ANSWERING REVIEWERS

November 30, 2014

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 14271-review.doc).

Title: Early activated hepatic stellate cell-derived molecules reverse acute hepatic injury

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Name of Journal: *World Journal of Gastroenterology*

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The manuscript has been improved according to the suggestions of reviewers:

1 Format has been updated

2 Revision has been made according to the suggestions of the reviewer

(1).Following the comments of peer review, we added some contents including:

1). We add page number throughout the manuscript.

2). We add the detailed information regarding the environment and medium compositions for growing HSCs in the Materials and Methods section.

3). We write the full name of the terms demonstrated in the text in the first time before using the abbreviations, such as DMEM, FBS, and alpha-SMA in the Materials and Methods section.

4). We define "blank conditioned medium" clearly for the control group in the Materials and Methods section.

5). We add the methods and detailed information for Figure 3 including the vehicle in the control group to demonstrate the effects of HSC lysate on the survival rate in the Materials and Methods section.

6). We are sorry the false statements statistical methods. Actually, we used non-parametric Mann–Whitney U-test, or one-way analysis of variance to perform comparisons between groups.

7) We add the part of "comments".

The above revises have been marked in the original text

(2). We revised the errors, typos, including:

1)"portal vein" in the fig legend, we add 1,and 2 means portal vein and center vein.

2) Figure 1-"A:HSC(24h)", "B:HSC(5d)" and "C:HSC(p3)" changed to "A: HSC (24h)", "B: HSC (5d)" and "C:HSC (p3)". 5, Figure 3- "HSC(5d)" and "HSC(p3)" changed to "HSC (5d)" and "HSC (p3)". 6, Figure 5- "HSC -CM(5d)" and "HSC -CM(p3)" should be changed to "HSC-CM (5d)" and "HSC-CM (p3)".

3) Label for figure 1 says SMA instead of MSA; Figure 4B: Statistical analysis of number of experiments/ standard deviation not provided for the graph, we add the standard deviation .

4) We revised the Fig 3, Fig 4, Fig6, which are split pictures including flow charts, line graphs, histograms, and graphs including text now.

(3) For some questions raised by reviewers, we modified one by one, including the following:

1) The Abstract of text provides reviewer with little information. For example, 'Different morphologies and phenotypes were observed between initiation HSCs and perpetuation HSCs'. Furthermore, the conclusion of

Abstract is redundant. As the suggestion of the reviewer, we have rewritten the part of "Abstract".

2) Some of the results were re-stated in the Discussion section, and little discussion was shown in the Discussion section. Please add more discussion regarding the comparisons between the previous and present studies as well as the possible mechanisms. As the suggestion of the reviewer, we revised the "Discussion section".

(4) For some questions raised by reviewers, we explained one by one, including the following:

1) The dose of cells administered was 2 × 106 per subject. The majority of experiments were performed with the optimal cell mass of 2 × 106 cells. Please provide additional information for the selection of dosage. 2. Acute liver injury was induced by intraperitoneal injection of APAP in phosphate-buffered saline (PBS) at the dose of 750 mg/kg. How to make sure the procedure is successful or not.

Answer: Actually, the dose–response graph of animal survival was done, which was showed in Figure 4B; we found that the effect of HSC (5d) concentrate abrogated at higher cell masses, indicating a therapeutic window of effectiveness. The best dose for mice survival was 2×10^6 cells. But the HSC-CM (P3) could not improve mice survival in all cell masses.

Acute liver injury was induced by intraperitoneal injection of APAP in phosphate-buffered saline (PBS) at the dose of 750 mg/kg. This dose was selected by our experience for observing C57 mice's liver injury and survival, which following the past classic studies (such as: Kofman AV, Morgan G, Kirschenbaum A, Osbeck J, Hussain M, Swenson S, Theise ND. Dose- and time-dependent oval cell reaction in acetaminophen-induced murine liver injury. Hepatology 2005;41:1252-1261. Liu ZX, Han D, Gunawan B, Kaplowitz N. *Neutrophil depletion protects against murine acetaminophen hepatotoxicity. Hepatology 2006;43:1220-1230.)

2) The authors had done a nice protein assay to show different molecules existed in the culture medium between HSC (5d) and HSC (P3). However, it would be great the authors could also show the dynamic expression of these significant molecules during each passage of HSC

Answer: This is a very good suggestion. We have used immunofluorescence and electron microscopy to analysis of the different stages of activation HSCS, and we found no obvious difference of biological morphology between primary cultured for 14 days and third generation of subcultured HSCs. Therefore, we hypothesized that cell secretory capacity of each passage may be similar. In this study, we did not observe the expression of molecules during each passage of HSC. Maybe, we will try to explore in subsequent studies.

3) Is there anyone who has ever used the methods concerning about HSC-CM and in vivo injection? Please list their articles in the reference. According to the knowledge of reviewer, HSC-CM should be processed by 0.22 μm filtration to avoid the bacterial contamination. Injection of HSC-CM without filtration probably leads to infection and sepsis

Answer: Based on our work, there is no study has ever used the methods concerning about HSC-CM and in vivo injection before. We are the first time report this method, but there is some consistent results between our research and other's research, which were described in the discussion section.

As sterile problem, actually, cell culture dish, condition medium concentrated device, and CM storage device used in this study were sterile. For HSC-CM, HSCs were cultured in serum-free DMEM supplemented with 0.05% bovine serum albumin. Supernatants were prepared by collecting serum-free medium after 24 h culture. The mediums were then concentrated approximately 25-fold using ultrafiltration units (Millipore, Bedford, MA, USA) with a 3 kDa molecular weight cut-off. Therefore, there was no bacterial contamination in HSC-CM, which need not be processed by 0.22 μm filtration to avoid the bacterial contamination.

4) As reported by figure 2 HSC5d treated mice start die after 24 h and continued till 72 h, but the authors reported hepatocytes necrotic analyses only at 24h, could be interesting to evaluate immuohistochemistry analyses of mice at 48, 72 and 96h to have a better understanding of the entire process. The Authors reported

no inflammatory infiltrate in liver of mice treated with HSC5d lysate at 24h, have the Authors evaluate infiltrate at different time point such as 48, 72 or 96? The HSC5d could delayed the recruitment?

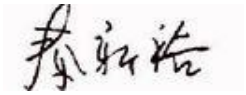
Answer: Your opinion is very good, we observed the APAP-induced liver injury level in experimental studies, and observed most severe liver damage at 24h after APAP injection, liver necrosis area will recovery, until to 96 hours necrosis disappeared and back into normal liver. This is consistent with previous findings (Liu ZX, Han D, Gunawan B, Kaplowitz N. *Neutrophil depletion protects against murine acetaminophen hepatotoxicity. Hepatology 2006;43:1220-1230.).

We further analyzed the serum transaminase levels at 12h, 24h, 36h, 48h after APAP injection. We found that serum transaminase levels were highest at 24h which was associated with the most severe liver damage. After the HSC-CM (5d) intervention, transaminase levels have a difference between HSCs (5d) group and vehicle at 24h point, however no difference was found on other time points. Based on these findings, we have chosen this point , 24h as the mainly observed time point.

3 References and typesetting were corrected

Thank you again for publishing our manuscript in the *World Journal of Gastroenterology*.

Sincerely yours,



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