis L. (Sobolák, 2000)), water (tertiary distilled water), and dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, U.S.A.). CSS was analyzed for its saikosaponin A, poncirin, paeoniflorin, narin, glycyrrhizinic acid, liquiritigenin, albiflorin, and hesperidin content using UPLC. CSS was analyzed for its saikosaponin A, poncirin, paeoniflorin, narin, glycyrrhizinic acid, liquiritigenin, albiflorin, and hesperidin content using UPLC. 

**Quantitative Analyses**

Quantitative analyses were based on retention time and quantities were determined by the peak area method (Tables 2, 3).

**Preparation of Cells and Cell Cultures**

Animal experiments have complied with the rules of the animal experiment ethics committee of Pusan National University (No. PNU-2018-2110). Small intestine of ICR mice (3–7 d) were taken out and removed the mucous membrane. Small intestinal muscle was equilibrated with Ca²⁺-free Hank’s solution. Cells were isolated by enzyme such as collagenase (Worthington Biochemical, Lakewood, NJ, U.S.A.), bovine serum albumin (BSA; Sigma-Aldrich) and trypsin inhibitor (Sigma-Aldrich) and then cultured at 37°C in a 95% O₂/5% CO₂ incubator in smooth muscle growth medium (SMGM) (Clonetics, San Diego, CA, U.S.A.) supplemented with 2% antibiotics/antimycotics (Gibco, Grand Island, NY, U.S.A.) and murine stem cell factor (5 ng/mL; Sigma-Aldrich). All other methods were carried out in a generally well-known manner.\(^{14,15,17-19}\)

**Patch Clamp Experiments**

Na⁺-Tyrode solution was used in bath and the pipette solution was KC1 140, MgCl₂ 5, K₂ATP 2.7, NaGTP 0.1, creatine phosphate disodium 2.5, N- (2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (HEPES) 5, and ethylene glycol bis(2-aminoethoxy) ether)-N, N', N'-, N'- tetracetic acid (EGTA) 0.1. Whole-cell patch clamp configuration techniques were conducted in a general method and results were analyzed using pClamp and Origin software (version 6.0, Microcal, U.S.A.).

**In Vivo Intestine Motility Measurements**

Male ICR mice (Samtako Bio Korea Co., Ltd., Osan, Republic of Korea) weighing 23–30 g were used to investigate the in vivo effects of CSS on the GI tract. After CSS administration, Evans Blue (5% (w/v), in distilled water (DW) was administered through the mouth. After 30 min, animals were sacrificed and ITR was measured Evans Blue as the length past in intestine. ITR was

### Table 1. Contents of Eight Marker Compounds of CSS by UPLC

| Compound               | Content (ppm) |
|------------------------|---------------|
| Saikosaponin A         | 11.65 ± 0.39  |
| Poncirin               | 0.57 ± 0.06   |
| Paeoniflorin           | 294.01 ± 8.07 |
| Naringin               | 33.81 ± 0.85  |
| Glycyrrhizinic acid    | 99.15 ± 0.91  |
| Liquiritigenin         | 1.96 ± 0.08   |
| Albiflorin             | 172.55 ± 6.28 |
| Hesperidin             | 109.13 ± 5.51 |

Values are expressed as the means ± standard deviation (S.D.) of three independent experiments. CSS was analyzed for its saikosaponin A, poncirin, paeoniflorin, naringin, glycyrrhizinic acid, liquiritigenin, albiflorin, and hesperidin content using UPLC. CSS: Chaihu-Shugan-San. UPLC: Ultra Performance Liquid Chromatography.

### Table 2. Mobile Phase Condition of UPLC

| Time (minutes) | 0.1% FA/water (%) | 0.1% FA/acetonitrile (%) | Flow rate (mL/min) |
|----------------|-------------------|--------------------------|-------------------|
| 0              | 98                | 2                        | 0.40              |
| 1.0            | 98                | 2                        | 0.40              |
| 3.0            | 85                | 15                       | 0.40              |
| 4.0            | 70                | 30                       | 0.40              |
| 6.0            | 60                | 40                       | 0.40              |
| 8.0            | 50                | 50                       | 0.40              |
| 9.0            | 20                | 80                       | 0.40              |
| 10.0           | 10                | 90                       | 0.40              |
| 12.0           | 100               | 100                      | 0.40              |
| 14.0           | 98                | 2                        | 0.40              |
| 16.0           | 98                | 2                        | 0.40              |

FA: Formic acids. UPLC: Ultra Performance Liquid Chromatography.

