Format for ANSWERING REVIEWERS

March 31, 2015

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 16030-review.doc).

Title:TLR4–HMGB1-, MyD88- and TRIF-dependent signaling in mouse intestinal ischemia/reperfusion injury

Author: Wang Jie, He Gui-zhen,Wang Yu-kang, Zhu Qian-kun, Chen Wei,Guo Tai

Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 16030

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

(1) As a scientific paper, it's really unprofessional for there was no page number. However, the study is overall well done, but the authors should clarify the reasons for choosing antibodies of HMGB1, MyD88 and TRIF but not inhibitors, for there are lots of efficient inhibitors of these proteins used in research. Is there any drug used in clinical to down-regulate HMGB1/TLR4/NF-κB pathway?

We are quite sorry for the negligence of page numbers and we have added the page number. Considering the stronger specificity of antibodies and therefore its better effect than inhibitors, we have chosen the antibodies rather than the inhibitors. We hope our experiment could provide the future clinical research with some possibilities to find target drugs related to the effects of HMGB1, MyD88, TRIF. To our knowledge, there is no drug used in clinical to down-regulate the HMGB1/TLR4/NF-κB pathway until now.

(2) A difference has been shown histologically and via measurements of inflammation. Do these differences manifest clinically. i.e. is there a survival difference?

To this end, there is no clinical research in this direction. Because of our limited amount of money, we did not do any survival experiment, and may conduct some researches in the future if possible. Thanks for your sincere suggestion!

(3) The exact site of lung and liver tissue collected for paraffin embedding was not mentioned in the Materials and Method

The exact site has been mentioned in the revised version. We really appreciate your concern.

(4) The source, location, purity of anti-HMGB1, -TRIF, - MyD88 should be provided.

The source, location and purity of anti-HMGB1, -TRIF, -MyD88 has been written in the new version. Thanks for your reminding.

(5) The source, location of SPSS 19.0 software should be provided.

Thanks for your reminding us of writing the source, location of SPSS19.0 software and it has been added to the newly revised article.

(6) Lung injury and intestinal injury should be graded according to the pathological results.

It has been added to the revision.

(7) A description of the method of animal sacrifice is required.

With regard to the description of the method of animal sacrifice, we have added to the new version "All animals were euthanized by barbiturate overdose (intravenous injection, 150 mg/kg pentobarbital sodium) for tissue collection."

(8) Consider a diagram detailing the effect of the pathway and the effect of antibodies on such changes to enhance the reading of the manuscript.

We have added it to the new version.

(9) Please correct " Results were analysed using on-way analysis of variance." “one-way or on-way?” in the last sentence of Abstract-Methods.

Indeed, it is “one-way”, not “on-way”. This is a written mistake.

(10) Please correct " The administration of anti-HMGB1, anti-MyD88, and anti-HMGB1 antibody could each significantly reduce the damage caused by I/R, and the role of anti-HMGB1 antibody was the most obvious" in the last sentence of Discussion.

We have corrected it in the last sentence of Discussion.

(11) This paper provides solid data into the role of TLR4/HMGB1 pathway in regulating intestinal I/R injury. My main concern is about its originality or novelty. It was known that anti-HMGB1 antibody has an effective role in alleviating intestinal I/R injury in rat (Kojima et al., J Surg Res, 2012). Thus, the main contribution of this work is confirmation of this pathway in mice.

As it is mentioned by the reviewer, Kojima et al in 2012 has demonstrated the effects of HMGB1 and its antibody in the model of ischemia-reperfusion injury in rat. However, our novelty and originality is to investigate the two downstream pathways of HMGB1, i.e., the MyD88-dependent pathway and the My88-independent pathway, and to discuss which pathway plays the main role in the model of ischemia-reperfusion injury in mice.

(12) The abstract part should presents the innovative and significant points related to the current study.

For the reason that there is a restriction of the number of words in the abstract, the innovative and significant points are written in the comment and in the introduction instead of in the abstract.

(13) Sufficiently detailed descriptions (manufacturer’s details) is needed for each antibody.

It has been described in the revision.

(14) The activity of GAPDH in the intestine will seriously changed after ischemia reperfusion (Sola et al., GUT, 1999). Is it appropriate to use GAPDH as internal control of qPCR in this study?

