



ESPS PEER REVIEW REPORT

Name of journal: World Journal of Gastroenterology

ESPS manuscript NO: 12829

Title: CNP signal pathway up-regulated in rectum of depressed rats and the interventional effect of Xiaoyaosan

Reviewer code: 02540150

Science editor: Jin-Lei Wang

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CLASSIFICATION	LANGUAGE EVALUATION	RECOMMENDATION	CONCLUSION
<input type="checkbox"/> Grade A: Excellent	<input type="checkbox"/> Grade A: Priority publishing	Google Search:	<input type="checkbox"/> Accept
<input type="checkbox"/> Grade B: Very good	<input checked="" type="checkbox"/> Grade B: Minor language polishing	<input type="checkbox"/> Existing	<input type="checkbox"/> High priority for publication
<input checked="" type="checkbox"/> Grade C: Good	<input type="checkbox"/> Grade C: A great deal of language polishing	<input type="checkbox"/> No records	<input type="checkbox"/> Rejection
<input type="checkbox"/> Grade D: Fair	<input type="checkbox"/> Grade D: Rejected	BPG Search:	<input type="checkbox"/> Minor revision
<input type="checkbox"/> Grade E: Poor		<input type="checkbox"/> Existing	<input type="checkbox"/> Major revision
		<input type="checkbox"/> No records	

COMMENTS TO AUTHORS

Comments for the authors The paper submitted by Ping Li et al examine the role of CNP/NPR-B pathway on depressed gastrointestinal motility disorders and the interventional effect of xiaoyasan. This is a interesting study but there is an important point that is necessary to emphasize. 1. Real-Time PCR is the benchmark method for detecting and quantifying mRNA expression because it allows sensitive, specific, and reliable results. An increasing number of reports have shown how the accuracy and reproducibility of Real-Time PCR data are closely dependent on appropriate normalization strategies to reduce the noise of the method (Bustin, 2000; Vandesompele, 2002; Huggett, 2005; Hendriks-Balk, 2007; Martino, 2011). Until now, housekeeping genes were adopted from literature as reference genes, i.e., transcripts stably expressed among different samples irrespective of their speci?c tissue-dependent behavior; however, recent studies have shown that the expression levels of traditional housekeeping genes can vary markedly across cells, tissues, metabolic conditions and between experimental treatments, emphasizing the need to adopt alternative reference genes or appropriate strategies for their selection (Schmittgen, 2000; Deindl, 2002; Dheda, 2005; Brattelid, 2007; de Jonge, 2007). The use of at least three reference genes for the correct normalization of Real-Time PCR data has been proposed by Vandesompele et al. (2002), and to date, it is considered the best approach for normalizing Real-Time PCR data. So, it appears to be of crucial importance to provide a set of reference genes specifically selected for the experimental



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conditions chosen, since the use of unvalidated reference genes can generate biased results if their expression is altered in the given circumstances. Following recent guidelines [Vandesompele J, *Genom Biol* 2002; Martino A, *J Biotechnol* 2011], more candidate reference genes, from among the most commonly cited in the literature, must be selected to normalize mRNA expression data obtained by RT-PCR and the geometric mean of the three most stably expressed genes settled must be used for normalization of Real-time PCR results. The use of only B-actin gene is not sufficient. 2. In abstract is necessary to enter the number of animals studied (N=...) 3. Results: The last sentence of the paragraph "The expression of CNP in rectum of depressed rats" must be corrected (higher significantly?)



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Reviewer code: 00290396

Science editor: Jin-Lei Wang

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CLASSIFICATION	LANGUAGE EVALUATION	RECOMMENDATION	CONCLUSION
<input type="checkbox"/> Grade A: Excellent	<input type="checkbox"/> Grade A: Priority publishing	Google Search:	<input type="checkbox"/> Accept
<input type="checkbox"/> Grade B: Very good	<input type="checkbox"/> Grade B: Minor language polishing	<input type="checkbox"/> Existing	<input type="checkbox"/> High priority for publication
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<input type="checkbox"/> Grade E: Poor		<input type="checkbox"/> Existing	<input type="checkbox"/> Major revision
		<input type="checkbox"/> No records	

COMMENTS TO AUTHORS

Please see attached pdf file.