### Table 3. Retention Time of Reference Standards

| Reference standard | Reference standard retention time (min) |
|--------------------|-----------------------------------------|
| Saikosaponin A     | 10.155                                  |
| Poncirin           | 8.024                                   |
| Paeoniflorin       | 5.432                                   |
| Naringin           | 6.476                                   |
| Glycyrrhizinic acid| 9.611                                   |
| Liquiritigenin     | 5.916                                   |
| Albiflorin         | 5.193                                   |
| Hesperidin         | 6.638                                   |

UPLC: Ultra Performance Liquid Chromatography.
measured the length of the entire length as a percentage of the length that it had passed.

**Western Blot Analysis** The total proteins of homogenates were determined using with RIPA buffer containing protease inhibitor (Roche, Indianapolis, IN, U.S.A.) and phosphatase inhibitor cocktail (Calbiochem, Darmstadt, Germany). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) electrophoresis by using 6% polyacrylamide gels and transferred to a polyvinylidene difluoride (PVDF) membrane. After 1 h treatment with blocking buffer in Tris-buffered saline (TBS) containing 5% non-fat milk at room temperature, PVDF membranes were analyzed by anti-β-actin (Santa Cruz Biotechnology, Dallas, TX, U.S.A.) antibodies, respectively. An enhanced chemiluminescence reagent kit (Advanzi, Menlo Park, CA, U.S.A.) antibodies, respectively. An enhanced chemiluminescence reagent kit (Advanzi, Menlo Park, CA, U.S.A.) was used for detection. All other procedures were generally carried out in a way.17)

**Drugs** All drugs were purchased from Sigma-Aldrich. Chemicals were dissolved in Na\(^+\)-Tyrode solution to their final concentrations immediately before use.

**Statistical Analysis** Results are expressed as the means ± standard error of the means (S.E.M.s). 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, U.S.A.) and Origin version 8.0 (OriginLab Corporation, Northampton, MA, U.S.A.). p Values of <0.05 were considered statistically significant. The n values reported in the text refer to the number of cells used in patch-clamp experiments.

**RESULTS**

**Analysis of CSS** CSS was analyzed for its saikosaponin A, poncirin, paoniflorin, naringin, glycyrrhizinic acid, liquiritigenin, albiblorin, and hesperidin content using UPLC. The concentrations of the eight compounds were calculated from a calibration curve of standards (Table 1, Fig. 1). The method validation confirmed its stability and reliability and resulted in consecutive separation of the eight major compounds in CSS.

**CSS Decreases the Amplitude of Pacemaker Potentials in Cultured ICCs** ICCs had a mean resting membrane potential of −57.3 ± 0.8 mV and produced electrical pacemaker potentials (n = 42). The frequency of this pacemaker potential was 18.2 ± 1.8 cycles/min with an amplitude of 23.7 ± 0.8 mV (n = 42; Fig. 2). The addition of CSS (5–100 mg/mL) decreased the amplitude of the pacemaker potentials (Figs. 2A–2E), but the resting membrane potentials hyperpolarized only in CSS 50 and 100 mg/mL (Figs. 2D, 2E). The amplitudes were 21.1 ± 0.6 mV at 5 mg/mL CSS (n = 7), 11.2 ± 0.7 mV at 10 mg/mL CSS (n = 6), 2.6 ± 0.5 mV at 30 mg/mL (n = 5), 2.5 ± 0.6 mV at 50 mg/mL (n = 7) and 1.4 ± 0.4 mV at 100 mg/mL (n = 5) (Fig. 2F). The resting membrane potentials were −56.8 ± 1.1 mV at 5 mg/mL, −57.2 ± 0.7 mV at 10 mg/mL, −58.1 ± 0.8 mV at 30 mg/mL, −64.1 ± 0.6 mV at 50 mg/mL, and −68.8 ± 1.7 mV at 100 mg/mL (Fig. 2G). CSS is mainly composed of isakosaponin A, poncirin, naringin, glycyrrhizinic acid, liquiritigenin, albiblorin, paoniflorin and hesperidin. Therefore, we examined the effects of these components on the pacemaker potentials of ICCs. Saikosaponin A depolarized pacemaker potential in a concentration-dependent manner (EC50 = 9.9 ± 1.4 µM).19) Liquiritigenin decreased pacemaker potential amplitudes in a concentration-dependent manner (Fig. 3D). In the presence of liquiritigenin, the mean amplitudes were 24.1 ± 0.8 mV at 1 µM, 24.7 ± 1.1 mV at 10 µM, 23.5 ± 1.7 mV at 50 µM, 22.1 ± 2.5 mV at 100 µM, 12.2 ± 2.1 mV at 300 µM and 3.5 ± 1.3 mV at 500 µM (IC50 = 303.4 ± 3.3 µM; Fig. 3I, n = 5). In addition, albiblorin also decreased pacemaker potential amplitudes in a concentration-dependent manner (Fig. 3E). In the presence of

