August 27, 2014

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 12829-review.doc).

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

Author: Ping Li, Xu-Dong Tang , Zheng- Xu Cai , Juan-Juan Qiu , Xue- Lian Lin , Tong Zhu , Lawrence Owusu , Hui- Shu Guo

Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 12829

The manuscript has been improved according to the suggestions of reviewers:

1 Format has been updated

2 Revision has been made according to the suggestions of the reviewer (We added line numbers and highlighted revision with yellow color in updated submission for reading conveniently)

The revision according to the suggestions of the first reviewer:

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 specify 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 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.

Answer: Thank you so much for your precious opinion. We must choose at least three stably reference genes in later experiment. Due to limited time, we added GAPDH as another reference gene. The result is in accordance with using β-actin as housekeeping gene. (Figure 4B and Figure 7B)

2. In abstract is necessary to enter the number of animals studied (N=…)

Answer: Thank you so much. I have added the number of animals studied (N=45) in abstract. (page 2, line 34)

3. Results: The last sentence of the paragraph “The expression of CNP in rectum of depressed rats” must be corrected (higher significantly?)

Answer: I am so sorry. It is slip of the pen. I have corrected it. (page 12, line 344-345)

The revision according to the suggestions of the other reviewer:

1. 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.

Answer: Thank you for your precious advice. Our manuscript has been revised carefully by a native English speaker, especially abstract part.

2. 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?

Answer: A large number of clinical cases showed that depression is often associated with gastrointestinal discomfort. So we choose the gastrointestinal tract as aimed parts, including the stomach, jejunum, ileum, colon, rectum and so on. In addition to the rectum, observation of other parts of the digestive tract will be further reflected in the subsequent study.

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.

Answer: I am so sorry about this mistake. It is slip of the pen. I have omitted it.

4. 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.

Answer: Thank you so much. According to your suggestion, the expression of AIM in abstract will be'To investigate the distribution and expression of CNP/NPR-B in rectal tissue of a rodent depression model and the effect of Xiaoyaosan'. (page 2, lines 32-33). Moreover, I state the hypothesis in the conclusion of the abstract (page 2,

lines 54-56)

5. 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).

Answer: Thank you so much for your suggestion. I have moved the text about Xiaoyaosan to the introduction according to your opinion. (page 5, lines 131-134) In addition, I had cited a reference about constituents and the pharmacological targets of Xiaoyaosan (Zhang Y, Han M, Liu Z, Wang J, He Q, Liu J. Chinese Herbal Formula Xiao Yao San for Treatment of Depression: A Systematic Review of Randomized Controlled Trials. Evidence-Based Complementary and Alternative Medicine.2012; 2012: 931636).

6. 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.

Answer: I am so sorry. The rats were sacrificed immediately after anesthetized. And the chloral hydrate will not be used as an animal anaesthetic again in future work.

7. Please state the strain of rat in the ‘Animals’ section.

Answer: Thank you so much for your suggestion. I had added the strain of rat in the‘Animals’section already. (page 6, lines 160)

8. 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’

Answer: Thank you very much. I have used ‘primary control’ instead of ‘normal

group' throughout the manuscript. Besides I amended it according to your suggestion. (page 2, lines37; page 6, lines 169)

9. Please provide brief details on the 'bondage, swim-induced fatigue, electrical stimulation, fasting and concussion' methods of animal treatment.

Answer: I have added some detail about the chronic mild unpredictable irritations methods.(page 6, lines 176-183)

10. 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.

Answer: Thank you for your question. Xiaoyaosan is a prescription, the therapeutic dose that patients are given has been proved over a long period in clinical practice. The therapeutic dose of Xiaoyaosan given to 70kg patients is 170g/d. (李晶晶,李晓红,王少贤,白明华,陈家旭. 逍遥散对肝郁脾虚证实验大鼠的治疗作用。吉林中医药杂志 . 2009 年 10 月第 29 卷第 10 期.) The dose conversion formula between 70kg human and 200g rat is A=KB，K=0.018. So the therapeutic dose given to rat is 15.3g/kg. On the basis, increase or decrease in doubling the xiaoyaosan dose of 15.3g/kg to set different dose gradient,7.65g/kg, 15.3g/kg, 30.6g/kg.

11. Please clarify what is meant by 'mental state' and 'flexibility'?

Answer: 'mental state'has been replaced with "mood" and 'flexibility'also replaced with irritability to reflect how the rats responded to stimuli (sound and/ or touch in this experiment). (page 7, line 191-192)

12. Please clarify what 'over three jaws crossed into adjacent grids' refers to?

Answer: I am so sorry. It is slip of the pen. The "jaws" should be "paws", I have

amended it. We want to know how many squares does the rat crossed, but only when all paws of rat crossed the line between two grids we counts a number.

13. Please clarify what controls for NPR-B and CNP antibody specificity were conducted?

Answer: NPR-B and CNP antibodies are purchased from Biosynthesis Biotechnology who has verified their specificity for antigens. They are rabbit polyclonal IgG purified by affinity.

14. 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.

