Author’s response to reviews

Title: Dissolved molecular hydrogen (H2) in Peritoneal Dialysis (PD) solution preserve mesothelial cells and peritoneal membrane integrity

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Author’s response to reviews:
Reply to Editor

Dear Editor and reviewers

Thank you very much for great reviewing to our manuscript.
We really appreciate your comments.
According to your suggestions, we fully revised the manuscript, and figures.
In addition, we changed the title of the original manuscript, as well.
Please see the reply to reviewer's comment below.
We hope the revised manuscript would be acceptable for BMC journal.

Masaaki Nakayama, MD, PhD
Tohoku University
Editor Comments:
1. Please include the appropriate information to the sections in the Declarations.
We cannot currently accept "Ethics (and consent to participate), Consent to publish, competing interests, authors’ contributions, and availability of data and materials are included in the manuscript."
2. The individual contributions of ALL authors to the manuscript should be specified in the Authors’ Contributions section. Guidance and criteria for authorship can be found here: http://www.biomedcentral.com/submissions/editorial-policies#authorship
3. Figures should be provided as separate files, and each figure of a manuscript should be submitted as a single file.
Reply: We did adequately in the revised section.

Reply to Comments by corresponding author (Masaaki Nakayama)

Reviewer reports:
Sandra Rieger (Reviewer 1): The manuscript by Zhu et al. investigates the potential positive effects of dihydrogen (H2) on mesothelial injury induced by peritoneal dialysis. Peritoneal dialysis can cause oxidative injury and H2 is thought to act as an antioxidant to reduce injury. In this study, the authors tested this idea. They find that H2 supplementation in dialysis bags has various positive effects on the mesothelium leading to reduced mesothelial thickening and infiltration of the mesothelium by macrophages, and H2 can also normalize cell death and proliferation.

1) The authors make the point that H2 acts as anti-oxidant, however no evidence is shown that it does. In the discussion, first paragraph the statement saying that the authors studied increased oxidative stress induced by FeCl3 is not supported. Could the authors assess oxidative stress markers by qPCR or use their microarray data to identify any stress-related genes that might be differentially regulated?
Reply:
Thank you very much for your critical comment. As you pointed out, the present study did not show any influences on oxidative stress. We did not have data on changes of oxidative markers,
and data of PCR. The main purpose of this study was to look at the potential of H2 for membrane protection in PD. Based on this status, we want to change the title as below, and, and changed the contents including discussion in the revised manuscript.

New title: Dissolved molecular hydrogen (H2) in Peritoneal Dialysis (PD) solution preserve mesothelial cells and peritoneal membrane integrity.

Short Title: PD solution with H2

2) The images in Figure 2 are too small and one cannot clearly distinguish individual cells with expression of the indicated genes. Please generate zoomed images and show them side-by-side.

Reply:
According to the request from reviewer, we zoomed images in order to make it easy for readers to recognize the findings in the revised manuscript.

3) It is unclear how cells were counted. In the Materials and Methods section it was mentioned that 5 randomly selected fields were quantified (how large are they?) but the graphs show that cells were counted per image (if "pic" means picture?). A standard way would be to express the cell numbers per area (e.g. cm2).

Reply:
We are very sorry for presenting the poorly described methods in the original version. In the revised manuscript, we added the sentences belo to explain the details.

“For the quantitative analysis, the number of positive cells in the peritoneal tissue sample was counted in five randomly selected fields. The positive cells of each picture were counted from per surface length. The surface length of peritoneum was measured by a free soft ware Image J. The average of the surface length (field) of peritoneum used in analysis corresponds to 9 pixels, e.g. 220µm. We selected 5 field randomly for quantified pictures. Regards to shedding cell analysis, surface length of peritoneal membrane with/without mesothelial cell covered were measured.”

4) Perhaps the authors could be more explanatory why PD increases proliferation and apoptosis at the same time? Is there evidence that the same cell type is affected or are these different cell types?
Reply:
The point you raised is very important in understanding the pathological process of membrane injury by PD treatment.
Therefore, we fully revised the discussion according to your point-out, as below.
In the revised discussion,
“...In this study, there were unique findings in PD group, e.g., increases in proliferation and apoptosis in the surface cells at the same time. At present, the exact mechanism of this finding remained to be elucidated. But we have following hypothesis: the mesothelial cells are constantly exposed to PD solution, which is potentially bio-incompatible. Therefore, mesothelial cells are in the state of the injury to be lost and the compensatory proliferation in order to restore membrane. The balance between the two directions could be crucial for peritoneal integrity preservation. As a matter of fact, the balance disruption by oxidative stress of FeCl3 resulted in severe mesothelial loss, accompanying membrane thickness (Fig.6B,C).
To note, in the present study, morphological differences were found in the surface cells of peritoneum in the PD group, e.g., cuboidal change of the cells. There were significant increase in vimentin staining in the PD group, as compared to the control and the H2PD groups, which may indicate the phenotypic alteration of mesothelial cell, to undergo epithelial-mesenchymal transition (EMT). However, unexpectedly, the gene expressions which modulate EMT were not different between the PD and the H2PD group, thus, it remained unclear whether the cells which represented apoptosis and proliferation, were the same mesothelial cells or not. This needs to be addressed including the possibility of infiltrating macrophages.