By reading some related papers, I admit that to choose GAPDH as internal control of qPCR is inappropriate in this study. After all, the GAPDH content in the intestine will seriously be altered in the state of the ischemia-reperfusion. However, in our experiment we strictly loaded equal amounts of tissue or material in the qPCR. Because that one of the functions of internal control is to indicate that the loading amount is equal and the internal control will be contracted during the calculation of the digits of the results, we sincerely request still put this qPCR results in this article. If it is not allowed, we will consider retracting the results of qPCR.

(15) Pathology score should be provide along with pathological changes.

This problem has been solved in question 6. Please refer to question 6.

(16) Disscussion should be more concise and logical.

It has been revised in the article.

(17) Dear Authors, your manuscript titled as "TLR4–HMGB1-, MyD88- and TRIF-dependent signalling in mouse intestinal ischemia/reperfusion injury", well organized research. But you can give more place to explain oxidative damage of I/R by using other paper which were done on several organs like muscle, urinary bladder, heart, kidney. You can find my few correction on manuscript as attachment. I wish you and your team further achievements.

It is revised according to your suggestion.

(18) Antibody isotype control group(s) are lacking in the experimental design, which is necessary to compare in conducting functional antibody blockade experiments.

This is our negligence. The “vehicle alone” in our experiment is referring to the “vehicle with the

control IgG antibody alone (abcam, Cambridge, UK)". It is corrected in the revision.

(19) Figures 2 and 3 are representative histological images by H&E staining. A more quantification method by a blinded observer is needed for the statement of 'obvious attenuation'.

Please see my answer to question 6.

(20) Table 2 and 3 showed the changes in mRNA of HMGB1, NFkB, MyD88, TRIF in lung and ileum. Why would a neutralizing antibody targeting proteins affect its own mRNA levels? Moreover, it is unreasonable that blocking TLR4-downstream pathways (MyD88 or TRIF) would even decrease the endogenous ligand HMGB1 mRNA levels.

We have seriously considered your sincere suggestion, and carefully scrutinized the results. The reason of this problem is as follows:

1. The neutralizing antibody targeting proteins will not affect its own mRNA levels. HMGB1 can stimulate the secretion of inflammatory factors, such as TNF-α, IL-6, IL-1β, and IL-8; or inflammatory factors secreted after HMGB1 stimulation could promote monocyte/macrophage secretion of HMGB1. Therefore, HMGB1 could form a positive feedback loop to cause inflammatory signal cascade amplification. Blocking the HMGB1-TLR4,MyD88/TIRAP and TRIF/TRAM pathways by injecting anti-HMGB1,anti-MyD88 and anti-TRIF antibodies, respectively, can inhibit the expression of inflammatory factors (e.g., NF-κB, IL-6, and TNF-α), and the reduction of levels of inflammatory factors can in some contexts attenuate levels of HMGB1 expression.

2. It is well known that the PCR technique is quite sensitive. For instance，even a tiny number of tissue contaminations may cause intolerable distinctive results. To this end, certain experimental errors may occur in the middle of the PCR experiment and therefore affect the result of the PCR. However, during the experiment course, we honestly are abiding by the principles of standard PCR process to prevent any contamination. So, we believe that the result of this PCR experiment is likely to be caused by some unknown regulatory mechanisms that are needed to be further explored.

(21)The authors should check the circulating LPS levels following I/R injury, as well as

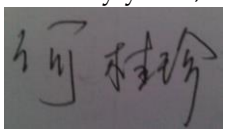
In terms of the LPS levels following I/R injury, it was in previous experiments that we tested the

LPS levels. It was published in *He G Z, Zhou K G, Zhang R, et al. World J Gastroenterol,2012*. Along with the results published by Moore EE(*Damle S S, Moore E E, Nydam T L, et al. J Surg Res,2007. Watkins A C, Caputo F J, Badami C, et al. J Trauma, 2008 Jordan J R, Moore E E, Sarin E L, et al.J Appl Physiol,2008*.), we generally consider that LPS will not play a leading role in the distant organ injury following I/R injury.

3 References and typesetting were corrected

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

Sincerely yours,



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