Point-by-point answers to the questions

September 7th, 2017

Dear Editor Qi,

Thank you very much for giving us an opportunity to revise our manuscript titled "Effects of Hemp seed soft capsule on colonic ion transport in rats" (Manuscript NO: 35258) by Xiaofang Lu, Mengdi Jia, Shengsheng Zhang and Luqing Zhao.

We have carefully made necessary revisions to the manuscript in response to the reviewer's comments and your suggestions. Hope these revisions will make it suitable for being published. The revised portions has been marked in yellow. Thanks for your consideration.

Best regards,

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Point-by-point answers to the reviewer's questions

Reviewer 1：

Question 1: HSSC was water soluble and diluted into aqueous solution, or insoluble and suspension was made? What concentration of HSSC was made for stock solution? Please clarify this in Method.

Answer: Thanks for your question and proposal. Indeed, we need to supplement the preparation of HSSC.

In our study, HSSC was water soluble and diluted into aqueous solution. The concentration of the prepared stock solution was 1g/ml.

This has been supplemented on page 7 in the revised manuscript.

Question 2: HSSC was added into the luminal bath or serosal? If HSSC in the one side increased I*sc*, how about HSSC in the other side? This is important, because the effective side (luminal or serosal) may demonstrate which side has signaling cascade in response to HSSC. For instance, only the serosal HSSC increased Isc, suggesting that HSSC or its ingredient works after its absorption. Please clarify this important issue.

Answer: Thanks for your question. We are sorry for the confusion that brought you because of our neglect.

In our study, HSSC was added into serosal side. This has been supplemented on page 8 in the revised manuscript.

In the pre-experiment, we had added the HSSC into both luminal or serosal side and found that when HSSC was applied to the luminal side, the Isc induced was tiny and no difference was found betweeen the experimental group (with HSSC added) and control group (with the same volume of normal saline added) at each concentration. So the response might be negligible. As you said, it's possible that the HSSC woks after its absorption.

The pre-experimental data about HSSC application to the luminal side as below (three different rats was used to repeat the procedure)：

Colonic mucosa secretion after HSSC application to the luminal side (means±SEM, %)

Group	N	0.1 mg/ml	0.5 mg/ml	1.0 mg/ml	2.5 mg/ml	5.0 mg/ml	10.0 mg/ml	25.0 mg/ml
Experimental group	3	95.93 ±4.46	93.56 ±6.94	100.70 ±3.14	105.32 ±9.33	110.52 ±9.93	117.54 ±11.23	138.19 ±21.65
Control group	3	96.51 ±3.86	98.89 ±10.07	97.09 ±11.49	94.35 ±9.16	96.67 ±7.03	91.73 ±3.89	94.64 ±8.60
P value		P>0.05	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05

Question 3: The representative I*sc* trace showing at least that HSSC increased I*sc* in time course should be demonstrated.

Answer: Thanks for your suggestion, we have attached the representative I*sc* trace in Figure 2 on page 22 in the revised manuscript. Details are as follows:

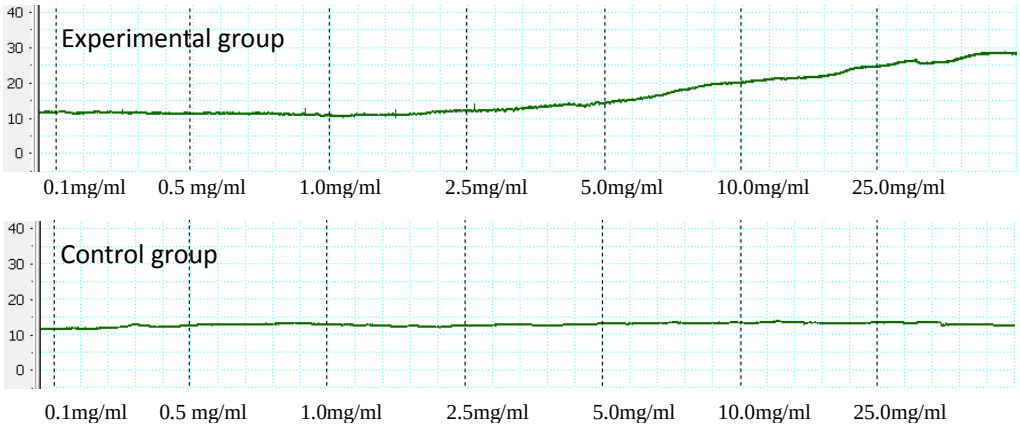

Figure Representative I*sc* trace in experimental group and control group.

