Dear Prof. Li Ma:

Enclosed please find the revised version of our manuscript (NO. 77808, Basic Study) titled “Mitochondria transfer from mesenchymal stem cells rescues injured glomerular endothelial cells *in vitro* and *in vivo*”.

We thank the Editors for careful examination of the manuscript and thank the Reviewers for their insightful comments, which were not only scientifically meritorious but also extremely helpful in directing our efforts to enhance the scientific quality of this manuscript. We have attempted to address the Reviewer’s concerns and believe that this has resulted in a significantly improved manuscript. A detailed point by point response is provided below. The modified parts have been marked with red.

Reviewer #1:

1. In the materials and methods on page 7, BMSCs passage 2-4 were used, while in the last sentence passage 2-3 were used. Which ones were used in the experiment? Please clarify and give reasoning for using multiple passage cells.

Response: We thank the reviewer for pointing out this problem. BMSCs passages 2-4 were used, and glomerular endothelial cells (GECs) passages 2-3 were used. Our cell culture found that the cell viability of BMSCs was still good after passage 5, while that of GECs decreased significantly after passage 3, and most of them could not continue to grow and to be passaged. Changes applied to the manuscript: Page 8, Paragraph 3 (revised manuscript).

2. Please add the location and country of the materials accordingly, some of them were not completely provided.

Response: We have now added that information to the materials. Such as, fetal bovine serum, penicillin-streptomycin, endothelial cell growth supplement, *etc*. Changes applied to the manuscript: Page 8, Paragraph 3; Page 9, Paragraph 1, 3; Page 10, Paragraph 4, 6; Page 11, Paragraph 3, 5; Page 12, Paragraph 4 (revised manuscript).

3. Page 17 line 9 "...figure 4E and 5F..." should be "...figure 4E and 4F...". Please revise accordingly.

Response: Our apologies for careless writing. We have made the revised accordingly. Changes applied to the manuscript: Page 18, Paragraph 3 (revised manuscript).

4. References number 40-43 are not available in the text, they can be added in the methodology.

Response: We have adjusted the position of this references number 40-43 and added in the "*RNA extraction and RT-qPCR*" section. Changes applied to the manuscript: Page 11, Paragraph 5 (revised manuscript). References number 21-24 is intended to be that citation number for the new reference above.

Reviewer #2:

5. Do the authors know what is the percentage of GEG cells that were transferred with BMSC mitochondria?

Response: Good point. We regret that we did not measure the percentage of GEC cells that were transferred with BMSC mitochondria during the experiment — that is something we hope to do in the future. Our next research content is to study how to promote mitochondrial transfer from BMSCs to GECs. Please allow me to show the result of percentage of GEC cells that were transferred with BMSC mitochondria in future research. We have elaborated this in the Discussion ("Limitations"). Changes applied to the manuscript: Page 22, Paragraph 2 (revised manuscript).

6. Did the authors noticed a stronger effect on the transferred cells (fig 1B and C) compared to non-transferred cells?

Response: Thanks for noting this. Mitochondria are important checkpoints of the apoptotic cell death. High glucose increased oxidative stress in GEC cells and induced apoptosis (fig. 1D and E). Some scholars claimed that mitochondria transfer protects damaged cells from apoptosis. Caspase-3, Bax and Bcl-2 are the common pathway

protein in multiple apoptotic mechanisms. The anti-apoptotic gene/protein (Bcl-2) was upregulated and pro-apoptotic gene/proteins (Bax, caspase-3) were found to be downregulated upon treatment with BMSC. The therapeutic effect correlated to mitochondrial transfer from BMSC to GECs. The stronger effect on the transferred cells compared to non-transferred cells, which reflects the anti-apoptotic ability of recipient cells after mitochondria transfer.

7. Figure 1: The ration Bcl-2/Bax should be included, because more informative about anti-apoptotic effect of BMSC on GEG cells.

Response: Thank you for your constructive suggestion. We have followed your suggestion to revise figure 1 and text. The ratio of Bcl-2/Bax was increased in the HG+MSC group compared to those in the HG group. However, these differences were small and did not reach statistical significance. The possible reasons for this might include the following two reasons: 1) a too large control (NC, NC+MSC) groups could lead to non-statistically significant differences in HG and HG+MSC groups; 2) we also had a small sample size, which may result in lower statistical power to detect differences between groups. Changes applied to the manuscript: Page 15, Paragraph 4; Page 21, Paragraph 1 (revised manuscript).

8. The authors refered to an old publication to explain the protocol to isolate the BMSC (reference 19). However, the authors must provide the source of BMSC. Are they rats? If so, is the treatment autologous or allogeneic? If the BMSC are humans, what is the IRB approved human protocol?

Response: We appreciate your comments. We have updated the reference 19 (Gong L, Chen B, Zhang J, et al. Human esc-sevs alleviate age-related bone loss by rejuvenating senescent bone marrow-derived mesenchymal stem cells. J Extracell Vesicles 2020; 9: 1800971 [PMID: PMC7480439 DOI: 10.1080/20013078.2020.1800971). The BMSC were isolated from Sprague-Dawley (SD) rats. Rat diabetic kidney disease models were treated by allogeneic rat BMSC. Changes applied to the manuscript: Page 8, Paragraph 3 (revised manuscript).

