Author's response to reviews

Title: Isoflurane Preconditioning at Clinically Relevant Doses Induce Protective Effects of Heme oxygenase-1 on Hepatic Ischemia Reperfusion in Rats

Authors:

Xin Lv (xinlvg@hotmail.com)
Liqun Yang (lqyang72721@hotmail.com)
Kunming Tao (tkmgood@163.com)
Yantao Liu (liuyangtao_218986@163.com)
Tian Yang (18917015805@189.cn)
Guozhong Chen (cgzssq2000@sina.com)
Weufeng Yu (ywf808@yeah.net)
Hao Lv (lhmousezhu@yahoo.com)
Feixiang Wu (feixiangwu@yahoo.com.cn)

Version: 3 Date: 8 December 2010

Author's response to reviews: see over
Dear editor:

Re: MS ID : 4576178403558461

Please find attached a revised version of our manuscript “Isoflurane Preconditioning at Clinically Relevant Doses Induce Protective Effects of Heme oxygenase-1 on Hepatic Ischemia Reperfusion in Rats”, which we would like to resubmit for publication in *BMC gastroenterology*.

Your comments and those of the reviewers are much appreciated and have enabled us to improve the quality of the manuscript. In the following pages we have outlined our point-by-point responses to each of the comments.

Revisions in the text are shown using blue highlight. In accordance with the reviewers’ suggestions, we have increased the Znpp group and also the sample size to 12 in each group and, consequently rearranged the statistical analysis and the figures accordingly. We also have using PCR-RT analysis to assess the expression of hepatic TNF-alpha instead of ELISA. Moreover, we assessed MDA activity as a marker for hepatic oxidative stress as suggested. Most of liver injury values were put into a table. Additional more detailed information is listed in the following letter and we shall look forward to hearing from you in due course.

Additionally, I need explain the changes in the list of authors. Since Dr. Tao KM, Dr. Yang T and Dr. Chen GZ finished most parts of the amendment study, and Dr. Chen GZ also give the foundation support for the amendment experiments from his Key Program of Nan Jing Military
District (No.07Z039), China. After discussing with all the co-authors, I decided to add them as
cowithers and also took Dr. Chen GZ as the co-correspondent author with me during this version
of submission.

Yours sincerely,

Wei-feng Yu, M.D., Ph.D.

Department of Anesthesia & Intensive Care, Eastern Hepatobiliary Surgery Hospital,

the Second Military Medical University, 225# Changhai Road, Shanghai, 200438, China

Reviewer 1:

Major Compulsory Revisions

The authors should reinforce in the introduction and objective sets the unpublished purpose of the
study. It was better defined in the abstract set.

Response: We have rewritten the background and reinforced our hypothesis and unpublished
purpose. The unpublished purpose of the study was also added in abstract set as suggest.

The hepatic TNF-alpha may be more representative using a PCR-RT analysis.

Response: We have measured the mRNA level of TNF alpha using RT-PCR and amended the
result in a table and the ELISA result of TNF alpha protein deleted accordingly.

The Heme oxygenase-1 pathway is related with oxidative stress, the authors should investigate
another markers of oxidative stress, as well as, nitrotyrosine, MDA, etc.
Response: The hepatic MDA level was assessed by biochemical kits purchased commercially as suggested (Nan-Jing Jiancheng Biochemicals Ltd, China). The detection range was 0–200 mmol.1⁻¹.

Minor Essential Revisions

There are excessive arrows in the figure 3

Response: Figures were rearranged and excessive arrows deleted.

The figure 7 presented H&E stain with a yellow background

Response: H&E staining photographs with yellow background were replaced with those in red.

Reviewer 2:

Major Compulsory Revisions

1. Effects of intervention on HO-1 enzymatic expression, Results, paragraph 1 and Fig. 1. The effects of IR and IR plus isoflurane compared to sham are relatively small. IR has induced an approximately 1.5-fold increase, and IR plus Iso in approximately 2-fold increase, compared to Sham. Given the magnitude of the error bars, it is not completely clear whether there should be or is a significant difference between IR and Sham. Again, taking account of the size of the indicated error bars, it is not completely clear whether there should be or is a significant difference between IR plus isoflurane and IR. Since this is really the central point of the whole paper, more convincing evidence for these changes should be provided.