Question 4: Which ingredient causes acute response in I*sc*? Although it is difficult to determine the effective ingredient(s) in HSSC in chronic treatment (7 days), I*sc* response should be acutely induced by certain receptor or transporter activation. Involvement of Cl^- channel, NKCC1, and NBC or anion exchanger cannot specify the signaling. Since submucosal plexus remains in the tissue of mucosa-submucosa preparations, the effect of TTX, atropine, or hexamethonium should be examined to rule out the neural pathway.

Answer:

Thanks for your suggestion. This provides a good idea for improving our study. We agree with you very much. We believe that the effective tragets of HSSC are complex. In our study, Cl^- and HCO_3^- were involved in the regulatory mechanism which mediated via Cl^- channel and anion exchanger or cotransporter (NKCC, Na^+-HCO_3^- cotransporter or Cl^-/HCO_3^- exchanger). This is an important and fundamental exploration for the further study. In the follow-up experiment, more receptors or transporters can be discussed.

As you said, the neural pathway may participate in the regulatory process. So to demonstrate the possible targets, we added some experiments as your suggestion and found that the submucosal neurons seemed not to play a key role in the secretagogue effect of Hemp seed soft capsule. The related method, result and discussion have been added in the revised manuscript on page 6, 8, 11, 26 in the revised manuscript. The main results displayed as follows:

The experimental mucosa was pretreated with the neural inhibitor Tetrodotoxin (TTX) (+ TTX group), muscarinic receptor inhibitor atropine (+ Atropine group) or nicotinic receptor antagonist hexamethonium (+ Hexamethonium group); the control mucosa was pretreated with the same volume of normal saline (- TTX group, - Atropine group, - Hexamethonium group, respectively). After that, the effective concentration of HSSC (1.0mg/ml, 2.5mg/ml, 5.0mg/ml, 10.0mg/ml) was added to solicit the Isc response. At each concentrations , there was no difference between the experimental group (+ TTX group, + Atropine group, + Hexamethonium group) and the control group (- TTX group, - Atropine group, - Hexamethonium group) (P>0.05).