9. One of the major concerns is the title. The authors claims that mitochondria transfer from MSC rescues the injured glomerular endothelial cells in vitro but also in vivo. However, the authors have no proof that the in vivo rescue is due to mitochondria transfer. Many other factors (paracrine, transfer of extracellular vesicles, exosomes, MSC differentiation) could be factors that rescue the kidney. The authors should modify the title, the discussion, and the conclusion, based on my comments.

Response: We appreciate your comments. We have followed your suggestion to revise all text. Revised title: Intercellular mitochondrial transfer as a means of revitalizing injured glomerular endothelial cells. Changes applied to the manuscript: Page 1, Title Section (revised manuscript). As for the lack of evidence of mitochondrial transfer *in vivo*, we have added this issue to the limitations paragraph of the Discussion section. Changes applied to the manuscript: Page 22, Paragraph 2, the second limitation (revised manuscript). The therapeutic effects of BMSC on DKD rats may be related to the mechanism of mitochondrial transfer. Changes applied to the manuscript: Page 4, Paragraph 3, the conclusion section; Page 23, Paragraph 1 (revised manuscript). We hope these modifications will make you satisfied.

10. It is well known the biodistribution of BMSC can be all over the body, after injection, especially in the lungs. Did the authors study the biodistribution of the BMSC, after injection and how many BMSC were alive in a time course manner after the injection?

Response: Thanks for the reviewer to bring up this question. Nakazaki and colleages found that intravenous infused MSCs, which do not themselves reach the injury site, release exosomes that can be taken up by M2-type macrophages at the lesion site. The reference is: Nakazaki M, Morita T, Lankford KL, Askenase PW, Kocsis JD. Small extracellular vesicles released by infused mesenchymal stromal cells target M2 macrophages and promote TGF-β upregulation, microvascular stabilization and functional recovery in a rodent model of severe spinal cord injury. J Extracell Vesicles. 2021 Sep;10(11):e12137. doi: 10.1002/jev2.12137. We regret that it is difficult to fill

two parts of the experiment in a short time. But we will study the biodistribution of the BMSC and the ratio/number of BMSC were alive in a time course manner after the injection in the next research. We have added this issue to the limitations paragraph of the Discussion section. Changes applied to the manuscript: Page 23, Paragraph 1 (revised manuscript).

11. This question is related to the previous one. In figure 4, the authors claimed TUNEL and histopathology analysis of tissues from all rats, but only the kidney was studied. Histopathology of other organs, such as heart and lungs, should be added to show the safety of the BMSC injection.

Response: Thanks for your nice suggestion. MSCs define a population of progenitor cells with low immunogenicity, ease of accessibility, broad differentiation potential and immunomodulatory effects, and therefore, they have become a good option for organ transplantation. One article reported that neither infusion of MSC induced significant fibrotic responses in organs (lungs, kidney, liver and spleen) which might cause safety concerns. The reference is: Nakazaki M, Morita T, Lankford KL, Askenase PW, Kocsis JD. Small extracellular vesicles released by infused mesenchymal stromal cells target M2 macrophages and promote TGF-β upregulation, microvascular stabilization and functional recovery in a rodent model of severe spinal cord injury. J Extracell Vesicles. 2021 Sep;10(11):e12137. doi: 10.1002/jev2.12137. Unfortunately, we did not reserve organs and tissues such as heart, lungs and brains, and the safety analysis experiment was difficult to supplement. But, we will analyze the safety of MSC injection while studying how to promote mitochondrial transfer from MSCs to GECs in the next research. We have added this issue to the limitations paragraph of the Discussion section. Changes applied to the manuscript: Page 23, Paragraph 1 (revised manuscript).

12. Did the author measured the level of liver injury by studying the level of AST and ALT in the blood, that could be associated with diabetic kidney diseases?

Response: Thanks for the reviewer's valuable comment. Renal damage in diabetic

nephropathy is associated with the damage of multiple organs. Revealing the damage of other organs is helpful to understand the theme of this study. Given the lack of understanding, we didn't analyze this issue. We will answer these questions in the next research report. We have made the revised in the Figure 4 title and added this issue to the limitations paragraph of the Discussion section. Changes applied to the manuscript: Page 23, Paragraph 1 (revised manuscript).

13. It is not clear if the injected BMSC were dyed or not. Can the authors specify it in the Materials and Methods section?

Response: Thank you for pointing out it. The injected BMSC were pre-labeled with MitoTracker Red CMXRos. We didn 't provide evidence of mitochondrial transfer *in vivo* because our laboratory conditions were unable to freeze renal tissue when they were obtained, the distribution of MSC mitochondria labeled with fluorescent in the tissue was not observed. We have made the revised accordingly. Changes applied to the manuscript: Page 13, Paragraph 2 (revised manuscript).

We have also made changes to the manuscript to comply with journal format requirements, including but not limited to the title and absence of references 34-37.

Thanks again for the above comment and suggestion.

We hope that the revised manuscript is suitable for publication.

Thank you for your consideration.

Best regards,

Hang Xu

July 9, 2022