Response: Thanks for your suggestions. Due to the limitation of sample size, in our original version, the difference between groups was actually not significant. We have increased the sample
size from 8 to 12, and then performed a statistical comparison between groups, consequently the power increased and statistical significant difference was achieved in HO-1 activity and liver injury values between groups, and updated result including those of Znpp control group were listed in figures and table. Hence, we think there was difference in HO-1 activity and liver injury between isoflurane treated group, IR and Sham groups.

2. Relating further to the above, the effect of Znpp. The bar indicated Iso plus Znpp. The authors claim that Znpp added prior to isoflurane pre-treatment completely blocks the action of isoflurane. It should be made clear in this graph that isoflurane treatment after Znpp involves both isoflurane treatment and IR.

Response: Since all these animals except in sham group subsequently underwent the same IR procedure as the first group. We decide to list the group bars as simple as Sham, IR, ISO, Znpp/ISO, Znpp and Hemin groups throughout this reversed manuscript. There is no control for the effect of Znpp on the effect of IR in absence Iso. Therefore it seems that the authors cannot conclude that Znpp has reduced the isoflurane effect. The decrease in the bar marked Iso plus Znpp could be due to an effect of Znpp on the IR action and not on isoflurane.

Response: We have added the Znpp control groups, and the differences between Znpp and Znpp/ISO and ISO have also been compared to IR. According the updated results, there was no difference between Znpp control, Znpp/ISO and IR. That means pretreatment of Znpp could not alleviative or aggravate liver injury after IR procedure but reduce the protective effect of isoflurane preconditioning.

3. Related further to the above comments, the description of the groups in the Methods under “4.
Experimental groups’ should be stated in list form much more clearly than it presently is. As mentioned, it seems that it would be necessary to add an additional group which is Znpp and IR without isoflurane pre-treatment.

Response: The Znpp control group has been added and the description of groups in Methods been improved.

4. Western blots, Fig. 2, Results, paragraph 2, effect of the different conditions on HO-1 protein experiment analyzed by Western blot. I believe two things are required for these experiments. Firstly, data on the quality of the primary antibody employed in Western blot should be shown with a full scale gel showing the position of the HO-1 band and molecular weight markers. How specific is the antibody? Secondly, the data should be quantitated by measuring the intensity of the spots by image analysis, number of experiments given and mean values for each condition given.

The argument above related to the effects of Znpp and the interpretation of these results also applies for Fig. 2 Western blot, Group D, Iso plus Znpp Group compared to Group C which is IR plus isoflurane.

Response: The western blots have been reanalyzed after adding the Znpp control group and sample size, and all the figures and figure legends were rearranged accordingly after statistical comparison.

5. Fig. 3, immunohistochemistry results and Results text, paragraph 2. The data should be scored quantitatively using an accepted form of scoring for injury (grading score), quantitative results expressed and statistics undertaken. Serafin et al. (2002) Am. J. Path. 161, 587 is one example of a grading system.

Response: The above data were reanalyzed using Image-Pro-Plus® Software (Media Cybernetics
Inc, Bethesda, MD) as below, the necrosis score was determined by dividing the measured necrotic area by the total area of the field, while area density of HO-1 positive tissues was analyzed in 6 random high powered (1× 400) microscopic fields. The figures were also rearranged according to the quantitative and statistics results as suggested.

6. Fig. 4, plasma ALT and AST concentrations, Results, Section 2. The changes in marker enzymes for liver damage, apart from the effect of IR compared to Sham, are small. The trend is there (as for also Figs. 5 and 6), but again the changes are very small. A confirmation on the statistical analysis is important. As indicated by others, in other studies effects of ischemic preconditioning, for example, or intermittent ischemia are not usually revealed as changes in liver marker enzyme concentration until about 6 h post beginning of reperfusion. (The same comment also may apply to histological changes.) With respect to liver marker enzymes, there is no data for effect of Znpp alone. Since Znpp plus Iso increases liver marker enzymes compared to IR plus Iso, it would be important to check that Znpp alone does not increase compared to Sham.

Response: Znpp control group was added and we have responded to the comment #1 and comment #2 from Reviewer #2, and please refer to that.

7. Title and relationship of this study to Reference 16. As indicated by the present authors, Schmidt et al. have previously shown that isoflurane will induce HO-1 in liver. The potential value of the present studies is the effects of isoflurane at clinically-relevant doses. It would be better to change the title to reflect this more explicitly. The term “1 MAC” may not be readily understandable to many readers. Something like “isoflurane preconditioning at clinically relevant doses induces protective effects of HO-1 …”. (There may be better wording.) Also in this connection a paper of Beck-Shimmer et al., Annals of Surgery, 248, December 2008, could also be
cited. This refers to clinical trials and possibly induction of HO-1.

