Manuscript ID: **31548**

Manuscript title: **Corticotropin-Releasing Factor Stimulates Colonic Motility via Muscarinic Receptors in the Rats**

Dear Editor in Chief,

Thank you for giving us the opportunity to revise and resubmit this manuscript.
We have revised the manuscript based on the comments of the reviewers, and it has certainly benefited from the insightful suggestions. Accordingly, we have uploaded the manuscript marked in red with all the changes made during the revision process.

Appended to this letter is our point-by-point response to the reviewers' comments. We would like to take this opportunity to express our sincere thanks to the reviewers who identified areas of our manuscript that needed corrections or modification.

All the authors declare that the work has not been published before (except in the form of an abstract or as part of a published lecture, review or thesis) - that the work is not under consideration elsewhere - that copyright has not been breached in seeking its publication – and that the publication has been approved by all the co-authors and responsible authorities at the institute where the work has been carried out.

We hope that the revised manuscript will now be accepted for publication in your Journal.

Sincerely,

Sei Jin Youn, MD Department of Internal Medicine Chungbuk National University College of Medicine 410 Seong bong-ro, Heungduk-gu, Cheongju, Chungcheongbuk-do 361-711, Korea Tel: +82-43-269-6357 Fax: +82-43-273-3252 E-mail: sjyoun@chungbuk.ac.kr

Comments to Author (required)

Reviewer #1

The theme of this paper is interesting; however, there are several issues that I believe should be addressed.

Major comments

I think it is necessary to explain the motility index in detail and to contextualize its pitfalls, since the number of contractions can interfere with presented values. In this context, the presentation of the frequency data is fragile (only in discussion) and not supported by other studies. Please verify that and discuss appropriately.

Response) We appreciate these comments, and have modified the METHODS section as follows: Motility indices (MI) were automatically calculated with the Medical Measurement Systems computer program (ML846 Powerlab 4/25, AD Inc., Australia) by multiplying the amplitude of each contractile wave by its recorded duration for the last 5 min of each 15 min interval, and were expressed as percentage changes over basal level.

We have also added the following to the DISCUSSION section: It is possible that the number of contractions on the tracings affected the values of MI obtained. However, MI was automatically measured with the Medical Measurement Systems computer program and expressed as percentage change over basal levels.

Comments) *Also, the bibliography should be updated and strengthen the discussion enormously.*

Response) We have added the 6 references and updated the bibliography, and we have changed about one third of the DISCUSSION SECTION in the revised

manuscript as follows:

For our experiments, we used the isolated rat colon, which may be an optimum model for investigating colonic motility.[12,16] Although the isolated bowel is free from control by the autonomic nervous system, the enteric nervous system and regulatory systems (endocrine, paracrine) remain functional.

The primary outcome of this study was the motility index (MI) at each concentration of CRF. CRF increased the motility index and tended to exert a more potent influence on the distal colon than the proximal colon, although the difference was not statistically significant. It is possible that the number of contractions on tracings affected the values of MI obtained. However, MI was automatically measured with the Medical Measurement Systems computer program and expressed as percentage change over basal levels.

Although the mechanical responses induced by infusion of CRF tended to be more potent in the distal colon in this study, a concentration-dependent MI increase was only observed in the proximal colon, and the correlation coefficient R was quite low. Since it is known that the CRF gene is more highly expressed in the distal rat colon,[17] a more vigorous response might have been expected in the distal colon. However, in our experiments, the difference in mechanical response to CRF between the distal and proximal colon was not significant, perhaps because of the small number of mice. Another explanation for the absence of a difference and the modest correlation between dose and response may have been a decline in CRF action at the highest doses in the distal colon due to an inhibitory effect of the CRF-R2 receptor [18]

Fast rhythmic activities at around 15-20 per minutes in our experiments were recorded in tracings of both the proximal and distal colon before administration of the test drugs. These activities seemed not to be influenced by phenotolamine, propranolol, hexamethonium, atropine or tetrodotoxin (Figs 5 and 6). Therefore, we speculate that they may originate in the stellate interstitial cells of Cajal near the myenteric border.[19]

Comments) *The number of animals (N) is extremely small and is not clear in the text. I*

think it was because protocol was extremely difficult; however the information about N should be clear for each group.

Response) We have now given the number of rats in the METHOD SECTION and in each figure as N= *.

