Author's response to reviews

Title: Early stage transplantation of bone marrow cells markedly ameliorates copper metabolism and liver function in Wilson disease mice

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Author's response to reviews: see over
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Dear Tim Shipley, PhD

Thank you very much for making us have a chance to revise our manuscript entitled “Early stage transplantation of bone marrow cells markedly ameliorates copper metabolism and liver function in Wilson disease mice” (4653053784230558). We also appreciate the reviewers for their critical comments. We have revised the manuscript following the reviewers’ suggestions and the corresponding modifications are listed at a point-by-point style. The locations where changes were made in the manuscript were stated with the corresponding page and line number in the revised manuscript. Relevant references are also listed.

Additionally, we had a native English speaker to review this manuscript and the revised version of the manuscript should be in much better shape. We would greatly appreciate your favorable consideration.

With best regards,

Sincerely yours,

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Reviewer #1

Point 1. The injection of saline does not represent an adequate control group. The transplantation of BM cells from Wilson disease mice (toxic milk mice) into Wilson disease mice, would be the right control in addition to the super control (saline).

Response: We appreciate the reviewer’s suggestion. Both tx mice and saline used as control would be the best. Since tx mice have a point mutation in ATP7B gene with the phenotype of Wilson disease, it would be theoretically ineffective to treat with tx bone marrow cells. Additionally, in other previous studies related cell transplant in tx mice [1, 2], buffer solution rather than tx bone marrow cells was selected as control.

Point 2. Since liver function and copper concentration are already improved one week after transplantation it is rather speculative whether the kind of BM cells is responsible for the improvement. This point should be elaborated in additional experiments.

Response: We agree with the reviewer’s suggestion. In the present study, we did found that liver function and copper concentration were corrected one week after transplantation compared to the control. Previously we have found that copper concentration and liver function were not significantly corrected until one week after transplantation (unpublished data). Consistently, this tendency is confirmed in other studies on bone marrow cell treatment study [3, 4].
**Point 3:** The statistical analysis should be recalculated by a testing appropriate for not normally distributed data (e.g. Mann-Whitney-U-Test). Alternatively the authors should show a normal distribution before using the Students t test. The exact N-number should be indicated in each single figure under each single column. All together the manuscript seems to be too descriptive since the effect of BM transplantation on the amelioration of liver function is far from being explained.

**Response:** We appreciate the reviewer’s suggestions. Data from the present study showed statistically normal distribution and Student’s t tests were used. The corresponding sentence “Data showed a normal distribution and Student’s t tests were used.” was added to the figure legends in the revised text. (Page 23, Line 9 to line 10; Page 23, Line 21 to line 22; Page 24, Line 7 to line 8; Page 24, Line 14 to line 15). We have also added the n values in the corresponding figure legends in the revised text.

**Point 4:** The authors should demonstrate whether liver fibrosis (collagen, hydroxyprolin or sirius red staining) is influenced by BM-transplantation in toxic milk mice.

**Response:** Thanks for the reviewer’s suggestion. Collagen, hydroxyprolin or sirius red staining are the methods to evaluate liver fibrosis. Besides, H-E staining could also be used as an appropriate measure to study liver fibrosis. We had ever completed H-E staining study. The results have been added to the results section as “no obvious liver fibrosis was observed by H-E staining”. (Page 11, Line 11 to line 12)

**Reviewer #2**

**Point 1:** The authors are very loose with their phraseology, often saying Wilson's
disease when they are referring to the mouse and other animal models of Wilson's disease. For example this happens in the abstract conclusions section. They should correct this throughout the paper.

**Response:** We feel sorry for these mistakes and made a revision throughout the manuscript.

**Point 2:** A serious misstatement occurs in the first paragraph of background where they imply that liver transplantation is useful for the neurologic symptoms of Wilson's. Transplantation will correct neurologic symptoms, but only to the extent that anticopper drugs would correct them, which is much cheaper and safer. Transplantation should never be done for neurologic reasons.

**Response:** We totally agree with the reviewer. These articles stated that the neurological symptoms were relieved after liver transplantation, which was secondary to the correction of copper metabolism. Presently liver transplantation is mainly suggested to patients with liver failure rather than neurological symptoms of Wilson disease. We agree that the present sentence easily makes misunderstanding and it has been revised as “Orthotopic liver transplantation allows the recipient to metabolize copper correctly, preventing progression of disease and it is especially suited to patients with liver failure”.(Page 5, Line 8 to line 10)

**Reviewer #3**

**Point 1:** The hepatic copper concentration in the early group is lower than control group at all time points. However, the control group presents a trend in reduction in
hepatic copper as well. Any explanation for this?