![Fig. 1. Ultra Performance Liquid Chromatography (UPLC) Chromatogram of the Eight Major Compounds Identified in Chaihu-Shugan-San (CSS)](image-url)
Fig. 2. The Effects of CSS on the Pacemaker Potentials of Cultured ICCs from Mouse Small Intestine

(A–E) Pacemaker potentials of interstitial cells of Cajal (ICCs) exposed to CSS (5–100 mg/mL). CSS decreased the amplitude of pacemaker potentials in a concentration-dependent manner. (F, G) Bar graph of the decrease of amplitude and the change of resting membrane potentials with CSS. Results are expressed as the mean ± standard error of the mean (S.E.M.). **p < 0.01. CSS, Chaihu-Shugan-San. CTRL, control. RMP, resting membrane potentials.

Fig. 3. Effects of Saikosaponin A, Poncirin, Naringin, Liquiritigenin, Albiflorin, Paeoniflorin and Hesperidin, the Major Components of CSS, on the Pacemaker Potentials of Cultured ICCs from Mouse Small Intestine

(A) Saikosaponin A depolarized pacemaker potential. (B and C) Poncirin and naringin had no effects on pacemaker potential. (D and E) Liquiritigenin and albiflorin decreased pacemaker potential amplitudes. (F) Paeoniflorin had no effects on pacemaker potential. (G) Hesperidine depolarized pacemaker potential. (H) Concentration–response curve with various saikosaponin A concentrations represents mean ± S.E. (I) Concentration–response curve with various liquiritigenin concentrations represents mean ± S.E. (J) Concentration–response curve with various albiflorin concentrations represents mean ± S.E. (K) Concentration–response curve with various hesperidin concentrations represents mean ± S.E. CSS, Chaihu-Shugan-San. EC_{50}, Half maximal effective concentration. IC_{50}, Half maximal inhibitory concentration.
albiflorin, the mean amplitudes were 24.6 ± 0.1 mV at 1 µM, 25.1 ± 1.1 mV at 10 µM, 18.5 ± 2.7 mV at 50 µM, 1.5 ± 1.4 mV at 100 µM, and 1.1 ± 1.0 mV at 500 µM (IC₅₀ = 58.3 ± 1.5 µM; Fig. 3I, n = 5). Hesperidine depolarized pacemaker potential in a concentration-dependent manner (Fig. 3G). In the presence of hesperidine, the mean amplitudes were 2.5 ± 1.0 mV at 1 µM, 5.5 ± 1.3 mV at 5 µM, 13.9 ± 1.5 mV at 10 µM and 24.7 ± 2.3 mV at 30 µM (EC₅₀ = 9.8 ± 1.3 µM; Fig. 3K, n = 6). However, poncirin, naringin and paeoniflorin had no effects on pacemaker potential (Figs. 3B, 3C, 3F). These results showed that CSS decreased the amplitude of ICC pacemaker potentials in a dose-dependent manner mainly through liquiritigenin and albiflorin.

No Involvement of Transient Receptor Potential Vanilloid 1 (TRPV1) Receptor on CSS-Induced Effects on Pacemaker Potentials in Cultured ICCs To check the involvement of various ion channels on CSS-induced effects on pacemaker potentials in cultured ICCs, we examined the relevance of TRPV1 channel. The inhibitory response of CSS (30 mg/mL) was not suppressed by the pretreatment of TRPV1 antagonist capsazepine (10 µM) (Fig. 4A). The amplitudes before and after the treatment of capsazepine with co-treatment of CSS were 23.7 ± 0.8 mV and 2.4 ± 0.5 mV (n = 6; Figs. 4A, 4B). The above results indicate that the TRPV1 channel is not involved in the response of the CSS.

Involvement of ATP Sensitive K⁺ Channels on CSS-Induced Effects on Pacemaker Potentials in Cultured ICCs Various types of K⁺ channel blockers were used to check the involvement of K⁺ channels in CSS-induced responses. In the presence of Ca²⁺-activated K⁺ channel blocker tetraethylammonium chloride (TEA; 10 mM), CSS inhibited pacemaker potential (Fig. 5A). In addition, CSS also inhibited pacemaker potential when co treated with transient voltage-dependent K⁺ channel blocker 4-aminopyridine or Ca²⁺-activated K⁺ channel blocker apamin (Figs. 5B, 5C). In a previous study, we found that ATP sensitive K⁺ channels may be involved in...
the regulation of pacemaker potentials in cultured ICCs.\textsuperscript{21,22} Therefore, we examined the effects of CSS on pacemaker potentials in the presence of an ATP sensitive K\textsuperscript+ channel blocker glibenclamide. CSS had no effects on pacemaker potentials after glibenclamide pretreatment (Fig. 5D) and CSS (30 mg/mL) reduced the amplitude of the pacemaker potentials, while the addition of glibenclamide (10 \(\mu\)M) reversed these effects (Fig. 5E). A summary of the effects of CSS and K\textsuperscript+ channel blockers on pacemaker potentials are provided in Fig. 5F. The results suggest that the inhibitory effects of CSS on pacemaker potentials in cultured ICCs are mediated by ATP sensitive K\textsuperscript+ channels.