In regards to the mechanism of H2 for peritoneal preservation, the exact mechanism also remained to be elucidated. But interestingly, as mentioned above, there were no differences in gene expressions, such as, EMT related genes (e.g. SNAIL, ECADHERIN aSMA), and anti-apototic and apoptotic gene (e.g. BCL2, BAX, BAD) between the PD and the H2PD groups (Fig.4). This fact may indicate the potential of indirect action of H2 in terms of cellular protection by H2. Recent studies have revealed the significant role of tissue macrophages in orchestrating the healing process in wound tissue, i.e., M1 macrophages for inflammatory actions, and M2 for remodeling/healing actions in damaged tissues [24]. In this study, there were significant differences in the infiltrated M2 macrophage sub-populations in the peritoneum...
between the PD and the H2PD groups, with M2 being dominant in the H2PD group. This may indicate enhanced healing of peritoneal tissue in the H2PD group. The question that H2 could facilitate macrophage functional switching to restore tissue injury, need to be clarified in future.”

5) It is unclear how the shedding cell analysis in Fig.6c was performed? There is no mentioning in the text.
Reply:
We are very sorry for not mentioning the data in the method section. We added the below sentences in the revised manuscript.
“Regards to shedding cell analysis, surface length of peritoneal membrane with/without mesothelial cell covered were measured by Image J in 5 randomly taken pictures, and the ratio of uncovered area was calculated by the following formula: (uncovered length / total surface length)onserved.”

6) The legend for Figure 6 does not match what is shown in Figure 6.
Reply:
We are very sorry for the mistakes. In the revised manuscript, we corrected as below:
Figure legends 6
“Representative findings of Masson and immunohistochemical staining (CD68) (Fig-A), and quantitative analysis of peritoneal morphology and immunohistochemical staining of peritoneum in respective groups (Fig-B-G) are shown. Peritoneal thickness (A), and shedding cells ratio of the peritoneal surface (B), immunostainings of mesenchymal maker: vimentin (B), proliferative marker: Ki-67 (C), apoptosis marker: M30 cyto-death (D), and total macrophage marker: CD68+, M1 macrophage: CD80+, and M2 macrophage: CD163+ (E), respectively.”

7) It is unclear how the array data was evaluated. What does the percentage difference between the two data sets mean in terms of gene expression and biology?
Reply:
We are very sorry for poor explanation.
The array was evaluated in one representative sample.
The percentage denotes the ratios of differences by Pearson' coefficient analysis between the test samples, e.g. 8.7% differences in total genes between PD and Control, and 3.7% differences between H2-PD and PD groups, respectively. This was described in the Result section.

In addition, In Fig5, we presented the differences of representative gene expression between PD and H2PD groups, in order to underline the differences. In the revised manuscript, we revised the figures in order to be understandable for readers.

8) The authors state "combined pathologies of EMT.....". It is unclear what that means. If it means that EMT is increased after PD, the authors can perhaps comment on the observation that the pro-EMT gene Snail is downregulated in PD rats, and is not rescued by H2. How does the H2 improve the phenotypes?
Reply:
As reviewer pointed out, it is very critical point, and, we cannot explain about the mechanism of H2-protection by EMT. Regarding the mechanism of H2 protection, it remained to be addressed. However, in the present study, we observed the fact that the profiles of macrophage were different by groups, and the ratio of M1/M2 were different between PD and H2PD, which indicated that H2 could facilitate macrophage functional switching to restore membrane injury. This is our hypothesis that could present at present. In the revised manuscript, we discussed this issue.

9) It would be helpful to know why the authors assessed the expression of the chosen genes, especially aSMA and cytokeratin.
Reply:
Thank you very much for the comment. In the revised manuscript, we touched on these molecules in discussion to be fully understandable for readers why we picked up these genes.

