

## Reviewer #1: Responses

In their manuscript, QiCheng Chen et al. Report how Xiangbinfang granule enhances gastric antrum motility and provides a link of the drug effect via ICC-IM—establishing how XBF mechanism and possible target cell in the gastric antrum would be beneficial in determining the drug effects in GI motility. Although key results should be explained in more detail and proper conclusions should be based on these observations.

Major points:

1- The paper can be edited to be more concise, and Grammarly corrected

**Response:** Thank you for your question. We will further revise the article to make it more concise and send it to the professional language polishing agency to correct the grammatical errors.

2- In the results section, the authors describe ICC-IM's absence, but figure 1 (ad and C) clearly shows some ICC-IM. The authors should clearly state these results and avoid using the “disappeared” terminology throughout the MS. “In the gastric antrum of W/W<sup>v</sup>, c-Kit immunoreactivity was significantly reduced, the ICC-MY network was sparse, and the ICC-IM disappeared” -The authors should discuss the reduction in ICC-IM that might also produce a functional response in GI tissues, similar to what been observed in the gastric fundus of W/W<sup>v</sup> mice “Responses to Enteric Motor Neurons in the Gastric Fundus of Mice With Reduced Intramuscular Interstitial Cells of Cajal; Sanders et al. J Neurogastroenterol Motil. 2014”

**Response:** Thank you for your question. We will use "significantly reduced" instead of "discovered" according to your review. In this study, the immunofluorescence showed very few isolated ICC-IM expression, they did not form a ICC-IM network structure. We agree with you and will further discuss the role of ICC-IM in antral motility.

3- The depolarization effects of XBF observed in WT, and W/W<sup>v</sup> mutants are not explained in the MS, and how XBF exerts these effects in w/wv animals with reduced ICC-IM? ...this observation argues against the involvement of ICC-IM in mediating the effects of XBF, or possibly XBF could have other targets?

**Response:** Thank you for your question. In this study, the contraction frequencies are different from the slow wave frequencies, that is likely because not all slow waves cause contraction. GI slow waves are mainly produced by ICC-MY, while ICC-IM responsible for the regenerative component of the slow wave. However, due to the lack of ICC-IM network in the antrum of W/W<sup>v</sup>, there is no regenerative component in W/W<sup>v</sup> antrum slow wave. Therefore, XBF can not regulate smooth muscle contraction through neural signals transduction mechanism. In Figure 6, the enhancement of XBF on gastric antrum in W/W<sup>v</sup> mice was significantly reduced. It is suggested that ICC-IM should be involved in the enhancement of antral motility by XBF. Of course, as XBF is a traditional Chinese medicine compound with complex

ingredients, there may be other pathways.

4- The concentration of Atropine is relatively high? Typically 1 microM is sufficient to inhibit muscarinic receptors effectively. An explanation of why to use 0.5 mM?

**Response:** Thank you for your question. As a traditional Chinese medicine for promoting gastrointestinal motility, perfusion of XBF usually leads to contraction of muscle strips and failure of intracellular recording. Even pretreatment with nicardipine to remove action potential, high concentrations of XBF can cause muscle contraction. Therefore, we will choose a larger dose of Atropine to inhibit tissue contraction. It was also reported that 0.5 mm atropine was used for intracellular recording. (Venkova K, Krier J. Postjunctional alpha 1- and beta-adrenoceptor effects of noradrenaline on electrical slow waves and phasic contractions of cat colon circular muscle. *Br J Pharmacol.* 1995 Dec;116(8):3265-73. doi: 10.1111/j.1476-5381.1995.tb15134.x. PMID: 8719806; PMCID: PMC1909195.)

5- Figure 4 is confusing, TTX had no effects on XBF mediated enhancement of gastric antrum motility, but atropine completely blocked these effects. This is puzzling; basically, XBF can indirectly release Ach and mediate its effects on ICC, but TTX treatment failed to support this hypothesis.

**Response:** Thank you for your question. Fig. 4 is the result of intracellular recording, which shows that atropine and TTX can not block the depolarization of slow wave induced by XBF. In strain gauge experiments, pretreatment with TTX, the enhancement effect of XBF on antral motility was weakened, but not completely blocked. We will chart the gastric antrum motion frequency, amplitude and dynamic index histogram before and after administration in Fig. 7, so as to make this part of the results clearer.