The Involvement of Nitric Oxide on CSS-Induced Effects on Pacemaker Potentials in Cultured ICCs The effects of N\textsuperscript{O}-nitro-l-arginine methyl ester (l-NAME) were examined to investigate the possible regulation of pacemaker potentials by CSS. l-NAME (10 \(\mu\)M) was pretreated for 10 min before application of CSS (30 mg/mL). The inhibitory effects of CSS were blocked by pretreatment of l-NAME (Fig. 6A). In the presence of l-NAME, the amplitude by CSS was 23.7 \(\pm\) 0.5 mV (\(n=5\); Fig. 6B). These results suggest that l-NAME had effects on pacemaker potentials.

Involvement of Guanylate Cyclase and Protein Kinase G (PKG) on CSS-Induced Effects on Pacemaker Potentials in Cultured ICCs Adenylate cyclase inhibitor (SQ-22536) and guanylate cyclase inhibitor (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; ODQ) were used to determine the relevance of CSS-induced response to cyclic nucleotide dependent pathway. In the presence of SQ-22536 (100 \(\mu\)M), CSS still inhibited pacemaker potentials (Fig. 7A). However, ODQ (100 \(\mu\)M) suppressed CSS-induced pacemaker potentials inhibition (Fig. 7B). In the presence of SQ-22536, the mean amplitude of CSS-induced pacemaker potentials was 2.4 \(\pm\) 0.5 mV (\(n=5\); Fig. 7E) and the ODQ corresponding value was 23.5 \(\pm\) 1.3 mV (\(n=6\); Fig. 7E). In addition, in the presence of KT-5720 (1 \(\mu\)M), CSS inhibited pacemaker potentials (Fig. 7C); however, preincubation with KT-5823 (a protein kinase G (PKG) inhibitor; 1 \(\mu\)M) suppressed CSS-induced pacemaker potentials inhibition (Fig. 7D). These results suggest that cGMP and PKG may play a vital role in CSS-induced responses.

CSS Inhibited Neostigmine-Induced Intestinal Hyperactivity Next, we looked at the effect of CSS on ITRs \textit{in vivo}. Previous researches have suggested that neostigmine increases intestinal motility.\textsuperscript{23,24} In neostigmine-administered mice, application of 0.1 g/kg or 1 g/kg CSS reduced the increase in ITR caused by neostigmine (Fig. 8).

Administration of CSS Treatment Decreased Protein Expression of c-Kit c-Kit is a transmembrane protein that represents the quantity and density of these ICCs.\textsuperscript{25} After treatment with neostigmine and CSS, the protein expression levels of c-Kit were checked by Western blot method. Western blot analysis results showed that the degree of expression levels of this were considerably higher after neostigmine treatment (Fig. 9A). The increased c-Kit expression level in neostigmine treatment mice significantly decreased by 30.7\% after treating the mice with CSS (\(p<0.05\)) (Fig. 9B).

DISCUSSION

In this study, we looked the efficacy of CSS on pacemaker potentials and related mechanisms in cultured ICCs from the mouse small intestine. Our observations suggest that CSS inhibits the pacemaker potentials of ICCs via NO/cGMP and ATP-sensitive K\textsuperscript+ channel dependent and TRPV1 independent pathways. These findings show that CSS offers a basis for the development of new treatments for GI motility dysfunction.
In China, an ancient traditional Chinese medicine (TCM) formula called CSS is efficiently applied to the treatment of various diseases; it is used for its neurologic impairment and depression treatment, anti-inflammatory with insulin signaling improvement, liver toxicity recovery effects, and nonalcoholic fatty liver regeneration effects, as well as to treat GI disorders with functional dyspepsia, gastric ulcers, diarrhea, and gastritis.6–11,13) Chronic stress can affect neuroendocrine and behavior and cause depression; however, administration of CSS under these conditions has been shown to regulate hypothalamic-pituitary-adrenocortical systems and exert antidepressive effects in rats.26) Additionally, CSS was traditionally used to treat chronic fatty liver diseases.7) Many studies have demonstrated that CSS protects against insulin resistance and lipid peroxidation.11,27) Some studies have also shown that it could cure a variety of GI diseases.5,12,13) Moreover, CSS exerts prokinetic properties on the GI tract.5) Additionally, CSS markedly accelerated gastric emptying (GE) and intestinal transit (IT) and significantly promoted ileum peristalsis in rats.5) However, the effects of CSS on ICCs have not been previously investigated.

