Answering reviewers

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ESPS Manuscript No: 33793

Title: Hwangryunhaedok-tang induces the depolarization of pacemaker potentials through 5-HT_3 and 5-HT_4 receptors in cultured murine small intestine interstitial cells of Cajal

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Editor

A1: Please provide one report:

Responses) We added the Scientific Research Process.

A2: Please revise and perfect your manuscript according to peer-reviewers comments.

Responses) We revised our manuscript according to peer-reviewers comments.

A3: You need to provide the grant application form(s) or certificate of funding agency for every grant, or we will delete the part of "**Supported by...**".

Responses) We provided the grant application form(s).

A4: Please finish them

Responses) We provided all them.

A5: AIM: No more than 20 words, and start with "To..."

Responses) We revised.

A6: METHODS: no less than 80 words

Responses) We revised.

A7: RESULTS: no less than 120 words

Responses) We revised.

A8: Please check that there are no repeated references!

Responses) We checked.

A9: Change it to 'e'

Responses) We changed.

A10: Change it to 'b'

Responses) We changed.

Reviewer1

The manuscript (NO: 33793) entitled Hwangryunhaedok-tang induces the depolarization of pacemaker potentials through 5-HT3 and 5-HT4 receptors in cultured murine small intestine interstitial cells of Cajal was investigated the effect of HHTE which is a traditional herbal medicine used to treat gastrointestinal (GI) disorders on ICC pacemaking potential. The author suggested that HHTE dose-dependently depolarizes ICC pacemaker potentials through 5-HT3 and 5-HT4 receptors via external and internal Ca2+ regulation and via G protein-, and MLCK-dependent and PKC- and Rho kinase-independent pathways. The work is concerned with traditional herbal medicine and its effect of promoting gastrointestinal motility via 5-HT3 and 5-HT4 receptors of ICC. This work is very interesting and has potential clinical application value.

Comments

1. The effect of HHTE on ICC pacemaking potential is very dramatically, so I think to know if the effect of HHTE on smooth muscle contraction is matched to the effect of HHTE on ICC.

Responses) We did not experiment on smooth muscle cells. However, we experimented the effects of HHTE on GI motility in mice *in vivo*. HHTE increased the GI motility in mice *in vivo*[26]. Therefore, we think that the effect of HHTE on smooth muscle contraction is matched to the effect of HHTE on ICC.

2. Does the author think HHTE may activates 5-HT3 and 5-HT4 receptors?

Responses) Yes. We think that HHTE may activate 5-HT3 and 5-HT4 receptors. Among them, we think that 5-HT4 receptors have main role to regulate the ICC and 5-HT3 receptors have supportive role of 5-HT4 receptors.

3. It is well known that ICC pacemaking currents elicited by ANO1 channel, so test the effect of ANO1 blocker on ICC pacemaking potential is very important.

Responses) We experimented the effect of ANO1 blocker (T16Ainh-A01, 10-30 μM) on ICC pacemaking potentials. In case of T16Ainh-A01, the ICC pacemaking potential was blocked about 50%.

Reviewer2

The authors investigated the effects of HHT (a traditional herbal medicine) on the pacemaker potentials of mouse interstitial cells of Cajal (ICCs), the results of study suggest that HHT dose-dependently depolarizes ICC pacemaker potentials through 5-HT3 and 5-HT4 receptors via external and internal Ca2+ regulation and via G protein-, and MLCK-dependent pathways. These data are somewhat interesting.

Minor comments:

1.The authors wrote that HHT dose-dependently depolarizes ICC pacemaker potentials via MLCK-dependent and PKC- and Rho kinase-independent pathways, but the results showed HHT-induced pacemaker potential depolarizations were inhibited by ML-7 (88.5% inhibition) , but not by calphostin or Y27632. So I am confused.

Responses) Thanks for your good comments. I am sorry but, we made mistakes. We deleted the ML-7 data. As we did not use cell sorting, there is a small possibility that other cell types may have been present in the small ICC networks that formed in culture. As such, the effect of ML-7 may have been due to the other cells types (e.g. smooth muscle cells).

2.The discussion paragraph should be rewritten, arrangement is not clear.

Responses) We rechecked the Discussion part and rewrote.

3.There are some grammar mistakes in the manuscript

Responses) We checked English language quality and grammar by American Journal Experts. We added the language certificate.

Reviewer3

Hwangryunhaedok-tang induces the depolarization of pacemaker potentials through 5-HT3 and 5-HT4 receptors in cultured murine small intestine interstitial cells of Cajal. The manuscript has important value to the interstitial cells field and the idea of exploring a traditional herbal medicine to treat gastrointestinal (GI) disorders. The authors examined the effects of the potential herbal drug Hwangryunhaedok-tang (HHT) on pacemaker potentials generated by ICCs. HHT depolarized ICC pacemaker potentials and the authors stated that this depolarization was due to activation of 5-HT3 and 5-HT4 receptors in ICC. It seems that G-protein coupled receptors, calcium influx and release mechanisms and MLCK are involved in mediating the HHT effects on ICC.

Major comments:

This hypothesis and conclusions are lacking evidence in the current form of the paper, as the authors did not establish clearly the link of how HHT active elements (Coptidis Rhizoma and Gardeniae

Fructus) can bind to 5-HT receptors on ICC. Furthermore, the data lacks the effects of HHT identified compounds (geniposide, berberine chloride, baicalin, and wogonin) on ICCs.