6- Although ICC-IM are important factor in regulating the MMC activity, it seems that the drug works via a neuronal pathway rather than directly affecting ICC. Results are confusing and proper explanations in the MS is needed

**Response:** Thank you for your comments. We agree with your opinion. We propose that motilin, enteric nervous system, vagus nerve and ICC-IM participate in the regulation.

7- Figure 5 shows that cyclic MMC-like events in WT animals (describe the propulsion of contents), But the effects of XBF under similar conditions caused increased phasic contractions and not MMC like activity. Can the authors explain the loss of MMC patterns after XBF? Is there a possibility that depolarization by XBF can cause an increase in the contractile status and make it harder to propel content?

**Response:** Thank you for your question. After treatment with XBF, gastric antrum causes high amplitude and high frequency contraction. In terms of frequency and amplitude, it is a MMC III like contraction. However, because this is only a single site record, it can not well describe the propulsion of gastrointestinal movement, which is

also the biggest regret of this paper. Strong and coordinated gastrointestinal movement can push gastric contents to the distal end. Our previous experiments in beagle dogs also showed that XBF can promote the coordinated movement of gastroduodenum.

(CHEN Zhiqiang, CAO Lixing, SHANG Wenfan, et al. The Effect of Xiangbin Fang on Gastrointestinal Motility of Dogs after Abdominal Operation[J]. J Tradit Chin Med. 2015, 56(22), 1953-1957. DOI:10.13288/j.11-2166/r.2015.22.018 (in Chinese)

) .

## Reviewer #2: Responses

This is a very interesting study. I will make comments to increase the value of the paper.

1. Abstract. Please use ICC-MP and not ICC-MY as has always been done by Komuro, Thuneberg, Fauussone Pellegrini, all the big names in ICC research.

**Response:** Thank you very much for your comments. I have changed all ICC-MY to ICC-MP in the revised version.

2. Abstract methods. Not train, but strain.

**Response:** Thank you for your comment, I will go over the whole text again and correct the spelling mistakes.

3. Resting membrane potential decreased

**Response:** Thank you for your comment, I have corrected all the same mistakes in the article.

4. XBF did not depolarize slow waves, it depolarized smooth muscle cells

**Response:** Thank you for your comment, I have corrected all the same mistakes in the article.

5. Similarly

**Response:** I have corrected the spelling error in the paper.

6. Why is the ICC-IM the cause? No direct evidence, please discuss more the interactions between vagus and ICC-IM and MMC (Hirst et al., 2002), and see below.

**Response :** Thank you. Based on your comments and related references. In this study, the Conclusion is "ICC-IM participates in the regulation of gastric antrum MMC in mice." We also discussed the interactions between vagus and ICC-IM and MMC. In "Discussion", we wrote "ICC-IM were also innervated and provided mechanisms of neural signals transduction to the gastric musculature[16]. ICC-IM were densely innervated by excitatory and inhibitory enteric motor neurons and in close contact with nerve terminals. ICC-IM played a role in both nitrergic inhibitor and cholinergic excitatory motor neurotransmission in the gastric antrum[17-18]. EJP

and IJP after intrinsic nerve stimulation were greatly attenuated in W/W<sup>v</sup> antrum, the reduced density of ICC-IM leads to reduced neural regulation in W/W<sup>v</sup> antrum. Hirst[19] have shown that excitatory vagal stimulation response resembles the regenerative response which is initiated in this tissue by ICC-IM. Regenerative responses were the dominant responses produced by neural stimulation, It suggested that ICC-IM is regulated by vagus nerve.”

7. The MMC is, as you correctly write, an interdigestive motor pattern. Important references here are Szurszewski (Szurszewski, 1969) and Diamant and Scott (Diamant & Scott, 1987), who recorded the MMC in rats. See also min (Rodriguez-Membrilla et al., 1995). I think that you are the first to record the MMC in the mouse. The true MMC you record in Figure 5A is really beautiful and happens every 15 min or so. You should carefully calculate all parameters of this MMC in all your wild type and WW<sup>v</sup> animals. I think this is the first really good demonstration of the MMC in wild type and WW<sup>v</sup> mice. Please compare with the two studies in rats . You will see that inside phase III of the MMC in rats and other animals we see a minute rhythm of activity. Unfortunately, Spencer et al and others have also called this minute rhythm “migrating motor complex”, but this is not the real MMC as was originally defined and as you show in figure 5A. So please expand on your description of the MMC as in figure 5A and see what effect XBF has on it.