ICCs act as gut pacemaker cells and coordinate peristaltic movements.14,15,17,18) In addition, hormones and neurotransmitters can modulate ICCs activity to influence gut motility.28,29) The results of the present study showed CSS decreased the amplitude of pacemaker potentials in a concentration-dependent manner in ICCs (Fig. 2) mainly through liquiritigenin and albiflorin (Fig. 3), and that these effects were mediated not in TRPV1 channels (Fig. 4), but in ATP-sensitive K+ channels (Fig. 5). Moreover, NO/cGMP pathways were involved in the effects of CSS (Figs. 6, 7). The TRPV1 channel is also called the capsaicin receptor, which stimulates the TRPV1 channel and generates various reactions. Immunohistochemical studies have shown that there are many TRPV1 channels in the GI tract.30,31) Therefore, this TRPV1 channel has become the most important pharmacological targets in GI studies, and capsaicin has been used to activate this TRPV1 channel in the GI tract.20) In this study, the inhibitory effect of CSS on pacemaker potentials was similar to that of capsaicin20) (Fig. 2). Therefore, we investigated the involvement of TRPV1 channels on CSS-induced effects on pacemaker potentials in cultured ICCs. However, similar to the capsaicin-induced effects,20) TRPV1 channels were not involved in CSS-induced effects on pacemaker potentials in ICCs (Fig. 4).

The K+ channel in smooth muscle has the function of regulating cell membrane potential and cell excitability. ATP-sensitive K+ channels stabilize the resting membrane potential and causes hyperpolarization of the cell membrane and reduces electrical excitability.32) ATP-sensitive K+ channels have also been reported in GI smooth muscle cells, and act as targets of neurotransmitters and peptides.33,34) In this study, CSS acted as neurotransmitters and peptides in ICCs and regulated...
the pacemaker potentials by modulating the ATP-sensitive K+ channels (Fig. 5). Therefore, we think that CSS acts on ATP-sensitive K+ channels of the intestinal ICCs. GI motility patterns are highly integrated and require coordination with smooth muscle cells, neurons, endocrinies and immune cells.35-36) Therefore, further investigations are needed to investigate the effects of CSS on other cells in the GI tract.

In this study, CSS exerts inhibitory effects on the pacemaker potentials of ICCs via NO/cGMP and ATP-sensitive K+ channel dependent pathways. Based on the findings described in this, we propose the following model of the effects of CSS in ICCs (Fig. 10). CSS is composed of saikosaponin A, poncirin, naringin, glycyrrhizic acid, liquiritigenin, albiflorin, paeoniflorin and hesperidin. These eight components showed generally the reduction of NO. Saikosaponin A or poncirin significantly inhibited the expression of inducible nitric-oxide synthase (iNOS) and finally resulted in the reduction of NO.35-38) Naringin could regulate the glutamate-nitric oxide cGMP pathway and enhanced the glutamine synthetase (GS) activity in hyperammonemic rats with neurotoxicity.39) Also, in rats with high blood pressure caused by glycyrrhizic acid, the renal protein expression of endothelial nitric oxide synthase (eNOS) and iNOS was increased and however, the cGMP production was not changed.40) Liquiritigenin or Albiflorin exerted anti-inflammatory effects, which results from the inhibition of nuclear factor (NF)-kappaB activation in macrophages, thereby decreasing production of iNOS.41) Paenoflorin, isolated from the root of *Paonia lactiflora* pall, protected RAW264.7 macrophages, thereby decreasing production of iNOS.42) Paeoniflorin, isolated from *Paeonia* pall, protected RAW264.7 macrophages, thereby decreasing production of iNOS.43) Hesperidin exerted antidepressant-like effects with the possible role of l-arginine–NO–cGMP pathway.44,45) However, the NO–cGMP pathway effects of these eight components in GI tract have not been known. In addition, these eight components did not have any the electrophysiological effects on ATP sensitive K+ channels, not TRPV1 channels. In addition, CSS also suppressed ITR increases caused by neostigmine in vivo. Further findings are needed to identify CSS as a basis for the development of new treatments for GI motility dysfunction.