Li, Cai, Lin, Zhu, Guo

CNP signal pathway up-regulated in rectum of depressed rats and the interventional effect of Xiaoyaosan

In a rat model of depression, Li *et al.* have examined the expression of NPR-B mRNA and protein using PCR and Western blotting and immunohistochemistry (IHC); and CNP mRNA and protein using PCR and IHC in rectal tissue. The effect of Xiaoyaosan (XYS) as a 'natural product' and apparent mixed herbal agent, was examined on such expression. Data are reported to show that NPR-B/CNP are upregulated in rectal tissue of the depression model and that YYS reduces CNP expression. The authors thus conclude that YYS may improve digestive disorders via CNP effects.

There are a number of issues with this manuscript that should be addressed, as;

General.

1. (minor). The English and grammar is poor in the abstract and reasonable (although still not up to the appropriate standard) throughout the rest of the manuscript. Please correct.

2. (major). For the authors' information and potentially for them to comment on in Discussion/Conclusion, it would have been of significant interest to compare the present rectal NPR-B and CNP expression data with that from the same models, but taken from further up the gut, such as in specific regions of the small and large intestine. Such an observation would lend credence to the potential extent of altered NPR-B/CNP signalling in the gut in general in the rat depression model, as opposed to just the rectum. Indeed, please state why the present study was limited to the rectum?

3. Please clarify the purpose of p. 19-20, 'Comments' text? These appear to be a previous critique of the manuscript and it is not appropriate that they are included in this submission.

Abstract.

4. (major). The English expression and grammar in the abstract need significant correction. In addition, the 'AIM', would be more appropriate as;

'To investigate the distribution and expression of CNP/NPR-B in rectal tissue of a rodent depression model and the effect of Xiaoyaosan'.

Further, please state the hypothesis/es in the abstract.

Introduction.

5. (minor). The Discussion refers to the constituent properties of Xiaoyaosan. Please move this text to the Introduction. Further, if the pharmacological targets (channels, receptors or stores) of Xiaoyaosan or its constituents are known (or hypothesized; with justification), please state what these are (with citation of references).

Methods.

6. (**major**). Regardless of your Institutional approval of experimental protocols in the present study (per statement on p. 6), use of chloral hydrate as an animal anaesthetic is not acceptable. As well as having several problematic issues, it is an irritant, a hypnotic and NOT an appropriate agent for anaesthesia (see for example, Baxter *et al.* 2009 *Anesthesiology* 111:209). **Regardless of whether you and others have published with this agent in the past, do not use it in future work.**

7. (minor). Please state the strain of rat in the 'Animals' section.

8. (minor). Please consider referring to the ‘normal’ group, as the ‘primary control’; as this is less confusing than the present classification. Please also correct the text, ‘Group N was fed normally’ to ‘Primary controls were fed normal chow, *ad lib*’.

9. (major; p. 5, 2nd last line). Please provide brief details on the ‘bondage, swim-induced fatigue, electrical stimulation, fasting and concussion’ methods of animal treatment.

10. (minor; p. 7-8). Please clarify and justify how the doses of Xiaoyaosan were selected? In addition, please briefly state how these compare to the apparent therapeutic dose/s of Xiaoyaosan that patients are given / take? On this latter point, it is noted that the Discussion (p. 17, line 15) states that the dose given to patients ‘has no dosage standard’. This is a problem for publication; and perhaps an issue for others to address, as it reflects a fundamental difference in ‘Western’ and traditional Chinese medicine.

11. (minor; p. 6, lines 7-8). Please clarify what is meant by ‘mental state’ and ‘flexibility’?

12. (minor; p. 6, line 16; and Figure 2, legend). Please clarify what ‘over three jaws crossed into adjacent grids’ refers to?

