February 24, 2023
Shengwen Calvin Li, and Cario Ventura
Editors-in-Chief
World Journal of Stem Cells

Dear Editors:

I wish to submit our revised manuscript entitled "Repetitive administration of cultured human CD34+ cells improves adenine-induced kidney injury in mice" (Manuscript No: 82212) for consideration of publication in *World Journal of Stem Cells*. We are grateful to the reviewers for their comments and suggestions, which have helped us to improve our manuscript. We have revised the manuscript based on their comments and have provided our point-by-point responses to each of their comments as written below.
We hope that the revised manuscript is now suitable for publication in your journal.

Thank you and I look forward to hearing from you.

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Replies to reviewers:

Reviewer #1:
Scientific Quality: Grade B (Very good)
Language Quality: Grade A (Priority publishing)
Conclusion: Minor revision
Specific Comments to Authors: The manuscript by Ohtake et al. investigates the ability of three weekly doses of cultured CD34+ cells to improve adenine-induced kidney injury in mice. The culture method, which the authors reported previously, greatly expand CD34+ cells and endothelial progenitor cells (EPC).

Overall the manuscript is well written and the work is well performed. I have a few recommendations for improving the manuscript prior to publication. I also have a few questions for the authors that don't necessarily need to be addressed experimentally now, but I would like the authors to consider addressing some of these concerns in the Discussion.

Dear the reviewer,
Thank you very much for your profound review and suggestions for our manuscript. I discussed about some concerns, which reviewer pointed, in the manuscript as written below. Corrected parts were written in red.

Questions for consideration:

1. The authors state that adenine consumption induces a chronic kidney disease. It may be too late to ask this question, since others have published on this model as well, but does a three-week feeding regimen of adenine really mimic CKD? This seems to be a very acute time frame.

Thank you very much for your valuable comment. Indeed, it seems to be rather short (three-week adenine ingestion) to say this is a model of chronic kidney disease (CKD). However, this model (usually 3-4 weeks' adenine ingestion) has been used for a model of CKD, as reviewer mentioned. The typical pathological findings in this model are prominent tubular damages including tubular dilatation and tubular atrophy accompanied by interstitial fibrosis, which mimic pathological findings of CKD in human. The model in our study showed CKD-like tubulointerstitial damages. However, we reconsider again, and agree with the reviewers' comment in consideration of a very short time frame. We deleted the word "chronic" from title, abstract, and conclusion.

2. The authors show very nicely that CD34 cells can blunt the effect of adenine on renal injury (Figure 4). However, it seems that renal injury as measured by serum Cr improves simply by stopping adenine administration in the control animals. Would the animals' renal function return to normal by 6-8 weeks after adenine is stopped if no cell therapy was given? Have the authors, or others, examined this?

   Adenine ingestion induces progressive tubulointerstitial injury in mice. In our preliminary study, serum creatinine and urea nitrogen continue to increase, and some mice (20-80%) die due to renal failure during day 21 to

day 28 if adenine diet would be continued. The article by Kamijo-Ikemori et al (Am J Physiol Renal physiol 2016: 310: F1366-F1376) used 0.2% adenine-containing diet for 4 weeks in mice, and changed to normal chow for 2 weeks. In their study, both blood urea nitrogen and serum creatinine levels at 2 weeks after stopping 4 weeks' adenine diet significantly improved compared to those at 4 weeks in adenine diet, but did not return to normal range. Stopping adenine diet directs kidney function to the recovery phase. However, we did not evaluate, and do not know whether renal function return to normal by 6-8 weeks after adenine is stopped if no cell therapy was given.

Ragarding switching to normal chow after stopping adenine diet, the article by kamijo-Ikemori as written above also switched from adenine diet to normal chow during the active treatment phase of drug intervention (Kamijo-Ikemori et al. Am J Physiol Renal physiol 2016: 310: F1366-F1376 Figure 1). We usually treat CKD patients by medication and diet control simultaneously. Therefore, it might be reasonable to stop adenine diet, change it to normal chow during the treatment phase by cell therapy, and follow the efficacy of treatment by cell therapy and diet control.

3. Similarly, can three doses of CD34 given during the period of adenine feeding which the renal injury is still developing be considered therapy for CKD? I suppose one could devise a clinical trial in which CD34 cells were given to earlier stage CKD to see if they prevented progression to ESRD, but a big question is do CD34 cells do anything for true CKD/ESRD, not CKD as it is developing as described in this manuscript. The authors should consider feeding with adenine to allow renal injury (CKD?) to fully develop, then investigate the effects of CD34 cells.

Thank you for your valuable comment. As reviewer pointed, this study examined the effects of cell therapy during the period of early progressive stage of CKD, not fully established stage such as end-stage renal failure (ESRD). Therefore, as reviewer commented, if we design a clinical trial on the basis on the results obtained from this study, CD34 cells would be given at earlier, but progressive, stages of CKD, not ESRD stage. As mentioned above in question 2, adenine diet induces CKD and promotes kidney injury to fully developed ESRD stage if adenine diet would be continued. As the reviewer commented, we might have to evaluate the effect of CD34 cells on

fully-developed, more advanced ESRD in a future study. (However, it may contain some difficulty because considerable rate of mice with fully developed ESRD by adenine ingestion may die due to renal failure. We might have to switch adenine diet to normal chow, not to lose mice, as used in this study.)