Answer: The staining index was calculated with the staining intensity and area according to the quantitative method (page 9, line 258-265) used by the reference (Di Martino E, Wild CP, Rotimi O, Darnton JS, Olliver RJ, Hardie LJ. IGFBP-3 and IGFBP-10 (CYR61) up-regulation during the development of Barrett's oesophagus and associated oesophageal adenocarcinoma: potential biomarkers of disease risk. Biomarkers 2006; 11: 547-561) instead of any image analysis software. The areas quantified were selected randomly. I have read the semi-quantitative DAB densitometry method in your paper, and we would like to try your quantitative method in my further studies.

15. 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?

Answer: A total of 45 rats were used for the whole experiment. Five groups are referred to N, M, D, Z and G (The capital letters represent primary control group,

depression model group, Low, Middle, High dose XYS groups)

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

Answer: I have added ‘A’, ‘B’, and ‘C’ to the respective Figure panels according to suggestion. (Figure 2)

17. 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.

Answer: Thanks for your suggestion; I have added the arrows to the respective panels. (Figure 3 and Figure 6)

18. 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.

Answer: Thanks for your suggestion, I have combined the figures. (Figure 3)

19. 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.

Answer: Thanks for your suggestion, I have combined the figures. (Figure 4)

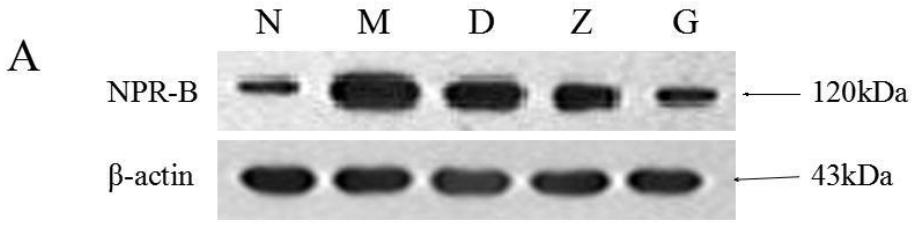
20. The example Western blot bands shown in Figures 5, 10 and 15 are very dense with sharp edges and thus appear to have been altered (via image processing software). Presumably such alteration was done consistently between bands and if so, that is acceptable, but this needs to be stated in the respective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

Answer: Thank you so much for your precious opinion. The western blotting pictures

used before have been altered via image processing software (Adobe Photoshop CS3) to get a cleaner background. This alteration was done consistently between bands and could make the bands looks very dense with sharp edges. In order to avoid misunderstandings, we showed original image without modification via processing software; (Figure 5, page 26, line 763-765)



We had added examples of full lane data to illustration. (page 26, line 761-763)

We also detect the expression using western blotting, but the data doesn't reach our expectation. The possible reason may be that CNP precursor (proCNP) is cut to CNP-53 under the action of intracellular protease. In some tissues, CNP-53 is decomposed to CNP22 (Wu C, Wu F, Pan J, Morser J, Wu Q. Furin-mediated processing of Pro-C-type natriuretic peptide. J Biol Chem. 2003;278:25847-52; Yeung VT, Ho SK, Nicholls MG, Cockram CS.Binding of CNP-22 and CNP-53 to cultured mouse astrocytes and effects on cyclic GMP. Peptides.1996;17:101-6). The CNP antibody that we purchased may be not specific to antigen. We will explore western blot experimental conditions of CNP in futher research work.

21. 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.

Answer: Thanks for your suggestion, I have combined the figures. (Figure 6)

22. 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.

Answer: Thanks for your suggestion, I have combined the figures. (Figure 7)

23. Criteria for the depression model should be in the Methods and not the Results. Please correct.

Answer: I am sorry for slip of the pen. These two paragraphs (page 11; 2nd and 3rd paragraphs) are the record results of depression rat model instead of Criteria for the depression model. The sentence "Criteria for the depression model" is inappropriate, I have omitted it.

24. Please round off rat weights to whole numbers.

Answer: Thank you so much. I revised weights according to your suggestion. (page 11, lines 312-313)

25. Similar to point 23, above, please state that the DAB signal examined was a 'brown' label in the Methods and not the Results.

Answer: Thank you so much. I have amended it according to your suggestion. (page 9, line 257-258)

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.

Answer: Thank you for your agreement.

27. 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.

Answer: Thank you so much. I have amended it according to your suggestion in the manuscript.

28. 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.

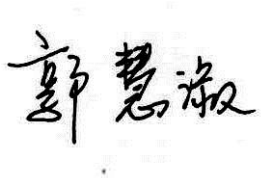
At present the Discussion starts with iii., above, and this is not appropriate. Please correct.

Answer: Thank you so much for your suggestion. I have amended the discussion according your opinion.

3 References and typesetting have been corrected

Thank you again for publishing our manuscript in the *World Journal of Gastroenterology*.

Sincerely yours,



Hui- Shu Guo, PhD

Central Laboratory,

The First Affiliated Hospital of Dalian Medical University, Dalian 116011, Liaoning Province, China.

Fax: +86-411-8362-2844

E-mail: guohuishu1@126.com