Responses) We will perform future experiments to determine the detailed effects of the HHT active elements (Coptidis Rhizoma and Gardeniae Fructus) and identified compounds (geniposide, berberine chloride, baicalin, and wogonin) on ICC pacemaking potential. This paper focused the effects of HHT (HHT was purchased from I-WORLD Pharmaceuticals (Incheon, Republic of Korea)) on ICC pacemaking potentials.

We added this part in Discussion.

A surprising results that HHTE-induced pacemaker potential depolarizations were inhibited by ML-7. These results are confusing as native ICCs are non-contractile and do not express MLCK. Therefore, the results obtained maybe due direct effects on smooth muscle cells contaminated with ICC cultures or there is potential change in ICC phenotype in culture. A more careful approach should be used to assess the data and interpretations.

Responses) Thanks for your good comments. I am sorry but, we made mistakes. We deleted the ML-7 data. As we did not use cell sorting, there is a small possibility that other cell types may have been present in the small ICC networks that formed in culture. As such, the effect of ML-7 may have been due to the other cells types (e.g. smooth muscle cells).

The authors demonstrated that the actions of HHTE is linked to G-protein coupled receptor mechanisms. Although 5HT3 receptors are cation channels that can be activated without G- protein mechanisms. No explanation was discussed to explain results of 5HT3 antagonist effects in relation to GDP—S effects.

Responses) Thanks for your good comments. The 5-HT_3 receptor channels are the only ionotropic receptors within the 5-HT receptor family[47-49]. 5-HT_3 receptors are involved in many pathophysiological processes, such as GI motility disorders and the development of nausea and vomiting[49-51]. Therefore, 5-HT_3 receptors play an important role in GI motility functions. In this paper, we found that HHTE may activate 5-HT_3 and 5-HT_4 receptors; among them, 5-HT_4 receptors may have the main role in regulating the ICC, whereas 5-HT_3 receptors may have a supportive role. Additionally, when GDP-β-S (1 mM) was applied in the pipette solution, we found that HHTE caused slight pacemaker potential depolarization (Figure 3), which might be due to the 5-HT_3 receptor signaling pathway.

We added this part in Discussion.

Minor comments:

-Please indicate if patched ICC were immunopostive for kit.

Responses) ICCs were identified immunologically using an anti-c-kit antibody[25].
We added this in Results.

- "Confirmation of HHTE receptor subtypes in ICCs". Please consider revising the sentence. For example HHTE mechanisms of action on ICC.
Responses) We revised the sentence.

-The conclusions that HHTE affects ICC may be through 5-HT3 and 5-HT4 receptors by the authors is weak and supported by data or discussed. Questions what and how exactly HHTE components binds to 5HT receptor are lacking.
Responses) Thanks for your good comments. We think that the detailed effects of HHT active elements (Coptidis Rhizoma and Gardeniae Fructus) and identified compounds (geniposide, berberine chloride, baicalin, and wogonin) on ICC pacemaking potential are different subjects. We are preparing the experiments about the effects of HHT active elements and identified compounds on ICC. This paper focused the effects of HHT (HHT was purchased from I-WORLD Pharmaceuticals (Incheon, Republic of Korea)) on ICC pacemaking potentials. Also, we added the explanation of 5-HT3 and 5-HT4 receptors in Discussion Part.
We added this in Discussion.

-Frequency of ICC pacemaker potentials should be analyzed for all drugs used to assess the effects of HHTE on pacemaker currents and provide valuable information on how these drugs affects motility.
Responses) We analyzed the frequency changes of ICC pacemaker potential for all drugs in figures and results part. Also we experimented the effects of HHTE on GI motility in mice *in vivo* and found that HHTE increased the GI motility in mice[26].

-The authors should explain and discuss the link between calcium signaling and 5HT receptor (5HT 3 and 4) activation and provide a model of the role of calcium influx and release mechanisms in mediating HHTE effects.
Responses) ICCs are pacemaker cells that appear to play important roles in the determination and regulation of GI motility[19,20]. HHTE depolarized the pacemaker potentials of ICCs in a G protein- and Ca^{2+}-dependent manner. Based on the findings described in this study, we propose the following model of the effects of HHTE in ICCs. HHTE binds 5-HT_3 and 5-HT_4 receptors, and HHTE-induced pacemaker potential depolarizations are G protein- and Ca^{2+}-dependent in ICCs. An increase in intracellular Ca^{2+} induces membrane depolarizations on ICCs[58,59]. In addition, these pacemaker potential depolarizations might be regulated by transient receptor potential melastatin 7 (TRPM7) and Ca^{2+}-activated Cl^- (ANO1) channels[20,60].

-Abstract: please include the drug action rather than drug name alone. For example the A 5-HT3 receptor antagonist (Y25130).

Responses) We changed the Abstract part.

- Please include the full name. For example HPLC (high performance liquid chromatography)

Responses) We included the full name.

- What is the definition of "standard compounds"? Should be included throughout the MS

Responses) Standard compounds are geniposide, berberine chloride, baicaline, and wogonin.

- Patch clamp temperature should be included in results section.

Response) We included patch clamp temperature in Results section