Comments) *Some differences between distal and proximal colon may not have been significant because of N. Please clarify that.*

Response) We agree and have added this comment to the DISCUSSION SECTION as follows: Since it is known that the CRF gene is more highly expressed in the distal rat colon,[17] a more vigorous response might have been expected in the distal colon. However, in our experiments, the difference in mechanical response to CRF between the distal and proximal colon was not significant, perhaps because of the small number of mice. Another explanation for the absence of a difference and the modest correlation between dose and response may have been a decline in CRF action at the highest doses in the distal colon due to an inhibitory effect of the CRF-R2 receptor [18]

Comments) *The R although significant is low indicating only a moderate correlation. Please clarify and explore that.*

Response) We agree with this comment and have added the following to the DISCUSSION SECTION:….. and the correlation coefficient R was quite low. Since it is known that the CRF gene is more highly expressed in the distal rat colon,[17] a more vigorous response might have been expected in the distal colon. However, in our experiments, the difference in mechanical response to CRF between the distal and proximal colon was not significant, perhaps because of the small number of mice. Another explanation for the absence of a difference and the modest correlation between dose and response may have been a decline in CRF action at the highest doses in the distal colon due to an inhibitory effect of the CRF-R2 receptor [18]

Minor comments

Review unnecessary statements such as in line 2 on page 11, confused statements such as in

lines 7 and 17 on page 6, without references as in line 12 on page 5 or with insufficient references and discussion such as in lines 1 and.

Response) We apologise for these statements; we have deleted line 2 on page 11 and made the statements clearer. We also deleted line 7 on page 13 and line 23 on page 12.

Comments) *without references as in line 12 on page 5*

Response) We have added the references as follows: Accordingly, CRF has emerged as a key mediator of functional bowel disorders and the effects of stress and inflammation on the gastrointestinal (GI) tract.[6] [7]

6 Larauche M, Kiank C, Tache Y. Corticotropin releasing factor signaling in colon and ileum: regulation by stress and pathophysiological implications. *J Physiol Pharmacol* 2009; **60 Suppl 7**: 33-46

7 Kiank C, Tache Y, Larauche M. Stress-related modulation of inflammation in experimental models of bowel disease and post-infectious irritable bowel syndrome: role of corticotropin-releasing factor receptors. *Brain Behav Immun* 2010; **24**: 41-48

Comments) *The sequence of presentation of the figures is very strange. On page 10 we jump from figure* 2 to figure 5. Please clarify.

Response) We have re-arranged the sequence of presentation of the figures on page 10 so that it is sequential.

Comments) *There is exceeding comma in line 4 on page 5.*

Response) We have deleted the comma.

Comments) *In spite of having understood the objective of showing the doses in the control CRF of figures 5 and 6, this differentiated presentation disrupts the comparative visualization. I suggest standardization or appropriate inserts.*

Response) We regret that we feel unable to accept this suggestion: if we changed the figures to allow comparative visualization as suggested, the number of figures

needed would be increased. Please excuse our reluctance in view of the objective of Figures 5 and 6.

Reviewer #2

Comments) *The paper is very interesting. I suggest you also mention other intestinal diseases: eg constipation, intestinal inflammation*

(Cirillo C, Capasso R. Constipation and Botanical Medicines: An Overview. Phytother Res. 2015 Oct;29(10):1488-93;

Pagano E, Capasso R, Piscitelli F, Romano B, Parisi OA, Finizio S, Lauritano A, Marzo VD, Izzo AA, Borrelli F. An Orally Active Cannabis Extract with High Content in Cannabidiol attenuates Chemically-induced Intestinal Inflammation and Hypermotility in the Mouse. Front Pharmacol. 2016 Oct 4;7:341.

Borrelli F, Romano B, Petrosino S, Pagano E, Capasso R, Coppola D, Battista G, Orlando P, Di Marzo V, Izzo AA. Palmitoylethanolamide, a naturally occurring lipid, is an orally effective intestinal anti-inflammatory agent. Br J Pharmacol. 2015 Jan;172(1):142-58.

Capasso R, Orlando P, Pagano E, Aveta T, Buono L, Borrelli F, Di Marzo V, Izzo AA. Palmitoylethanolamide normalizes intestinal motility in a model of post-inflammatory accelerated transit: involvement of CB? receptors and TRPV1 channels. Br J Pharmacol. 2014 Sep;171(17):4026-37.

Response) We appreciate the suggestion, and would have liked to follow it. However this article focuses on corticotrophin releasing factors, so we regret that we are unable to cite the articles suggested.

Reviewer #3

Comments) *Congratulations! Very well written manuscript and well done basic research study.*

Response) We appreciate the comment.