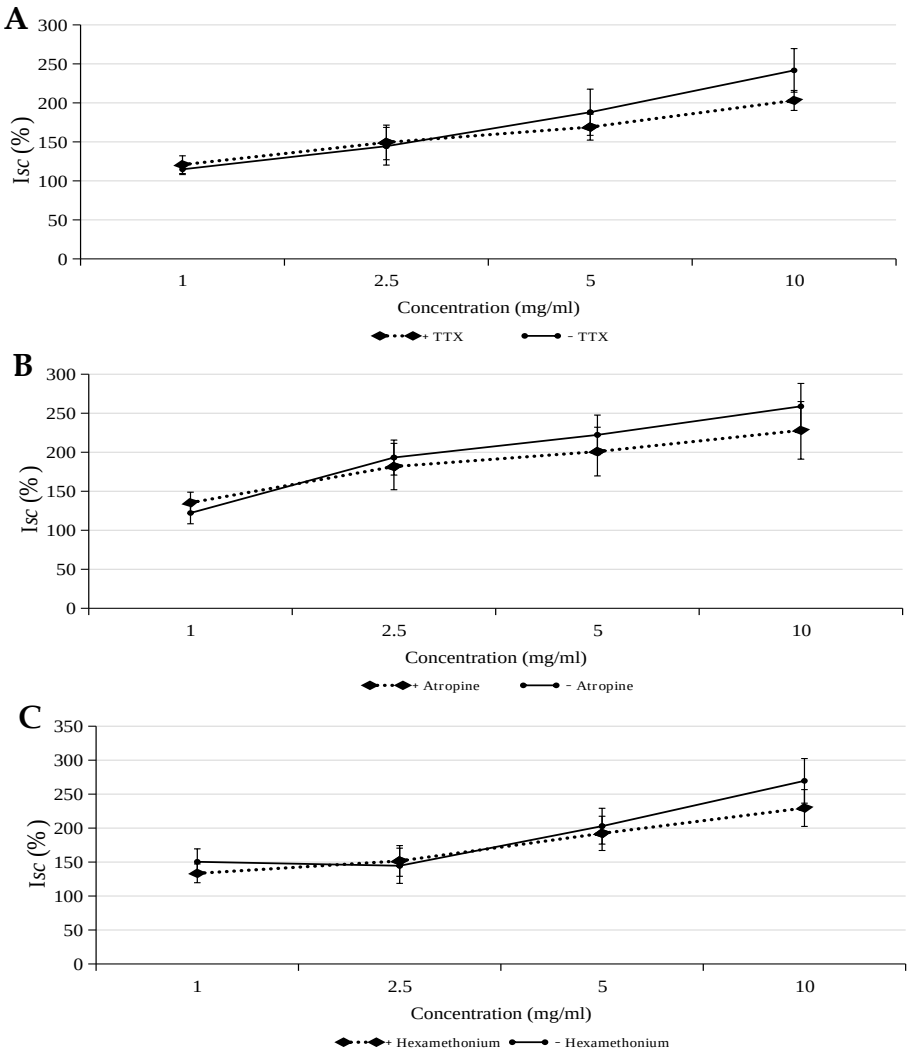

Figure Effects of neural pathway on the secretagogue role of Hemp seed soft capsule (means ± SE, n = 6). A: Pretreated with neural inhibitor Tetrodotoxin; **B:** Pretreated with muscarinic receptor inhibitor Atropine; **C:** Pretreated with nicotinic receptor antagonist Hexamethonium.

Minor Revision:

1. Method 6; R = U/I should be R = V/I.

2. Method 7, Results 4; What is the cAMP-independent Cl^- channel or Ca^{2+}-independent Cl^- channel? These should be cAMP-dependent and Ca^{2+}-dependent, such as CFTR and CaCC. Please correct.

Answer: Thanks for your careful review. We had corrected the aboved mistakes in our revised manuscript.

Reviewer 2:

Question 1: Why one dosage of HSSC was used in the HSSC group?

Answer: Thanks for your question. In the clinical practice, HSSC is one of the most frequently-used chinese patent medicines. So the dosage of HSSC used in the study was based on the clinical effective dose and only one dosage of HSSC was discussed.

Question 2: The discussion paragraphs should be modified, they are not clear to explain the results.

Answer: Thanks for your suggestion. We have carefully modified the discussion. Hope the revised manuscript is qualified to be published.

Other important revisions that should be explained:

1. About the author

Dear editor Qi, approved by all the original authors, there are some fine-tuning of the author list, and "Xiongfei Chang" has been took out. In the Copyright_Assignment, the related contents have been revised. Thanks for your permission and consideration.

2. About the "Institutional animal care and use committee statement"

Dear editor Qi, we have corrected the Institutional animal care and use committee statement (All procedures involving animals were reviewed and

approved by the Institute of Basic Theory, China Academy of Chinese Medical Sciences Ethics Committee (No: 20160905)). This is my negligence that I miswrite the department. I am sorry for the simple mistake. The ethical form has been attached in the uploading statement.

3. About the supplementary references

Dear editor Qi, we have supplemented four new references in the revised manuscript. Reference 4-5 are used to clarify the limitation of current treatment methods. And Reference 24-25 are used to demonstrate the neurons especially the cholinergic neurons on colonic secretion.

4. About the notes in illustrations

Dear editor Qi, according to the "Guidelines for Manuscript Preparation and Submission: Basic Study", we have added the superscripted alphabetical letterings "a" "b" to replace the "*" "#", which was used to denote statistical significance in the figure.

5. The other marked revisions are mainly about the language editing, which are not listed here one by one.