Author's response to reviews

Title: Intravenous pretreatment with emulsified isoflurane preconditioning protects kidneys against ischemia/reperfusion injury in rats.

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Author's response to reviews: see over
Dear Dr. or Prof. Costoy,

Thank you very much for your letter and advice. We have revised the manuscript, and would like to re-submit it for your consideration. We have addressed the comments raised by the reviewers, and the amendments are highlighted in red in the revised manuscript. Point by point responses to the reviewers’ comments are listed below this letter.

We hope that the revised version of the manuscript is now acceptable for publication in your journal.

I look forward to hearing from you soon.

With my best wishes,

Yours sincerely,

En Lv

Director of Department of Anesthesiology, Three Gorges University People’s Hospital, China.
We would like to express our sincere thanks to the reviewers for the constructive and positive comments.

**Replies to Reviewer Anja Slikkerveer**

**Major Compulsory Revisions**

1. The Use of the words “a prospective randomized study” in the title suggest that this is a clinical study. Please change the title. I suggest: Intravenous pretreatment with emulsified isoflurane preconditioning protects kidneys against ischemia/reperfusion injury in rats.

   Answer: The title has been changed according to the suggestion in the revised version.

2. Page 11, line 7-13: the description of the histopathology needs to be improved. It does not indicate clearly in which part of the tubules the effect has been observed (if possible the S segment can be added), which cells were seen to be necrotic and in which way the findings form an understandable pathophysiology: did the bleeding or inflammation occur in conjunction with the necrosis? There is usually a logical sequence of events? Since this is a known pathology, it is highly advisable to describe the observations in the temporal order of occurrence.
Answer: The description of the histopathology has been improved in the revised version (Page 11, line 7-17).

3. Page 13, line 2: the term “acute renal failure” covers a wide variety of clinical entities. Please replace by more exact term.

Answer: The term “acute renal failure” has been replaced by the term “renal I/R injury” in the revised version (Page 13, line 12).

Minor Essential Revisions

1. The authors seem to have focussed their assessment on the proximal tubule. Have the other parts of the kidneys (glomeruli, podocytes, distal tubule and collecting duct) also been included in the histopathology review? It would be beneficial for the article if such information could also be described. Quantification as for the proximal tubule is not considered necessary.

Answer: The degree of renal necrotic injury is most severe in the proximal tubules located in the renal cortex area after I/R injury. Therefore, we have showed this area in the figures and focussed our assessment on the proximal tubule. Actually, there are also glomerular atrophy, distal tubular dilatation, tubular epithelial cell swelling, necrosis and sloughing, tubular luminal obstruction in some kidneys after I/R injury.
These histopathological changes are not included in our demonstrated figures, so we have not described the parts other than the proximal tubule of kidneys in the histopathology review.

Quantification as for the proximal tubule has been used in many studies about kidney I/R injury, so we use a grading scales to assess necrotic injury of the proximal tubules in order to better understand damage degree.

2. Page 5: line 10: “regimens”. Suggest to replace by “doses”. Only one regimen has been tested.

Answer: “Regimens”, in fact is “strategies”, has been replaced by “doses” in the revised version (Page 5, line 10).

3. Page 8: can the authors indicate the rationale for the selecting of renal markers? Has there been a consideration to include novel markers like Kim-1, GST-alpha or delta?

Answer: Serum Cr and BUN are the classical and the most commonly used renal markers, which have been used to determine renal function in many experimental studies. Serum level of CYC could reflect glomerular filtration rate very accurately, even in cases where there was only a minor reduction in glomerular filtration rate. It can be used as a reliable endogenous marker for renal damage. Therefore, we have
selected the three variables to reflect kidney function. Both urinary Kim-1 and GST-alpha or delta are sensitive and novel markers for the early detection of acute kidney injury. Because we have not collected urine samples in the study design, these novel markers were not considered to detect. In the next study we will use these novel markers like Kim-1, GST-alpha or delta.

4. Suggestion to use more standardized names for the paragraphs: e.g on Page 9: line 21: Renal Function parameters, page 10, line 9 markers of inflammation and line 18: markers of oxidative stress; page 11 line 6: Renal histopathology.

Answer: These standardized names have been used in the revised version (Page 9, line 21; Page 10, line 9; Page 10, line 18; Page 11, line 6).

5. Page 11, line 7: delete “however” since the contrast here is expected

Answer: “However” has been deleted in the revised version.

6. Page 11, lines 14-16: please add the value of the histopathological score to the Text.

Answer: The value of the histopathological score has been added to the text in the revised version (Page 11, line 18-21).
7. Page 12: please add to the discussion in which respect this study is new. If my understanding is correct, then this is the first study on kidney I/R in the rat. But also the intravenous administration has not been done often. It is good to describe what is special about your study.

Answer: A sentence “To our knowledge, this is the first study to show that intravenous administration of EIso could produce renal protection.” has been added to the discussion in the revised version to address this issue (Page 12, line 12-13).

8. Page 15. Line 13 this sentence is incomplete: I suggest “..... with 2 or 4 ml, partly protected against .....”

Answer: The sentence has been changed in the revised version (Page 16, line 1-2).


Answer: “The” has been replaced by “A” in the revised version (Page 16, line 5).

10. Page 15, line 19: please delete “our”.

Answer: “Our” has been deleted in the revised version.
11. Page 16, line 2: please delete „but EIso reduced... current study”. Seems unnecessary repetition in this place.

**Answer:** „But EIso reduced... current study” has been deleted in the revised version.

12. Page 16, line 13-16: please rephrase into a clear statement. I suggest „Eiso can be administrated intravenously, which makes its clinical application more practical than inhalation of isoflurane. For this reason intravenous Eiso may gain more wider acceptance as a treatment option for ...”