**Response :**I read the articles of Szurszewski (Szurszewski, 1969) and Diamant and Scott (Diamant & Scott, 1987) carefully. Szurszewski describes the MMC movement of the canine small intestine, while Diamant and Scott use three pairs of bipolar jejunal electrodes spaced 2.5 cm apart and with a jejunostomy tube for motility recording. Therefore, this article will compare with other results of using stress sensors to record rat gastric antrum movement in the paper below.

(Takayama I, Seto E, Zai H, Ohno S, Tezuka H, Daigo Y, Fujino MA. Changes of in vivo gastrointestinal motor pattern in pacemaker-deficient (WsRC-Ws/Ws) rats. Dig Dis Sci. 2000 Oct;45(10):1901-6.

Taniguchi H, Ariga H, Zheng J, Ludwig K, Takahashi T. Effects of ghrelin on interdigestive contractions of the rat gastrointestinal tract. World J Gastroenterol. 2008 Nov 7;14(41):6299-302.

)

8. Page 4. Not pacemaker activity and slow waves. Produce slow waves that function as pacemaker activity

**Response:**Thank you for your reminder, I have corrected all the same mistakes in the article.

9. No one has shown the real MMC in mice except your study. The “migrating motor complex” in mice shown by Spencer et al, shows a rhythmic motor pattern that is not the same as the MMC as described by Szurszewski et al. Please also see

(Malysz et al., 1996) (Huizinga et al., 1995). (Huizinga et al., 1998).

**Response:** Thank you for your comments. I will read these articles carefully and describe the characteristics of MMC in rat gastric antrum.

10. The MMC in the stomach is orchestrated by the vagus (see studies from Nick Diamant), the enteric nervous system and motilin. The ICC-IM are involved in vagal activation of motility (see work from Hirst) so in that way they can influence the MMC. The ICC-IM are also involved in the sensory part vagal activity, see work from Powley. (Chung et al., 1994; Hall et al., 1983; Hall et al., 1986; Powley et al., 2008; Powley et al., 2016)

**Response:** Thank you for your reminder. We totally agree that MMC in the stomach is orchestrated by the vagus, ENS and motilin. In this paper, we discussed the effect of motilin on MMC. We wrote "The MMC has been found to be a complex system that may be regulated by motilin[20], enteric nervous system and vagal nerve. Mondal, et al[21] found that treatment with motilin induced phase III contractions in vivo and in vitro, while motilin antagonists can abolish the occurrence of spontaneous gastric phase III contractions. Their other experiment showed that that motilin-induced gastric contractions were mediated through the myenteric plexus in a vagus-independent manner[22]."

We also discussed the effect of ICC-IM in vagal activation. We wrote "ICC-IM were also innervated and provided mechanisms of neural signals transduction to the gastric musculature[16]. ICC-IM were densely innervated by excitatory and inhibitory enteric motor neurons and in close contact with nerve terminals. ICC-IM played a role in both nitroergic inhibitor and cholinergic excitatory motor neurotransmission in the gastric antrum[17-18]. EJP and IJP after intrinsic nerve stimulation were greatly attenuated in W/W<sup>v</sup> antrum, the reduced density of ICC-IM leads to reduced neural regulation in W/W<sup>v</sup> antrum. Hirst[19] have shown that excitatory vagal stimulation response resembles the regenerative response which is initiated in this tissue by ICC-IM. Regenerative responses were the dominant responses produced by neural stimulation, It suggested that ICC-IM is regulated by vagus nerve."

11. ICC not ICCs, ICC is both single and plural

**Response:** Thank you for your reminder, I have corrected all the same mistakes in the article.

12. ICC do not have synapses, that is reserved for neurons

**Response:** Thank you for your reminder, I have corrected all the same mistakes in the article.

13. Page 10, which parameters were significantly different? For example, was the frequency different or were all parameters different?