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

Conflict of Interest The authors declare no conflict of interest.

REFERENCES

1) Yanai M, Mochiki E, Ogawa A, Morita H, Toyomasu Y, Ozata K, Tabec Y, Ando H, Ohto T, Asao T, Aomori T, Fujita Y, Kiuwano H, Intragastric administration of rikkunshito stimulates upper gastrointestinal motility and gastric emptying in conscious dogs. J. Gastroenterol., **48**, 611–619 (2013).

2) Bent S. Herbal medicine in the United States: review of efficacy, safety, and regulation: grand rounds at University of California, San Francisco Medical Center. J. Gen. Intern. Med., **23**, 854–859 (2008).

3) Comar KM, Kirby DF. Herbal remedies in gastroenterology. J. Clin. Gastroenterol., **39**, 457–468 (2005).

4) Li H, Qu Y, Zhang J, Zhang J, Gao W. Spasmolytic activity of *Aurariae Lignum Resinatum* extract on gastrointestinal motility involves muscarinic receptors, calcium channels and NO release. Pharm. Biol., **56**, 559–566 (2018).

5) Qiu XJ, Huang X, Chen ZQ, Ren P, Huang W, Qin F, Hu SH, Huang J, He J, Liu ZQ, Zhou HH. Pharmacokinetic study of the prokinetic compounds meranzin hydrate and ferulic acid following oral administration of Chaihu-Shugan-san to patients with functional dyspepsia. *J. Ethnopharmacol.*, **137**, 205–213 (2011).

6) Huang X, Guo Y, Huang WH, Zhang W, Tan ZR, Peng JB, Wang YC, Hu DL, Ouyang DS, Xiao J, Wang Y, Luo M, Chen Y. Searching the cytochrome p450 enzymes for the metabolism of meranzin hydrate: a prospective antidepressant originating from Chaihu-Shugan-san. *PLoS ONE*, **9**, e13819 (2014).

7) Yang QH, Xu YJ, Liu YZ, Liang YJ, Peng GF, Zhang YP, Xing HJ, Yan HZ, Li YY. Effects of Chaihu-Shugan-san and Shen-Ling-Bai-Zhi-San on p38 MAPK Pathway in Kupffer Cells of Nonalcoholic Steatohepatitis. *Evid. Based Complement. Alternat. Med.*, **2014**, 67013 (2014).

8) Wang Y, Fan R, Huang X. Meta-analysis of the clinical effectiveness of traditional Chinese medicine formula Chaihu-Shugan-san in depression. *J. Ethnopharmacol.*, **141**, 571–577 (2012).

9) Jia HM, Yu M, Ma LY, Zhang WH, Zou ZM. Chaihu-Shu-Gan-san regulates phospholipids and bile acid metabolism against hepatic injury induced by chronic unpredictable stress in rat. *J. Chromatogr. B Analyt. Technol. Biomed. Sci.*, **1064**, 14–21 (2017).

10) Jia K, Pan SM, Ding H, Liu JH, Zheng YJ, Wang SJ, Pan Y, Kong LD. Chaihu-shugan san inhibits inflammatory response to improve insulin signaling in liver and prefrontal cortex of CUMS rats with glucose intolerance. *Biomed. Pharmacother.*, **103**, 1415–1428 (2018).

11) Jiang WN, Li D, Jiang T, Guo J, Chen YF, Wang J, Zhou Y, Yang CY, Tang CP. Protective effects of Chaihu Shugan San (CSS) on nonalcoholic fatty liver disease in rats with insulin resistance. *Chin. J. Integr. Med.*, **24**, 125–132 (2018).

12) Qin F, Huang X, Ren P. Chinese herbal medicine modified xiaoyao san for functional dyspepsia: a meta-analysis of randomized controlled trials. *J. Gastroenterol. Hepatol.*, **24**, 1320–1325 (2009).

13) Qin F, Liu JY, Yuan JH. Chaihu-Shugan-San, an oriental herbal medicine modified xiaoyao san for functional dyspepsia: a meta-analysis of randomized controlled trials. *J. Ethnopharmacol.*, **146**, 433–439 (2013).

14) Kim HJ, Kim BJ. Naringenin inhibits pacemaking activity in intestinal cells of rat from murine small intestine. *Integr. Med. Res.*, **6**, 149–155 (2017).

15) 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*, **6**, e24928 (2011).

16) He CL, Sofier EE, Ferris CD, Walsh RM, Szurszewski JH, Farrugia G. Loss of interstitial cells of cajal and inhibitory innervation in insulin-dependent diabetes. *Gastroenterology*, **121**, 427–434 (2001).

