April 25, 2015

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 17160-review.doc).

Title: Overexpression of pim-3 and its protective role in LPS-stimulated rat HSCs

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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) Abstract section has been rewritten according the format and the suggestions of the reviewers.

(2) Materials and Methods, and Results sections have been improved.

(3) The manuscript has been polished in English language by a native English speaking expert (certificate is attached)

(4) The specific responses to the reviewers are attached as follows.

3 References and typesetting were checked and corrected.

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

Sincerely yours,

Ji-Xiang Zhang, MD

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Reviewed by 02439020

1. The "Abstract" is too simple and not well managed. Please write the "abstract" section properly and include all the important information in your study.

Response: We have revised the Abstract according to the format of WJG.

2. A reference is needed regarding the rat hepatic stellate cell lines HSC-T6 gifted from Scott L. Friedman'

Response: We have added the reference.

3. The "Material and Methods" section is too simple, no mention of many methods, such as flow cytometry analysis.

Response: We have given more details in this section, including the methods used.

4. The "Results" section is very confusing and hard to understand, and needs to be reorganized and properly written to enable a better understanding of the data. In the third paragraph of "Results" section, the title is misleading, please check it and correct it.

Response: We have rewritten the Results section.

5. More fresh references within 5 years are more persuasive.

Response: We have cited more references published within recent 5 years.

6. The English of the manuscript is pretty ordinary, and not very scientific.

Response: We have had our manuscript polished in English language by a native English speaking expert. from the editing service company recommended by WJG.

Reviewed by 02822399

1 The calculation of OD is strange. Is it 52.33? Or it is percentage of results calculated in relation to control? Why the control in Tables 1 and 2 has values other than 100%.

Response: The OD of 52.33% means 0.5233 instead of the ratio of the calculated results related to control.

2 In the Discussion: "Normal cells induced by special stimulus can also up-regulate expression of pim-3 kinase, such as anoxia/reoxgenation injury or ischem/reperfusion injury or cytokine or LPS treatment [9,10]". It has been reported previously that pim3 is overexpressed by LPS? What is the novelty of your manuscript.

Response: LPS could up-regulate pim-3 in intestinal mucosa stimulated by LPS, which was reported by our laboratory several years ago from an animal experiment. However, there is no study about hepatic stellate cells, and in this study we focused on cells instead of animal or tissue. Thus, we believe that this may represent the novelty of our manuscript.

3 Why did you starve your cells in the culture protocol. This starvation will reduce viability and increase apoptosis, while your results illustrated the opposite.

Response: It is common to starve hepatic stellate cells in the culture protocol, and the culture protocol has been presented by another article. For example, to determine the effect of LPS on proliferation of activated HSCs in mice, activated HSCs were cultured in 10% FBS, and cells experienced a lower concentration of 1% FBS, and were stimulated with LPS as shown by the Reference below:

Brun P, Castagliuolo I, Pinzani M, Palu G, Martines D. Exposure to bacterial cell wall products triggers an inflammatory phenotype in hepatic stellate cells. Am J Physiol Gastrointest Liver Physiol. 2005. 289(3): G571-8

4. Regarding the significance signs in the figures: - In Figure 2, you used: *P<0.05 vs 0h and 3h. **P<0.01 vs 0h. You should use different signs for different time points. In addition, it appears that in Figure 2D, there is a significant difference between 0h and 3h. - In Figure 3, you did not measure the significance against LPS group. - In Figure 4b, you did not measure the significance of LPS against control group.

Response: Yes, we have made revisions in all these figures as you suggested.

Reviewed by 02447901

1 The spontaneous apoptosis of HSC-T6 cells over 16% was strange. Generally, cell lines grew well. As said in Materials and Methods "For experiment, HSC-T6 cells were seeded into 96-well plates at 1 × 104 cells/well. After being cultured in complete medium for 12 h, HSC-T6 cells were starved for 24 h in DMEM supplemented with 0.75% fetal bovine serum, then the culture medium was replaced with DMEM complete medium." Why the cells should be starved prior to experiments? How about the outcomes of regular cells culturing growth media?