**Response:** We thank the reviewer for the critical comments and the main reason is that tx mice has a self-rescue trend [5, 6]. Consistent with previous studies [6], we have previously revealed that copper accumulation under physiological condition of tx mice reaches to the peak by 4 months of age, which is equal to 8 weeks post transplant in 2-month transplant group. Then liver concentration decreases gradually to half of the peak concentration by 15-19 months old [5]. The control group consistently presented a trend in reduction in hepatic copper at 12 weeks post transplant in the present study. The sentences as above have been added in the Discussion section. (Page 14, Line 5 to Line 11)

**Point 2:** Why is hepatic copper level decreasing in the late group, both bone marrow transplanted and control?

**Response:** We thank the reviewer for the comments. It has been well documented that tx mice is a proper murine model for WD but with a self-rescue trend. In the nature pathogenesis, liver copper concentration reached to the peak by 4 months old and decreased gradually [5, 6]. So in the late group, the hepatic copper level decreased both in the bone marrow transplant and control group, but much more significantly in the transplant group at the 12th week post-transplant.

**Point 3.** Similarly, why ceruloplasmin tends to increase in the late control group?

**Response:** Thanks for the reviewer. The ceruloplasmin tended to naturally improve with time after 4 months old [5]. Therefore, it tended to increase in the late control group.
Point 4. Why AST levels decrease in the late control group?

Response: Thanks for the reviewer. AST was with the same self-rescue trend after the peak time (5 months) [5], so it decreased in the late control group.

Other critiques:

the manuscript requires a major revision of the English language. For example:

Point 5: I recommend the use of “Wilson disease” instead of “Wilson's disease”.

Response: We appreciate the reviewer’s suggestion and have replaced “Wilson's disease” with “Wilson disease” throughout the manuscript.

Point 6: Background, line 3: variable or various organs?

Response: We feel sorry for this mistake. In the revised manuscript, we have corrected it. (Page 5, Line 4)

Point 7: Methods: “...were allocated to one of four groups as followed” or as follows

Response: We feel sorry for this mistake. In the revised manuscript, we have corrected it. (Page 7, Line 8)

Point 8: Methods: please specify DNA method extraction

DNA extraction was specified and added in the Methods as follows: “Genomic DNA was extracted according to the protocol provided by the kit. Briefly, under deep anesthesia with 10% chloral hydrate (5 ml/kg body weight), fresh liver tissue was extracted from the female recipient mice, minced and placed in lysis buffer (50 mmol/l Tris, pH 7.5, 100 mmol/l EDTA, 100 mmol/l NaCl, 1% sodium dodecylsulfate containing proteinase K (0.5 mg/ml) and incubated at 55°C overnight. DNA was bound to the spin column membrane and the remaining lysate was removed
by centrifugation. A filtration column was used to remove cell debris. After washing to remove contaminants, the DNA was eluted with buffer into a collection tube. The purified DNA was prepared for PCR.” (Page 8, Line 17 to Page 9, Line 2)

**Point 9:** Results, page 11, line 1: 2nd week or 4th week?

**Response:** We feel sorry for this mistake. In the revised manuscript, we have corrected it.

**Point 10:** Discussion: “we found that BM cells transplantation starting at 2 months of age corrected liver injury”. I do not think the authors can make this conclusion. Their study is not really looking at parameters of liver injury but essentially only at metabolic parameters.

**Response:** We totally agree with the reviewer that it is subjective to draw this conclusion because our present data is not sufficient enough to support a direct association between bone marrow cell transplantation and liver injury correction. However, our data do suggest correcting the metabolic parameters. So we revised the corresponding sentence as “We found that BM cells transplantation starting at 2 months of age corrected copper concentration, ceruloplasmin and AST in tx mice through 1 to 12 weeks”. (Page 13, Line 5 to Line 7)

**Reviewer #4**

**Point 1:** The writing of the manuscript needs to be edited for English language, currently it is difficult to understand, with several words used incorrectly.

**Response:** We feel sorry for these mistakes. In the revised manuscript, we have
corrected them throughout the manuscript and we had a native English speaker to review the manuscript.