Response: Thanks for your efforts to improve the quality of the title. The title you suggested have now been defined as “Isoflurane Preconditioning at Clinically Relevant Doses Induce Protective Effects of Heme oxygenase-1 on Hepatic Ischemia Reperfusion in Rats” the this version.

Minor Essential Revisions

1. Methods, paragraph 2, hepatic ischemia reperfusion. Rats were anaesthetized with sodium pentobarbital and remain under anesthesia for at least 5 h. Some comment on effectiveness of anesthesia during this time period should be included.

Response: Actually, maintain of anesthesia throughout the entire experiment was: Anesthesia was induced by intraperitoneal injection of pentobarbitone (50 mg.kg-1 body weight, Dainabot, Osaka, Japan) and maintained by repeat doses of 25 mg.kg-1 if necessary, based on animal movement. The above sentence was added in reversed manuscript.

2. Methods, hepatic HO-1 activity assay, homogenisation. More details of ultrasonic homogenisation should be given. Also a reference for measurement of bilirubin, the principle.

Response: the details of method related were amended accordingly.

3. Methods, Section 9, transaminases TNF and myeloperoxidase. Where appropriate, principle of the assay should be given, rather than just saying, for example, “using a kit from ….”

Response: the details of method related principle of the assay were also amended accordingly.

4. Results, paragraph 2, histology, Fig. 3. The statement “based on morphological characteristics HO-1 was expressed mostly in hepatocytes”. This requires more detail in order for the reader to be convinced that HO-1 is actually expressed principally in hepatocytes.

Response: This statement was deleted as it was not closely relevant to our major results and
conclusion.

5. Results, Section 3, effects of intervention on cell morphology, Fig. 7, second to last paragraph before Discussion. The statement “integrity of the overall structure of hepatic lobules were seriously compromised” should be clarified and explained better.

Response: the statement about effects of intervention on cell morphology rectified as: At 4 hrs after reperfusion, the liver histology in IR group exhibited much areas of necrosis and structural derangement around the central vein as compared to sham.

6. In the Discussion, first three paragraphs. To a great extent these paragraphs refer to reasons why the experiments were performed, especially relating to Schmidt et al. reference 16 and the aim of the present study which is to use lower clinically-relevant doses. This material should be expressed more concisely that it is now but in the Introduction.

Response: The objective of performing present study, the using clinical relevant dose of volatile anesthetics was explained in the Introduction.

7. Discussion, paragraph 4, referring to results relating to “As Znpp is not the specific inhibitor of HO-1, it completely blocked the increase of HO-1 activity”. As mentioned, cannot really see clearly from the data the effects of Znpp. Also the differentiation between effects of Znpp on activity of the enzyme which is present in the cells and effects of Znpp on gene expression of the enzyme should be more clearly described. These issues should be made more clear.

Response: According to the results update, we believed that using the inhibitor of HO-1 Znpp, could completely blocked the increase of HO-1 activity and expression compared with isoflurane pretreatment group. The effects of isoflurane on all measures of liver damage were also
completely abolished by Znpp, strongly suggesting that the protective effects of isoflurane were mediated by an increase in HO-1 activity and expression. Effects of pretreatment with the HO-1 inducer hemin produced beneficial effects similar to isoflurane, further indicating a pivotal role of HO-1 in the cytoprotective effects under IR.

The issues have been clearly described in Result and Discussion during the version of submission.

8. Later in Discussion, two paragraphs on “HO-1 ubiquitous enzyme and among the three mammalian isoforms of HO-1 etc”. Should be expressed more concisely in Introduction and Discussion shortened.

Response: rectified accordingly.

9. Second to last paragraph of Discussion, comment that “IR damage through phenomenon called preconditioning”. This should be modified to say “through the same mechanism as preconditioning”. The phenomenon, although it is a form of preconditioning, is not the same.

Response: Rectified accordingly.

10. Fig. 1, for some statistics, there is no symbol described in the legend.

Response: Figures have been rearranged, and the symbols have been described in figure legends.

Discretionary Revisions

1. Methods, Section 7, Western blot, chemiluminescence agents, source should be indicated.

Response: Membranes were again washed as above and then incubated with ECL Plus (Amersham Pharmacia, Piscataway, NJ) and visualized on film.