Response: The spontaneous apoptosis of HSC-T6 cells is often high, and according to some literatures, it can reach about 20%.

(Wang Y, Gao J, Zhang D, Zhang J, Ma J, Jiang H. New insights into the antifibrotic effects of sorafenib on hepatic stellate cells and liver fibrosis. J Hepatol. 2010. 53(1): 132-44.(DOI: 10.1016/j.jhep.2010.02.027; PMID: 20447716)

And the protocol that HSC was subjected to starve prior to experiments were also presented in other previous studies. For example, to determine the effect of LPS on the proliferation of activated HSCs in mice, activated HSCs were cultured in 10% FBS, and cells experienced a lower concentration of 1% FBS, and were stimulated with LPS.

(Brun P, Castagliuolo I, Pinzani M, Palu G, Martines D. Exposure to bacterial cell wall products triggers an inflammatory phenotype in hepatic stellate cells. Am J Physiol Gastrointest Liver Physiol. 2005. 289(3): G571-8)

2. In Tables 1 and 2, the value in control was 50.33+/-2.3%. How to get this value?

Response: The value of 50.33+/-2.3% in the control means 0.5033+/-0.0233.

3. The figure legends should state the experimental conditions.

Response: Yes, we have added these in the figure legends.

4 Normally, LPS shows mitogenic and fibrogenic effects on hepatic stellate cells. In addition to proliferation and apoptosis, the changes in fibrogenic genes should be investigated.

Response: Pim-3 is a gene of pro-proliferation and anti-apoptosis, we did not investigate its fibrogenic effects on hepatic stellate cells stimulated by LPS. And anti-proliferation and pro-apoptosis of HSCs is one of the approaches for inhibiting hepatic fibrosis.

5. In Figure 2, paired control should be included in time course study.

Response: Figure 2 shows that LPS up-regulated mRNA and protein expression of pim-3. In the time course study, the samples with different LPS-stimulated course were collected and treated at different time points within designated duration. As shown in the text, the cells without stimulation served as controls. The data are expressed in line chart in order to reflect the expression change over time. Data are expressed as mean ± SD (n = 3). $^{a}P <$ 0.05 *versus* control, 0 and 3 h. $^{b}P < 0.01$ *versus* 0 h.

6. According to Table 1, HSC-T6 cells grew well at 24 and 48 h after seeding. If the cells kept growing, the role of spontaneous apoptosis was bare.

Response: MTT reflects the cell proliferation by the enzyme activity of mitochondria, and OD of MTT can indirectly represent cell number. The cell number depends on cell growth and cell spontaneous apoptosis. When cells quickly step into cell cycle and complete cell cycle, cell growth is considered well; at the same time, partial cells show spontaneous apoptosis. Thus, a good condition of cell growth did not mean bare apoptosis. And cell proliferation was not necessarily associated with cell apoptosis.

7. In Table 2, the silence of pim-3 attenuated LPS-stimulated proliferation. However, si-pim3 plus LPS-treated cells still showed better viability than si-pim3-treated cells. These findings suggest that LPS also showed proliferative potential in si-pim3-treated cells. Then, prosurvival mediators other than pim-3 might present.

Response: Yes, we believed that there are other pro-survival mediators besides pim-3.

8. As said in Discussion "However, about the proliferation of HSC in vitro stimulated by LPS, different cells and different detection assays have different outcomes. The proliferation from primary HSC assessed by [3H] Thymidine incorporation is unchanged[5], while that from HSC lines assessed by MTT is increased[16]." If so, what is the conclusion after this study?

Response:

1). We focus on the pim-3 expression and its role in LPS-stimulated rat HSC, and not on the proliferation and apoptosis of HSCs induced by LPS, and have found that overexpression of pim-3 plays a protective role in LPS-stimulated HSC-T6 cells

2). Results from MTT assays and [3H] Thymidine incorporation were not contradictory. MTT results indirectly reflect the cell number, which is the net outcomes of cell proliferation and cell apoptosis, however, the assays of [3H] Thymidine incorporation reflect the proliferation by means of DNA synthesis, which represents the cell number of entering cell cycle.