Point 2: The Authors only look in the liver for donor cell engraftment. Why not look at other sites as well to ensure that the liver is the major site of engraftment? The bone marrow, blood and spleen for example.

Response: We appreciate the reviewer for the suggestion and donor cell engraftment in other sites as well as liver should be detected. We have also observed donor cell engraftment in kidney and brain in the present study. Few fluorescence of CM-DiI was detected in kidney and brain (Data were not showed). This sentence has been added in the revised text. (Page 11, Line 10 to Line 11)

Point 3: The Authors determine donor cell engraftment in the female recipient mice only. How many of these female mice were engrafted? What about the male mice?

Response: We appreciate the reviewer for the suggestion. For every time point, 10 mice (female: male = 3:2) were studied in transplant and the control group respectively. Since can only the Sry gene found from the female recipient make senses, in PCR assay part we studied the female recipients (n=6). In other parts of the present study, data came from both the female and male mice recipients (n=10). The corresponding sentences were added in the Methods section as follows: “Female recipient mice (n=6) from each group were sacrificed at 1, 4, 8 and 12 weeks after transplantation for DNA extraction.” (Page 8, Line 15 to Line 17). “Recipient mice (n=10) from each group were sacrificed at the corresponding time points for the analysis as follows” (Page 9, Line 2 to Line 3)
**Point 4:** The best method to determine cell engraftment is to use PCR of the WND gene on all the recipient mice. This method should be used on all the samples. The SRY gene could then be used as a secondary method to confirm the results.

**Response:** We agree that PCR of WND gene is a good method to determine the cell engraftment. Besides, PCR of SRY gene in the female recipient was also widely used as an acceptable method to confirm cell populations in donor cell engrafted organs in the sex-mismatched study [7].

**Point 5.** What is the sensitivity of the SRY PCR? There are very nice bands in the gel photos, suggesting very high levels of liver engraftment, but how much is it? Could the Authors show a positive control, negative control and some mixing studies to give an estimate of the amount of cell engraftment and sensitivity of the method? Could they use real-time PCR?

**Response:** We agree with the reviewer’s points. In previous study, a positive control, negative control and some mixing studies have been performed to confirm the sensitivity of the method [2]. Unfortunately, the sensitivity of the SRY PCR was not determined in the present study. In the further study, these points should be seriously considered. For electrophoresis, products of PCR (20µl) were loaded. In the present study, we aimed to estimate the engraftment using SRY PCR assay and real-time PCR was not performed.

**Point 6.** Is it possible to look for CM-DiI fluorescence in BM cells isolated from the recipient mice by flow cytometry?

**Response:** We appreciate the reviewer. Flow cytometry assessment may be used to
trace CM-DiI fluorescence in BM cells. However, CM-DiI is a fluorescent dye and is mostly studied by fluorescent detection [8, 9]. Consistently, we used immuno-staining method to further trace the engrafted cells.

**Point 7.** How many mice were in each group? The Authors say 40 mice/group with (3:2 female to male ratio), but there are no n values on the graphs or listed anywhere to know exactly how many mice were analysed for each test.

**Response:** We thank the reviewer for the critical comments. We have added the n values in the corresponding figure legends in the revised text.

**Point 8.** How many bone marrow cells were transplanted? The methods section does not mention lysis of the red blood cells, so how many bone marrow cells were transplanted and how many red blood cells? Final concentration in the methods sections states 6x10^7/ml, but then they transplant 0.2ml of 1.2x10^7 cells/mL =2.4 x 10^7 cells transplanted, or should this be 1.2x10^7 cells?

**Response:** We are sorry for this misunderstanding. Red blood cells lysis had been performed and the corresponding sentence has been added to the BM cells extraction and transplantation part of the method section as “Red blood cells were lysed by the addition of blood cell lysis buffer (Solarbio Company, China) for 4 times volume of cells. Tubes were placed on ice for 15 min, then centrifuged at 450 g for 10 min at 4°C” (Page 7, Line 17 to Line 20). We have transplant 0.2 ml of 6 x 10^7 cells/ml (totally 1.2 x 10^7 cells) into the corresponding animals. This sentence was replaced with “Resuspended BM cells with a dose of 0.2 ml were intravenously injected into 2- or 5-month old tx mice”. (Page 8, Line 9 to Line 10)
**Point 9.** Were the mice perfused prior to sample collection? Could the Authors comment on whether they think the cells may just be in the blood rather than in the liver and how they came to this conclusion?