2. Immunohistochemistry, reference for the principle of measurement of HO-1 immunoreactivity should be given.

Response: Added as below, area density of HO-1 positive tissues was analyzed in 6 random high
powered (1× 400) microscopic fields using Image-Pro-Plus® Software.

3. Methods, Section 8, towards the end of the paragraph, the word “polymerise” in relation to anti-rabbit IgG is probably not correct. Possibly covalently-linked.

Response: the word “polymerise” has been deleted.

4. Discussion, beginning of paragraph 2, the term “hepatic flux” possibly means blood flow?

Response: It is actually the liver blood supply as you postulated, we replaced with it in manuscript.

Level of interest: An article of importance in its field

Quality of written English: Acceptable

Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.

Declaration of competing interests:

I declare that I have no competing interests

Reviewer 3:

In the present study, Win Lv and co-authors investigate the role of preconditioning with isoflurane on Heme oxygenase 1-dependent liver protection during ischemia reperfusion injury.

Major comments:

1.) The authors need to include arterial blood pressure values for both groups. I suspect that the induction and HO-1 protection are induced by hypotension/ischemia - like ischemic preconditioning - by isoflurane induced decrease in blood pressure.

Response: Actually, we have done hemodynamic monitor (MAP, HR) during experiments. After
exposure to light concentration (1.4%) of isoflurane, the MAP and HR did not fluctuate markedly even in ischemia and reperfusion procedure. Moreover, hemodynamic values were not listed in previously studies which even using higher concentration of volatile anesthetics in murine according to. (Ann Surg 2007; 245: 931-42; Anesthesiology 2002; 97: 1318-21; Anesthesiology 2006; 104: 101-9), That was the reason we did not put the hemodynamic data inside.

2.) The authors need to provide genetic evidence for a role of HO-1 in their responses.

Response: Role of HO-1 in organ protection was provided in Background accordingly.

3.) The paper needs to be re-written in better English.

Response: We have asked a native English anesthesiologist for improved the manuscript as suggested.

Reviewer 4

Xin et al, shows novelty of Isoflurane Preconditioning anesthesia and Heme oxygenase-1 relationship. Though prolong surgery Isoflurane is not recommended because slow recovery rate. However the finding is novel and is suitable for publication. I will be convinced and the paper may give good look if the authors are followed this suggestion.

1. The authors should mention the HO-1 activity in ischemia followed reperfusion.

Introduction should be start with HO-1

Response: Thank for your reasonable suggestion, we have rewritten the Introduction accordingly.

2. Ischemia followed reperfusion should be noted uniformly IR or I/R through out the paper.

Response: All the difference between IR and I/R have be uniformed into IR in reversed manuscript.

3. The authors also suggested the IL-6 and IL-10 level along with neutrophil accumulation.
Response: It is technically difficult to quantify neutrophil infiltration in every H&E stained field. We have assessed MPO activity as a marker for hepatic neutrophil infiltration in order to more accurately quantify neutrophil infiltration. It was not necessary to measure the cytokines levels as TNF alpha was assessed already.

4. Was any necrotic cells and any hyperplasia was observed? Should mentioned in histopathology.
Response: Histology results and figures were arranged and described, all the necrotic and inflammatory infiltration changes among groups were also been defined in figure legends.

5. What was the viability of hepatocytes?
Response: we apologize for the misusing of term about hepatocyte cultures in vitro, it has been deleted during this submission.

6. In research paper Phenobarbitone should not use in Ischemia group. May be Phenobarbitone effect participate Isoflurane action
Response: The preconditioning effect of volitale anesthetic is actually unrelated to phenobarbitone, and it was commonly accepted that phenobarbitone did not have protective effect. That is the reason using phenobarbitone in the animals of control groups in many studies (seen in Anesthesiology 2004; 101: 918-23; Am J Physiol Heart Circ Physiol 2006; 291: H979-83; Am J Physiol Renal Physiol 2007; 293: F713-22; Am J Physiol Renal Physiol 2008; 295: F128-36…), otherwise, we can not exclude isoflurane action if we still using isofluare in IR group.

7. The paper has novelty with relation HO-1 expression and anesthesia. The paper can be published with improving English and above said suggestion
Response: Thanks, we have asked a native English anesthesiologist to improve the quality of manuscript.