**Answer:** The paragraph has been rephrased in the revised version (Page 17, line 2-4).

13. Page 16, line 21: please replace “renal damage” by the more accurate proximal tubular damage”

**Answer:** “Renal damage” has been replaced by “renal proximal tubular dilatation, tubular epithelial cells swelling, necrosis and sloughing, and interstitial hemorrhage and edema” in the revised version (Page 17, line 9-10).

**Discretionary Revisions**

1. Page 4: Line 13: add some relevant review references
Answer: Two relevant review references (reference 13 and 14) have been added in the revised version (Page 4, Line 12).

2. Page 6 Line 20: “bestowed” : suggest replacement by donated

Answer: “Bestowed” has been replaced by “donated” in the revised version (Page 6, line 20).

3. Page 13, line 12 “please check if “1 alveolar concentration” is the correct term.

Answer: “1 minimum alveolar concentration” in the original version is the correct term, please see the reference 9 (Page 14, line 1).

4. Page 14, line 16: please delete “in a previous study”. it does not add to the Discussion.

Answer: “In a previous study” has been deleted in the revised version.

5. Page 15, line 14-16: is there an understanding why the lowest dose is ineffective? If so please add some considerations.
Inhalation of 0.25 MAC isoflurane for 30 min can protect myocardium against infarction in dog. And higher concentrations of isoflurane may have greater efficacy to protect myocardium. It shows that isoflurane decreases myocardial infarct size in a dose-dependent fashion. In our study 1 ml/kg EIso did not produce renal protection, whereas 2 or 4 ml/kg EIso did. The similar result was found in the rat myocardial injury model. These results indicate that there is also a dose-dependent effect of EIso for kidney protection. There are some considerations in the original text (Page 16, line 5).

6. Page 26-28: I suggest to combine table 2,3 and 4. The message from these table is the same, so they can be easily combined. I also suggest to avoid that the wording below the tables is a copy of the main body text. Please shorten.

Answer: Table 2, 3 and 4 have been combined into Table 2. The wording below the table has been shorten in the revised version (Page 27-28).

Replies to Reviewer Bin Yang

Majors:

1. How to explain that the serum level of IL-10 was increased by I/R if IL-10 is an antiinflammatory cytokine?

Answer:
Multiple studies have demonstrated the increase of IL-10 during human myocardial I/R injury and during cardiopulmonary bypass, also in animal models of reperfused liver, brain, kidney, or heart. A study showed that free radical-mediated nuclear factor-κB activation was responsible for IL-10 production from the reperfused liver (Le Moine O, Louis H, Stordeur P, Collet JM, Goldman M, Deviere J. Role of reactive oxygen intermediates in interleukin-10 release after cold liver ischemia and reperfusion in mice. Gastroenterology. 1997; 113: 1701–1706.). Endogenously produced IL-10 serves to suppress the production of TNF-α in the reperfusion phase (see reference 32). Therefore, both inflammatory and antiinflammatory cytokines are concurrently increased during I/R injury in order to inhibit inflammatory response.

2. As the authors mentioned this the time of reperfusion is only 3 h, so many changes will be not detected and the longer time point will be necessary to fully evaluate the effect of EIso precondition on the renal I/R injury.

We accept Dr. Bin Yang’s comment. Though the time of reperfusion is only 3 h, our results showed that the renal I/R injury model was successfully established, and pretreatment of EIso with 2 or 4 ml/kg did reduced kidney damage. It indicates that the observation duration is acceptable. Of course, the longer time point such as 24 h or 48 h could be better to observe the effect of EIso precondition on the renal I/R injury. In the next study we will observe the change at different time points to
dynamically evaluate the effect of EIso precondition on the kidney I/R injury.

3. Inflammatory cell infiltration and apoptosis could be assessed, the data of this study will be more valuable.

Answer: We accept Dr. Bin Yang’s comment. Because our observation duration is short (only 3 h), and it is on the early stage of acute inflammatory response, we have not observed obvious inflammatory cell infiltration and apoptosis in the injured area.

4. The authors did mentioned that serum concentration of CYC could reflect glomerular filtration rate very accurately, even in cases where there was only a minor reduction in glomerular filtration rate. However, this point needs to be compared with Scr and discussed properly.

Answer: We accept Dr. Bin Yang’s comment. Several sentences have been added in the discussion in the revised version to address this issue (Page 13, lines 3-7, add reference 25 and 26).

Minors:

1. The abstract might be better organized to avoid the repetition of the same Terminologies.
Answer: The abstract has been reorganized in the revised version.

2. Table 2: The format of cystain C at “EIso 4ml/kg” need to be adjusted.

Answer: The format of cystain C at “EIso 4ml/kg” has been adjusted, and Table 2, 3 and 4 have been combined into Table 2 in the revised version (Page 27-28).

3. Table 2, 3 & 4: “EIso 4ml/kg” may also be changed to “EIso 4 ml/kg” on the top row, the same for other 2 dosages.

Answer: “EIso 1, 2 & 4ml/kg” on the top row in Table 2, 3 & 4 have been changed to “EIso 1, 2 & 4 ml/kg”, and Table 2, 3 and 4 have been combined into Table 2 in the revised version (Page 27-28).

4. Figure 1: the labeling of letters on the pictures should be more visible such as color contrast.

Answer: The labeling of letters on the pictures in Figure 1 have been changed for more visible in the revised version.

5. References: The format of references needs to be adjusted such as 14 and 23.
Answer: The format of references such as 14 and 23 have been adjusted in the revised version.