17) Kim BJ, Lim HH, Yang DK, Jun JY, Chang JY, Park CS, So I, Stanfield PR, Kim KW. Melastatin-type transient receptor potential channel 7 is required for intestinal pacemaking activity. *Gastroenterology*, **129**, 1504–1517 (2005).
25) Sanders KM, Koh SD, Ro S, Ward SM. Regulation of gastrointestinal depolarizations of pacemaking activity in interstitial cells of Cajal from murine small intestine. *Integr. Med. Res.*, 2, 62–69 (2013).

24) Law NM, Bharucha AE, Undale AS, Zinsmeister AR. Cholinergic stimulation enhances colonic motor activity, transit, and sensation in humans. *Gastroenterology*, 153, 1787–1792 (2007).

23) Corsetti M, Gevers AM, Caenepeel P, Tack J. The role of tension receptors in colon motor sensitivity in humans. *Gastroenterology*, 153, 1793 (2007).

20) Choi S, Sun JM, Shahi PK, Zuo DC, Kim HI, Jun JY. Capsaicin induces pacemaker potential inhibition via cGMP-dependent ATP-sensitive K+ channels by stimulating mu/delta opioid receptors in cultured interstitial cells of Cajal from mouse small intestine. *Mol. Med.*, 16, 265–273 (2010).

21) Kim BJ, Chae H, Kwon YK, Choi S, Jun JY, Jeon JH, So I, Kim SJ. Effects of inomatin mesylate in interstitial cells of Cajal from murine small intestine. *Biol. Pharm. Bull.*, 33, 993–997 (2010).

22) Kim HJ, Kim H, Jung MH, Kwon YK, Kim BJ. Berberine induces pacemaker potential inhibition via cGMP-dependent ATP-sensitive K+ channels by stimulating mu/delta opioid receptors in cultured interstitial cells of Cajal from mouse small intestine. *Mol. Med. Rep.*, 14, 3985–3991 (2016).

23) Corsetti M, Gevers AM, Caenepeel P, Tack J. The role of tension receptors in colon motor sensitivity in humans. *Gastroenterology*, 153, 1787–1792 (2007).

24) Law NM, Bharucha AE, Undale AS, Zinsmeister AR. Cholinergic stimulation enhances colonic motor activity, transit, and sensation in humans. *Gastroenterology*, 153, 1793 (2007).

25) Sanders KM, Koh SD, Ro S, Ward SM. Regulation of gastrointestinal motility: insights from smooth muscle biology. *Nat. Rev. Gastroenterol. Hepatol.*, 9, 633–645 (2012).

26) Chen XQ, Li CF, Chen SJ, Jiang WN, Wang M, Wang SS, Dong SQ, Yi LT, Li CD. The antidepressant-like effects of Chaihu Shugan San. Dependent on the hippocampal BDNF-TrkB-ERK/Akt signaling activation in perimenopausal depression-like rats. *Biomed. Pharmacother.*, 105, 45–52 (2018).

27) Li SQ, Su ZH, Peng JB, Zou ZM, Yu CY. In vitro and in vivo antioxidative effects and the possible relationship between the antidepressant efficacy of traditional Chinese medicine formulation Chaihu Shugan San. *Chin. J. Nat. Med.*, 8, 353–361 (2010).

28) Kim BJ, Nam JH, Kim KH, Joo M, Ha TS, Weon KY, Choi S, Jun JY, Park EW, Ji W, So I, Nah SY. Characteristics of gintonin-mediated membrane depolarization of pacemaker activity in cultured interstitial cells of Cajal. *Cell. Physiol. Biochem.*, 34, 873–890 (2014).

29) Kim BJ, Nam JH, Kim KH, Joo M, Ha TS, Weon KY, Choi S, Jun JY, Park EW, Ji W, So I, Nah SY. Characteristics of gintonin-mediated membrane depolarization of pacemaker activity in cultured interstitial cells of Cajal. *Cell. Physiol. Biochem.*, 34, 873–890 (2014).

30) Horie S, Yamamoto H, Michael GJ, Uchida M, Belai A, Watanabe K, Priestley JV, Murayama T. Protective role of vanilloid receptor type 1 in HCl-induced gastric mucosal lesions in rats. *Scand. J. Gastroenterol.*, 39, 318–324 (2004).

31) Patterson LM, Zheng H, Ward SM, Berthoud HR. Vanilloid receptor (VR1) expression in vagal afferent neurons innervating the gastrointestinal tract. *Cell Tissue Res.*, 311, 277–287 (2003).

32) Quayle JM, Nelson MT, Standen NB. ATP-sensitive and inwardly rectifying potassium channels in smooth muscle. *Physiol. Rev.*, 77, 1165–1232 (1997).