13. (major; and Results/Discussion). Please clarify what controls for NPR-B and CNP antibody specificity were conducted?

14. (minor; p. 8, ~line 21). Please state what the image analysis software used was, and clarify how the areas examined / quantified were selected? Were they random or selected regions of positive staining? Presumably the positive regions were selected as the DAB staining is brown. Did the software enable selecting the comparative ‘brown’ label and if so, how? My laboratory has published semi-quantitative DAB densitometry recently (in *FASEB J*) and the only way we found that we could do this was to trace the comparative DAB positive regions by hand using ImageJ.

15. (major). Please clarify how many animals were used for each observation? For example, at p. 8, line 20, ‘five groups’ are referred to. Are these the control, M, D, Z, G groups?

Results.

There are currently 17 separate Figures in this manuscript and this is excessive and unnecessary. Having examined the Figures in detail, panels of several Figures are repeated in subsequent Figures and this is not appropriate. In this light the Results section needs to be reordered and rewritten. Some suggestions to correct this issue are detailed below.

16. (minor). Figure 2. Please add ‘A’, ‘B’, and ‘C’ to the respective Figure panels.

17. (minor). Figure 3 (also Figures 6, 8, 11, 13, 16). Please add arrows / labels to the respective immunohistochemistry (IHC) panels indicating the respective tissue / cell layers shown in the panels, such as the smooth muscle, mucosal / serosal layers, granular cells, and where the positive staining is located.

18. (major). Please combine the data in Figures 3, 8 and 13. At present Figures 8 and 13 are a repetition of part of Figure 3 and this is inappropriate.

19. (major). Please combine the data in Figures 4, 9 and 14. At present Figures 9 and 14 are a repetition of part of Figure 4 and this is inappropriate.

20. (major). The example Western blot bands shown in Figures 5, 10 and 15 are very dense with sharp edges and thus appear to have been altered (via image processing software). Presumably such alteration was done consistently between bands and if so, that is acceptable, but this needs to be stated in the respective Figure legends. Please correct.

Further, please include examples of full lane data, inclusive of molecular weight markers for NPR-B; noting that it is not necessary to provide such full lane data for actin.

In addition, only NPR-B blotting data is presented. Please clarify why CNP blot data was not included? If this was due to issues with the antibody (which are common and acceptable), please state this.

21. (major). Please combine the data in Figures 6, 11 and 16. At present Figures 11 and 16 are a repetition of part of Figure 6 and this is inappropriate.

22. (major). Please combine the data in Figures 7, 12 and 17. At present Figures 12 and 17 are a repetition of part of Figure 7 and this is inappropriate.

23. (major; p. 11; 2nd and 3rd paragraphs). Criteria for the depression model should be in the Methods and not the Results. Please correct.

24. (very minor; p. 11; 4th paragraph). Please round off rat weights to whole numbers.

25. (minor; p. 11; last paragraph). Similar to point 23, above, please state that the DAB signal examined was a 'brown' label in the Methods and not the Results.

26. For noting only. It is interesting that NPR-B mRNA data were apparently consistent with NRP-B IHC and blotting protein data. Such a clear-cut positive correlation is unusual.

27. (major; p. 15). The stated conclusion that XYS 'inhibited the expression of NPR-B in a dose-dependent manner' is not appropriate. Such terminology is only appropriate on a dose-response curve, and given that only three concentrations of XYS were examined, it is not possible to construct such a curve. It may be appropriate to state that the higher dose examined produced the largest effect, but this is not a dose-dependent effect, per the accepted pharmacological definition. Please correct.

Discussion.

28. (major). **i.** In the 1st paragraph of the Discussion, please summarize your major / primary findings. **ii.** In the subsequent paragraph/s, please discuss these findings in the context of the specific literature on the topic. **iii.** After that, please relate your findings to the more general aspects of the topic.

At present the Discussion starts with **iii.**, above, and this is not appropriate. Please correct.