**Response:** Animals were intracardiacally perfused with 0.9% saline prior to sample collection so as to ensure removal of transplanted cell in the blood. This sentence was added in the Methods section. (Page 8, Line 14 to Line 15)

**Point 10.** Para 3 of the Background section suggests that bone marrow cells have been “proved to differentiate into hepatocytes”. Nothing can be proved. There discussion only mentions cell fusion as the mechanism of how the BM cells engraft in the liver…not differentiation. There is still a lot of controversy in the field as to the mechanism and thus the Authors should discuss both mechanisms and then suggest that their work did not attempt to discern between the two theories.

**Response:** We are sorry for the mistake and it was corrected in the revised version. At present, it is controversy how transplanted cells engraft in the recipient liver. We have modified the words as “Hepatocyte can be replaced by bone marrow cells” (Page 5, Line 21). Meanwhile, we have discussed the potential mechanisms and stated that the present study did not aim to investigate the underlying mechanisms in the Discuss section in the revised manuscript. “Therefore, transplanted BM cells may engraft the recipient liver and function to improve liver injury by differentiation or cell fusion mechanisms. However, the underlying mechanisms did not aim to be investigated in the present study. Further study need to elucidate the cell type involved in partial

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disease correction as well as cell fusion in Wilson disease.” (Page 15, Line 16 to Line 21)

**Point 11.** To test whether the donor cells are active they could look for WND gene mRNA. Given the recipient cells do not have the gene it would give direct evidence that the donor cells are active within the recipient mice.

**Response:** Thanks for the critical suggestion. It will be of great values to test WND gene mRNA to give the direct evidence that transplanted bone marrow cells are active within the recipient mice. Although WND gene mRNA was not evaluated, parameters such as serum ceruloplasmin oxidase activity and AST, as well as copper concentration were alternatively assessed, which may imply the activity of donor cells.

**Point 12.** Ref 14 is incorrect it should be Buck et al. 2007, Hepatol Int.

**Response:** We are very sorry for this mistake and it has been corrected in the revised text.

**Point 13.** Please check all the references are correct. There are several incorrectly referenced. For example Ref # 23 does not mention Wilson’s disease mice.

**Response:** We thanks for the suggestion and have checked the references throughout the revised manuscript.

**Point 16.** What is in D-hanks?

**Response:** D-hanks contains NaCl 8.00g, KCl 0.40g, Na₂HPO₄·12H₂O 0.12g, KH₂PO₄ 0.06g and anhydrous dextrose 1.00g in 1L distilled water. We added the corresponding sentence in Method section in the revised text. (Page 7, Line 14 to Line 22)
**Point 15.** The BM cells are transplanted in D-hanks, but the sham transplant control is saline. The authors need to explain why they did not use the D-hanks as the correct sham transplantation control. Or explain why the cells were not in saline.

**Response:** Thanks for the reviewer’s criticism. Setting control as the reviewer’s suggestion will be better. Both D-hanks and saline are mineral salt water and other research work also use buffer to resolve cells while saline as control [10].

**Point 16.** Figure 1 shows some images of 4 weeks post transplant. Which group was this mouse from? How much engraftment did it have? Do the values and number of cells detected correlate?

**Response:** The represent photograph came from the early stage (2 month) treated group. In the present study, recipient mice were transplanted with the same dose of bone marrow cells. Thus the correlation between them cannot be done.

**Point 17.** If the error bars are overlapping, how can the results be significantly different?

**Response:** Data were present as mean ± SEM and the significant differences were marked in the corresponding figures.

**Point 18.** Need to add n values to all the graphs.

**Response:** We have added the n values in the corresponding figure legends in the revised text.

**Point 19.** Figure 2: A) has a final copper concentration of about 800, whilst B has an initial copper concentration of 1100…shouldn’t they be the same, or very similar?
Response: In our previous study [5], during the nature illness course of tx mice, copper concentration changes with time, peaking at 4 months and gradually decreasing thereafter. Therefore, the copper concentration at 5 months is higher than that at 2 months old.

Point 20. The abstract suggests that there is “significantly improved copper accumulation”, which is the opposite of what the results show and what the aim of the experiments were to do, could the authors re-word this sentence.