33) Choi S, Parajuli SP, Yeum CH, Park CG, Kim MY, Kim YD, Cha KH, Park YB, Park JS, Jeong HS, Jun JY. Calcitonin gene-related peptide suppresses pacemaker currents by nitric oxide/cGMP-dependent activation of ATP-sensitive K+ channels in cultured interstitial cells of Cajal from the mouse small intestine. *Mol. Cells*, 26, 181–185 (2008).

34) 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.*, 39, 314–321 (2015).

35) Kim JB, Han AR, Park EY, Kim JY, Cho W, Lee J, Seo EK, Lee KT. Inhibition of LPS-induced iNOS, COX-2 and cytokines expression by poncirin through the NF-kappaB inactivation in RAW264.7 macrophage cells. *Biol. Pharm. Bull.*, 30, 2345–2351 (2007).

36) Lu CN, Yuan ZG, Zhang XL, Yan R, Zhao YQ, Liao M, Chen JX. Saikosaponin a and its epimer saikosaponin d exhibit anti-inflammatory activity by suppressing activation of NF-κB signaling pathway. *Int. Immunopharmacol.*, 14, 121–126 (2012).

37) Ma X, Dong C, Kang H, Dai Z, Lin S, Guan H, Liu X, Wang X, Hui W. Saikosaponin-D reduces cisplatin-induced nephrotoxicity by repressing ROS-mediated activation of MAPK and NF-κB signaling pathways. *Int. Immunopharmacol.*, 28, 399–408 (2015).

38) Shin JS, Im HT, Lee KT, Im HT, Lee KT. Saikosaponin B2 suppresses inflammatory responses through IKK-IκBα/NF-κB signaling inactivation in LPS-induced RAW 264.7 macrophages. *Inflammation*, 42, 342–353 (2019).

39) Ramakrishnan A, Vijayakumar N, Renuka M. Naringin regulates glutamate-nitric oxide cGMP pathway in ammonium chloride induced neurotoxicity. *Biomed. Pharmacother.*, 84, 1717–1726 (2016).

40) Ma SK, Bae EH, Kim IJ, Choi KC, Kim SH, Lee J, Kim SW. Increased renal expression of nitric oxide synthase and atrial natriuretic peptide in rats with glycyrhizic acid-induced hypertension. *Phytother. Res.*, 23, 206–211 (2009).

41) Kim YW, Zhao RJ, Park SJ, Lee JR, Cho JJ, Yang CH, Kim SG, Kim SC. Anti-inflammatory effects of liquiritigenin as a consequence of the inhibition of NF-kappaB-dependent iNOS and pro-inflammatory cytokines production. *Br. J. Pharmacol.*, 154, 165–173 (2008).

42) Kim ID, Ha BJ. Paeoniflorin protects RAW264.7 macrophages from LPS-induced cytotoxicity and genotoxicity. *Toxicol. In Vitro*, 23, 1014–1019 (2009).

43) Ji Q, Yang L, Zhou J, Lin R, Zhang J, Lin Q, Wang W, Zhang K. Protective effects of paeoniflorin against cobalt chloride-induced apoptosis of endothelial cells via HIF-1α pathway. *Toxicol. In Vitro*, 26, 455–461 (2012).

44) Qian GQ, Ding J, Zhang X, Yin X, Gao Y, Zhao GP. Preconditioning with glycyrhizin, feralic, paeoniflorin, cinnamic prevents rat hearts from ischemia/reperfusion injury via endothelial nitric oxide pathway. *Pharmacogn. Mag.*, 11, 292–296 (2015).

45) Kumar A, Lalitha S, Mishra J. Possible nitric oxide mechanism in the protective effect of hesperidin against pentyleneetetrazole (PTE)-induced kindling and associated cognitive dysfunction in mice. *Epilepsy Behav.*, 29, 103–111 (2013).

46) Donato F, Borges Filho C, Giacomeli R, Alvater EE, Del Fabbro L, Antunes Mda S, de Gomes MG, Goes AT, Souza LC, Boeira SP, Jesse CR. Evidence for the involvement of potassium channel inhibition in the antidepressant-like effects of hesperidin in the tail suspension test in mice. *J. Med. Food*, 18, 818–823 (2015).

47) Donato F, de Gomes MG, Goes AT, Filho CB, Del Fabbro L, Antunes MS, Souza LC, Boeira SP, Jesse CR. Hesperidin exerts antidepressant-like effects in acute and chronic treatments in mice: possible role of l-arginine–NO–cGMP pathway and BDNF levels. *Brain Res. Bull.*, 104, 19–26 (2014).