"**CARBON MONOXIDE CONTRIBUTES TO CONSTIPATING EFFECTS OF GRANISETRON IN RAT COLON**" by Nacci et al. (**WJG ms code#28018)**

**REVIEWERS' COMMENTS AND REPLIES BY THE AUTHORS**

**Reviewer's code:** 03647717

Comments to the Author Re: Carbon monoxide contributes to constipating effects of granisetron in rat colon
Dear sir, thank you very much for your effort to describe this manuscript**.**

*We thank the Reviewer for his/her positive comments and appreciation of our work.*

1. MATERIALS AND METHODS: Mention about age of rat.
*Authors' reply: As requested, the age of rats has been added in the Materials and Methods section*

2. MATERIALS AND METHODS: You should describe meaning of abbreviations of i.p., s.c..
*Authors' reply: As requested, the abbreviations i.p. (intraperitoneal) and s.c. (subcutaneous) have been explained at first mention in the abstract and in the main body of the manuscript.*

3. Result: Following sentences are not Result. Put it in Introduction. ? In line with our previous study[9], acute administration of granisetron increased the time to first defecation. ? In a previous work we did not observe any significant effect of granisetron at concentrations of 0.1, 0.3 and 1 μM[9]; therefore, a 3 μM concentration of granisetron was chosen for the present investigation.
*Authors' reply: In our mind, the sentence "In line with our previous study, acute administration of granisetron increased the time to first defecation" was supposed to link our present results obtained in vivo with those observed in the past; therefore, we feel it is important to leave the sentence in the Results section. However, for better clarity, we have rephrased it as follows: "Consistent with results obtained in our previous study[9], acute administration of granisetron increased the time to first defecation".*
*On the other hand, the sentence "In a previous work we did not observe any significant effect of granisetron at concentrations of 0.1, 0.3 and 1 μM[9]; therefore, a 3 μM concentration of granisetron was chosen for the present investigation." has been moved to Introduction section as suggested by the reviewer.*

4. Illustrate relation of HO/CO pathway, Ach, granisetron and other elements with figures.
*Authors' reply: We thank the Reviewer for this suggestion. To increase the comprehension of the work, we have now included a new figure (Fig. 10) illustrating the relations between HO/CO pathway, ACh and granisetron*

**Reviewer's code:** 00699919

*We thank the reviewer for his/her thoughtful analysis of our manuscript and for his/her important proposals and comments*

Major comments

1. What are the effects of the granisetron/ZnPPIX treatment within the serotonergic system? Is the colon response to granisetron/ZnPPIX treatment related to changes in the serotonergic system?

*Authors' reply: We agree with the Reviewer concerns related to the likelihood that the observed effects might depend, at least partially, on interferences with the serotoninergic system. Future studies will be planned to address this possibility, which at the moment is beyond the scope of our current investigation. However, although our approach does not allow to completely exclude this possibility, the results obtained in the present study strongly suggest that the constipating effect of granisetron is only indirectly affected by ZnPPIX, which acts through reduction of EFS-induced acetylcholine release. This limitation is now clearly indicated in the discussion.*

2. It seems necessary to study if alleviation of granisetron-induced constipation does not lead to reduced antiemetic effects of this drug.

*Authors' reply: Although the Reviewer raises an important point, studies evaluating the antiemetic effect of a drug would require a specific animal model and a completely different experimental approach, that are both not available for us at this time. However, our perception is that alleviation of granisetron-induced constipation does not interfere with its antiemetic potential, since this last effect depends on granisetron activity at the CNS site. On this regard, it has been reported that ZnPPIX does not cross the blood brain barrier (Li X and Clark JD, Anesth Analg 90: 677 – 682, 2000; Wu L. and Wang R., Pharmacol Rev 57: 585 – 630, 2005). Thus, it is plausible that the effects of ZnPPIX to reduce granisetron-induced constipation are related to peripheral mechanisms not involving the CTZ.*

3. Gastrointestinal transit (GIT) should be measured with the use of a nonabsorbable, colored marker given intragastrically. The method to measure GIT used by authors is obviously erroneous.

*Authors' reply: We do agree with Reviewer that intragastric administration of non-absorbable, colored marker is a widely used method to measure gastrointestinal transit (GIT). On the other hand, administering a non-absorbable marker by gavage would increase the stress in animals already subjected to administration of drugs by the same procedure. On this regard, a recent paper suggests that evaluation of the time between the introduction of colored* ***food*** *and the observation of the colored fecal pellet may represent an alternative, reliable non-invasive method evaluating transit behaviour in rats (Bove G, J Pharmacol Toxicol Methods 2015, 74: 1-6). To evaluate the modulating effects of drugs acting on CO/HO system, we considered that in rat administered with granisetron, due to its constipating effect, the time to first defecation was statistically increased with respect to rats administered with vehicle. In other words, treatment with granisetron was considered a positive control on the parameter "time to first defecation" after food ingestion. This limitation has been addressed in the discussion.*

Moreover, how do the authors include to the statistics the differences in the amount of food consumed?

*Authors' reply: The amount of eaten food was evaluated between groups and no significant difference was observed between treatments. These data have now been included in the Result section.*

In addition, this method does not allow to distinguish differences in the passage through the small intestine and colon.
*Authors' reply: We do agree that this method can give an assessment of whole-gut transit, and not specifically of colon transit. Thus, the sentence "Following drug administration, each rat was monitored every 10 min for 180 min, and the time to first defecation was assumed as an index of colon emptying[24-26]" was rephrased as "Following drug administration, each rat was monitored every 10 min for 180 min, and the time to first defecation was assumed as an index of whole-gut transit."*

4. The 180 min observation to first defecation seems be to shorter due to "ceiling effect" of granisetron (most of the animals do not defecated at this time).
*Authors' reply: We apologize with the Reviewer for the lack of details on this aspect. In a pilot study, we observed that in rats injected with vehicle (in order to mimic the stress of the procedure) the average time to first defecation was between 80 - 110 min (median 105 min; interquartile range 90 – 110; full range 80 – 180). Of the 8 rats included in the experimental set, only 1 rat did not defecate during the observation period of 180 min (Figure 1). Based on these observations, we chose to set the cut-off time at 180 min for the following investigations. These details have now been included in the Methods section.*

These are all fairly general statements, but at least some mechanisms and direct cause - effect data must be added to the manuscript.
*Authors' reply: We thank the Reviewer for this suggestion. To increase the comprehension of the work, we have now included a new figure (Fig. 10) illustrating the relations between HO/CO pathway, ACh and granisetron*

Minor Comments

1. Introduction – phrase (see[15] for references) should be … (see[15] for review)
*Authors' reply: as suggested, phrase (see[15] for references) has been changed to … (see[15] for review)*

2. The NG-nitro-L-Arginine (L-NNA) and atropine effects are not introduced in the Abstract and Introduction sections - please do so.
*Authors' reply: As requested, the NG-nitro-L-Arginine (L-NNA) and atropine effects have been introduced in the Abstract and Introduction sections*

3. Results section should be clarified and rearranged mainly in the aspect of statist
*Authors' reply: Results section has been rearranged in order to make it more clear with particular regard to the statistical presentation of the results.*