Response: We are sorry for this mistake and re-worded the corresponding sentence as “BM cells transplant at early stage significantly corrected copper accumulation, AST across the observed time points, and serum ceruloplasmin oxidase activity through 4 to 12 weeks in tx mice compared with those treated with saline (P<0.05).” (Page 3, Line 17 to Line 20)

Point 21. What sizes are the PCR products produced in the SRY assay?

Response: The PCR product of SRY was 444 bp. The corresponding sentence was added in the Method section. “The primer sequences for Sry gene were 5’-TGGGACTGGTGACAATTGTC-3’ (forward) and 5’-GAGTACAGGTTGTGCACCTCT-3’ (reverse), with a predicted product of 444 bp.” (Page 9, Line 13 to Line 15). “The primer sequences for β-actin gene were 5’-ATGGATGACGATATCGCT-3’ (forward) and 5’-ATGAGGTAGTCTCTGTCAGG-3’ (reverse), with a predicted product of 1110 bp.” (Page 9, Line 16 to Line 18)

Point 22. Methods section: Statistical analysis provides a probability value rather than a possibility value, please fix wording.
Response: We corrected the word in the revised text. (Page 10, Line 20)

Point 23. What % viability did the cell preparation need to be for transplantation? Minimum of 90% viable cells?

Response: Thanks for the criticism and we added the sentence in the revised manuscript as following: “Before transplantation, the number and viability of cells were estimated by trypan blue exclusion test as the standard protocol. Viable cells accounted for 98%.” (Page 8, Line 4 to Line 6)

Point 24. What computer program was used to estimate the ratio of SRY and β-actin?

Response: The ratios of SRY to β-actin bands were analyzed with Image pro plus image analysis software. The corresponding sentence was added in the Method section in the revised text. (Page 9, Line 21 to Line 22)

Point 25. “Additional tissue from liver, brain and kidney were sorted”, should read “Additional tissue from liver, brain and kidney were stored”.

Response: We replaced the word “sorted” with the word “stored” in the revised text. (Page 9, Line 6; Page 10, Line 11)

Point 26. What sort of blood tubes were the blood samples collected in?

Response: Blood samples were collected in heparin-containing tubes and the corresponding sentence was added in the revised text. (Page 9, Line 7)

Point 27. The Background section (para 1) says the sites of copper accumulation are ‘variable’. This is incorrect, I think the authors have used the wrong word, maybe a better word would be ‘various’?

Response: We replaced the word ‘variable’ with ‘various’ in the revised text. (Page 5,
Line 4)

**Point 28.** Para 2 of the Background section should highlight that the recent evidence has been derived from rat experiments.

**Response:** We have highlighted that the recent evidence has been derived from rat experiments in Background. The revised sentence is as follow “Recent evidence has indicated that hepatocyte transplantation not only provides temporary liver function but also is able to cure certain metabolic conditions in Wilson disease rats.” (Page 5, Line 12 to Line 14)

**Point 29.** Figure legend 1 compares the amount of engraftment in the two groups to the saline group. The saline group would not be expected to have any cells, so the analysis is incorrect. I think this may be a typographical error rather than a data analysis problem.

**Response:** We are sorry for this mistake and correct this sentence in the revised text as “*P<0.05, compared with animals from 5 months of age group.” (Page 23, Line 10 to Line 11)

**Point 30.** Please provide the error values for the liver copper results in the text.

**Response:** Thanks for this suggestion and we have added the corresponding error values in the revised text. The corresponding sentence was corrected as “Liver copper was decreased by approximate to 21.2 ± 8.2 % at 1\textsuperscript{st} week, 20.3 ± 5.9 % at 2\textsuperscript{nd} week, 19.7 ± 7.4 % at 8\textsuperscript{th} week and 29.7 ± 8.3 % at 12\textsuperscript{th} week after in mice treated with BM cells transplantation at 2 months of age compared to those in age-matched mice with saline transplantation respectively (all *P<0.05).” (Page 11, Line 14 to Line 17)
**Point 31.** Figure 1 mentions green arrows…they are white.

**Response:** We are sorry for this misunderstanding and correct this sentence as “Representative immunofluorescence staining for CK-18 in the recipient liver shows CM-DiI labeled donor BM cells (red) stained positive for the hepatocellular antigen CK-18 (green) at 4 weeks post-transplant (Merged fluorescence was indicated by arrow).” (Page 23, Line 11 to Line 14)

**References**