**Response:** Thank you for your question. The RMP, amplitude and frequency were significantly different. To avoid ambiguity, We amend the sentence to "The difference of RMP, amplitude and frequency were statistically significant between WT and W/W<sup>v</sup>."

14. A RMP decreases or increases. The cell depolarizes or hyperpolarizes

**Response:** Thank you for your reminder, I have corrected all the same mistakes in the article.

15. If atropine abolishes cholinergic neural activity, that should also be abolished by TTX, right? So I do not quite understand why TTX would not mimic the action of atropine

**Response:** Thank you for your question. This is what we are puzzled. It is difficult to use an accurate dose of TTX in living animals. In the past, TTX only partially blocked the gastrointestinal motility enhancement of XBF in conscious dogs. XBF is a compound of Chinese medicine, but not a single compound. Other substances contained in XBF can promote the motility through other pathways.

16. Strain gauge experiments: the contraction frequencies are different from the slow wave frequencies, that is likely because not all slow waves cause contraction. But the contractions at 2 or 4 cpm are very likely associated with ICC-MP and since they are sparse in WWV mice, they are lower in amplitude and frequency.

**Response:** Thank you for your comments. The frequency of gastric antrum contraction recorded by strain gauger is lower than that of slow wave. We also believe that not all slow waves cause contraction. In W/W<sup>v</sup> mice, the slow wave frequency was higher than WT mice. But Strain gauge experiments shown the lower amplitude and frequency in W/W<sup>v</sup>.

17. Your data on Phase III of the MMC are very interesting and important. Unfortunately you only recorded 30 min, which is a short time for measuring MMC phase III. I hope you sometimes recorded longer and you can tell us about this. You really have to discuss the relationship between ICC-IM and the vagus and the relationship between the vagus and stomach MMC.

**Response:** Thank you for your comments. In fact, we recorded more than 2 hours, but because of the effects of anesthesia, the time of waking up was shorter. Moreover, all MMC was record after full consciousness. We plan to record MMC simultaneously in the stomach and small intestine for a longer time, so as to understand the transmission of MMC in mice. You suggest that we discuss the relationship between ICC-IM, vagus nerve and gastric MMC. We agree with your comments and make a discussion.

18. You also should expand phase III or the MMC and study what kind of contraction

patterns there are inside phase III and Phase II just like Diamant and Scott did in the rat.

**Response:** Thank you for your comments. In “Characteristics of the gastric antrum motility in WT and W/W<sup>v</sup> ”, we describe the contraction patterns of phase III and Phase II.

19. You wrote “The periodic MMC movement turned into a high frequency and high amplitude MMC III phase contraction.” But you did not calculate the specific effects of phase III. Instead you wrote about the fast contractions that are mediated by slow waves. So did the MMC actually disappear and we now see the feeding pattern?

**Response:** Thank you for your comments. In our study, XBF induced a strong and persistent contraction after gavage. These contractions are stronger than that of MMC III phase. At the fasting stage, the duration of III phase in WT mice was about 3min. XBF mediated contraction lasted more than 10 minutes. This is due to the depolarization of slow waves by XBF, resulting in more action potentials. When the effect of XBF disappeared, the antral motility returned to the normal MMC cycle. So what we're seeing now is not the feeding pattern.

20. The page numbers disappeared. Making it difficult to review and comment

**Response:** Thank you for your comments. I'm sorry for the trouble. We will deal with this in the revised version.

21. You wrote: The results indicated that the I phase contraction of MMC occurred in the antrum” . I do not understand this.

**Response:** Thank you for your question. I will modify it to “The results indicated that the contractions were inhibited in the antrum of WT and W/W<sup>v</sup> after intraperitoneal injection of 0.1 mg/kg of atropine”.

22. You wrote: Subsequent treatment with 5 mg of XBF induced the MMC III phase contraction in the gastric antrum of WT mice, which was also observed in W/W<sup>v</sup> mice (Fig. 7B, D),” This is very interesting, so you have to better analyse and quantify the effects of the medicine on phase III of the MMC in all conditions, not just calculate the slow wave driven contractions, although that is also important.

**Response:** Thank you for your question. We will analyse and quantify the effects of the medicine on conditions of gastric antrum in Figure 7.