10) How were the dialysis solutions administered to rats?
Reply:
We are very sorry for not mentioning the method properly in the original version. Dialysate was infused intraperitoneally by direct injection every time. Rats were mildly anesthetized in order to be injected safely. We added this sentences in the revised manuscript.

11) Please also indicate the statistical significance of the PD and P/H2 groups in Figure 3A-D, if there is any?

Reply:
In statistical analysis, we employed one-way ANOVA, followed by post-hoc test. The final statistical analysis was noted in figures. We explained in figure legends in the revised manuscript.

Carl Öberg (Reviewer 2): In this manuscript the effects of using a PD solution with dissolved hydrogen gas (H2) are studied in Sprague-Dawley rats in order to investigate effects on peritoneal histology and gene expression assessed by RT-PCR. Additionally, the effects of adding FeCl3 to the dialysis fluid are examined in order to evaluate tentative effects of dissolved H2 treatment on oxidative stress. This is an important subject with clinical relevance. The methods appear to be appropriate. The conclusions are however not supported by the results and should be stated much more carefully. A key issue in histological studies is the lack of quantitative measurements. Differences that may appear striking visually can be due to chance or be difficult to quantitate. From the current quantifiable measurements, there were no differences between the PD group and the H2PD group except for the effects on infiltrating M1/M2 macrophage sub-populations. Thus, in my opinion, the main finding is not that H2 ameliorates injury caused by the PD fluid alone (which is the clinically relevant comparison) but rather that H2 ameliorates injuries caused by 5 uM FeCl3. While this latter finding is interesting, it cannot be used to draw the conclusion that dissolved H2 ameliorates injury in conventional PD treatment. Lastly, the risks of using highly flammable and explosive hydrogen gas in the clinic must be addressed.

Reply:
Thank you very much for reviewing the manuscript and giving us a lot of crucial and important points. Firstly, we are very sorry for poor explanations of methods section, which had made a lot of confusion and doubt. We explained the method of the quantification process of histological
Secondly, as reviewer pointed out, the present study did not show any influences on oxidative stress. We did not have data on changes of oxidative markers, and data of PCR. The main purpose of this study was to look at the potential of H2 for membrane protection in PD. Based on this status, we want to change the title as below, and, changed the contents including discussion in the revised manuscript. Challenges of FeCl3 is an important part, but we intended to show the possible H2 ability for membrane protection. Therefore, we want to show the result of FeCl3 in the context of this idea (protection by H2).

Based on this idea and reviewer’s suggestion, we changed the title as follows:

New title: Dissolved molecular hydrogen (H2) in Peritoneal Dialysis (PD) solution preserve mesothelial cells and peritoneal membrane integrity.

Short Title: PD solution with H2

Lastly, the risks of using highly flammable and explosive hydrogen gas in the clinic must be addressed.

Reply:
We inserted the sentences below in the discussion section.

“In doing the clinical trial, risks of flammable H2 gas need to take into account. However, the explosive levels of H2 is at the vicinity of 4% (40 x103 ppm), while the levels of H2 in bags are less than 0.4 ppm, and H2 levels of H2-oversaturated water manufactured by the present method are 1.6 ppm at maximum. Therefore, we think it is safe to conduct the clinical trial by employing this system.”

Title: Should be changed to e.g. "Peritoneal dialysis fluid with dissolved hydrogen gas (H2) ameliorates oxidative stress caused by FeCl3"

Reply:
I mentioned my reply in the above section.

Abstract: The abstract must be revised to reflect the main findings vide supra. Also replace "maintenance therapy" with e.g. "renal replacement therapy".

Reply:
According to the comment from reviewer, we revised the abstract.

The Methods section is well written and the statistical procedure is appropriate although the authors should consider doing non-parametric tests due to the low number of animals in each group.
Reply:
In statistical analysis, we primarily employed one-way ANOVA, followed by post-hoc test. The final statistical analysis was noted in figures. We explained in figure legends in the revised manuscript. In addition, in case of non-parametric cases, we examined two factors respectively by employing Mann-Whitney, however, they all did not reach the significance.

Background, results and discussion are mostly well-written but must undergo revision to reflect the actual findings in your study.
Reply:
Thank you very much. According to reviewer’s comment, we revised the manuscript, rearranged theb presentation, and discussed the pathophysiology of membrane damage, and the mechanisms of protective effect of H2, based on the findings of ours. They are rewritten in red in the revised manuscript.

Minor comments:
My copy of the ms was not paginated.
Fig. 3 and Fig. 6: Immunohistochemistry misspelled.
Reply:
Misspelled words were corrected in the revised manuscript.