4. Furthermore, are the effects of the cell therapy long-lasting? It is impossible to tell from these studies since the animals were sacrificed only one week after the last dose of cells. Have the authors, or others, waited longer after the last dose of cells before sacrifice? Does the Cr ever return to and remain at baseline, or does renal function worsen again with more time after the last cell infusion? Likewise, do the histologic improvements last?

Thank you for your valuable comment. Regretfully, we did not wait longer after the last dose of cells before sacrifice. Therefore, we could not evaluate whether the effect of CD34 cells are long-lasting or not.

5. Again, I don't necessarily think the authors need to do more experiments to address questions 1-4 above for this manuscript (unless they already have obtained some data), but perhaps in the Discussion where limitations of the study are reviewed the authors can discuss some of these concerns. It would be acceptable to discuss these topics and say they will be addressed in future research.

Thank you for reviewers' comment. I discussed about issues, which reviewer pointed, in study limitation in discussion.

Recommended changes prior to publication:

1. Can the authors comment on the cell dose used? A cell dose of 10^6/mouse in a 20g mouse is equivalent to about 50 x 10^6 cells/kg in a human, an amount that is probably not achievable clinically, at least in adults.

As reviewer pointed, a cell dose of $1x10^6$ cells/20 g mouse is equivalent to about $50x10^6$/kg in humans, which is indeed not achievable clinically. It is known that in case of cell administration via the tail vein, many cells are trapped in the lung, spleen, and bone marrow (although the model was different, we previously confirmed extra-renal trap of administered cells via tail vein in our own study, Cell Transplantation 2018: 27: 520-530). When we think a clinical trial in the future, we should consider the route of cell administration.

To get beneficial effects in human clinical trial, cells might be desired to administer via arterial injection to directly deliver the cells to the target organ. We added comments about this issue in discussion.

2. Results, top of page 10, referring to Figure 2B: The text says a 59% vs. a 1% difference; shouldn't this be 59 vs. 1 fold difference, not %?

Thank you for pointing it. It is not percent but fold as reviewer pointed. We corrected the part.

3. I recommend combining Figures 2 and 3 (e.g., make Figure 3 Figure 2C) since both figures are showing analyses of the cultured CD34 cells.

We understand reviewers' suggestion. Indeed, it is better to understand to combine Figure 2 and 3. We corrected Figure 2 (2A, 2B, and 2C). Figure 2C was originally Figure 3. As we changed figure2, serial figure numbers were changed.

4. I'm not certain that Table 1 is needed, can just describe in the text.

We deleted Table 1.

5. The Y axes in the figures need to be labelled better; labels are either missing (Figures 2, 3) or just a small % sign is given at the top of the Y axis.

We added labels of Y axes in the figures as reviewer suggested.

6. I would consider amending the title to something like "Repetitive administration of human cultured CD34+ cells improve the time course of adenine-induced [chronic – delete?] kidney injury in mice" to better reflect the short-term nature of the experiments as noted in my questions above.

Interstitial fibrosis is a chronic reaction responding to tubular epithelial cell damage and interstitial inflammation. Interstitial fibrosis is usually used as one important marker of chronic kidney damage. Adenine-induced tubulointerstitial injury is widely used as a model of CKD. Recent studies also use this model as a progressive CKD. In article by Hao et al. (Frontiers in Cell and Developmental Biology 2021: 9: 603802) used 21 days' adenine ingestion to induce CKD model in mice. Makhluofi et al. (TH Open 2020: 16: e66-e76) used 2 weeks adenine ingestion to induce CKD model in mice. We also used "chronic" in our original manuscript. The model in our study showed CKD-like tubulointerstitial damages. However, we reconsider again, and agree with the reviewers' comment. In consideration of rather short-term nature of the experiments, we delete the word "chronic" from the title, abstract, and conclusion.

Reviewer #2:

Scientific Quality: Grade C (Good)

Language Quality: Grade B (Minor language polishing)

Conclusion: Minor revision

Specific Comments to Authors: Takayasu Ohtake et al., showed that repetitive administration of cultured human UCB-CD34+ cells significantly improved chronic tubulointerstitial damage in adenine-induced CKD model in mice via their vasculoprotective and anti-inflammatory effects. The data were interesting and have potential future application in cell therapy, but several points should be addressed, 1) Specific statistical methods used in data analysis of each figure should be mentioned. 2) The language is insufficient and in some parts incomprehensible.

Dear the Reviewer 2,

Thank you for reviewing our manuscript.

Comparison between 2 groups were performed using Mann-Whitney *U* test (Figure 2A, 2B, 2C, 3B, 4C, 5B, 6B). Differences in time-course of serum creatinine levels during the experimental period between the control group and cell therapy group were analyzed using repeated measures ANOVA (Figure 3A). These comments were written in statistical analysis in Method (page 9) and each figure legend.

Our manuscript underwent English proofreading before we submitted our first manuscript. However, as reviewer pointed, English writing might not be correctly written in its present form. Therefore, we re-underwent second English proofreading by other external company, and corrected manuscript according to the suggestion. If our revised manuscript is still insufficient as a response to reviewers' suggestion, and incomprehensive in some parts, please kindly indicate the part. We are willing to reconsider and revise the pointed part. Corrected parts were written in red.