23. I am not sure about giving TTX, if you gave enough to kill all nervous activity. Should that not kill the animal?

**Response:** Thank you for your question. This is also the biggest difficulty for us to use TTX. TTX can kill animal easily. We can only ensure that the maximum dose of TTX can be used when the animal is alive to achieve blocking effect.

24. In the discussion you wrote that ICC-MP are present in WWv mice 60%, but in the text you say that they are sparse. Please be more precise and consistent throughout the manuscript.

**Response:** Thank you for your question. We will revise according to your opinion to make the article more precise and consistent.

25. You wrote: but no regular contraction and typical MMC movement were found in conscious W/Wv mice.” . I am not sure, you did find MMC phase III and you did record only for 30 min, so maybe the MMC is there but very low frequency. Do not say that WWv mice do not produce MMCs, because you have measured them!!

**Response:** Thank you for your comments. We reviewed the experimental data. In the gastric antrum of W/Wv , the duration of MMC phase III-like was  $123.67 \pm 2.96$  s, the amplitude was  $194.12 \pm 4.76$  mg, and during the 30 min observation, no phase III-like contractions were found twice in the same W/Wv. Compared to WT, the duration and amplitude of MMC III phase of gastric antrum was significantly reduced in W/Wv ( $P= 0.0117$  and  $0.0020$ , respectively), suggested that the MMC is very rare and weak in the gastric antrum of W/Wv mice.

26. You write: “In W/Wv mouse intestine, MMC is enhanced by the inhibition of nitric oxide (NO) synthesis [5].” , but this is not the same MMC that you record. They call it MMC but it is NOT the real MMC, also the minute rhythm in WWv mice is shown to be blocked by nitric oxide synthesis inhibition.

**Response:** Thank you for your comments. We fully agree with you. In the revised version, we wrote: “Spencer et al[5] tried to record MMC in the isolated small intestine of mice. They found the interval between MMCs in mice small intestine was  $5 \pm 1$  min, and the durations of MMC contractions was about 30s. Maybe these periodic contractions are not really MMC. The MMC was regulated by motilin, enteric nervous system and vagal nerve. In vitro, motilin-induced contractions are much less potent than that of in vivo[28]. The complex regulatory system of MMC was not complete in vitro.”

27. In your study, the typical MMC did NOT disappear in WWv mice, it was just not seen very often, for reasons I do not know for sure, one is the short recording time may be.

**Response:** Thank you for your comments. We reviewed the experimental data and found the MMC was very rare and weak in the gastric antrum of W/Wv mice.

28. You wrote: “of gastric antral through the cholinergic pathway of ICC-IM, rather than the enteric nervous system” . Hence it is not rather then, but a combination of ENS and ICC

**Response:** Thank you for your comments. We agree with your opinion. We propose that motilin, enteric nervous system, vagus nerve and ICC-IM participate in the regulation.



29. You wrote: “XBF depolarizes SMCs and initiates an action potential, resulting in a rhythmic MMC III phase contraction of gastric antrum in mice.” You did not show that XBF induces a regular MMC phase III, please make more and better calculations.

**Response:** Thank you for your comments. In our study, XBF induced a strong and persistent contraction after gavage. These contractions are stronger than that of MMC III phase. The duration of III phase in WT mice was about 3min. XBF mediated contraction lasted more than 10 minutes. Therefore, we believe that XBF can mediate the MMC III-like contraction pattern.

30. Figure 4 F, why did slow wave amplitude not decrease when the depolarization happens?

**Response:** Thank you for your question. In Figure J, when the membrane potential is depolarized, the slow wave amplitude has been reduced, and the difference is statistically significant.

31. I do not understand the Y axis of Figure 6B

**Response:** Thank you for your question. The Y axis of Figure 6B Refers to the amplitude index (sum of amplitudes within 20 minutes). We have mentioned in the article "Recording of MMC in the gastric antrum". Calculation of the parameter will attach in Fig 6.

32. This is a very interesting study, and I hope you can make it better because of my comments.

**Response:** Thank you very much for giving us so many valuable comments. You have corrected many conceptual errors for us. The questions you have raised are important to improve this article. We are glad that this article has been reviewed by you.