Manuscript NO.: 53046

Title: Calpain-2 activity promotes aberrant endoplasmic reticulum stress-related apoptosis in hepatocytes

Dear Editor:

We thank you for your encouragement and advice. We would like to resubmit the manuscript for your further consideration as an original research article for publication in *World Journal of Gastroenterology*. In response to the reviewer's advice, we have modified the manuscript title as "**Calpain-2 activity promotes aberrant endoplasmic reticulum stress-related apoptosis in hepatocytes"** in the revision**.**

We also thank the reviewers for their constructive comments. Accordingly, we have made the necessary changes in the manuscript to acknowledge the concerns raised by you and reviewers, as well as to fully meet the requirements of the publishing policies of *World Journal of Gastroenterology*. As a result, we believe that we have addressed all of the questions and the quality of the manuscript is greatly strengthened. The major changes in the revision have been marked in red and please see our point-by-point responses below. In addition, we have carefully checked every sentence in the revision to eliminate/reduce any potential syntax error. This revised manuscript has been proofread by two native English biologists from *Medjaden*, a professional publication service company. We think that this manuscript is easily understood in terms of a scientific story and its language writing.

If I can be of any assistance regarding the process of this manuscript please contact me. We look forward to hearing from you soon.

Sincerely,

Dr. Bing Han
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First of all, we would like to express our sincere gratitude to the reviewers for their helpful and valuable comments.

Replies to the Reviewer 1,

Specific Comments

1. The title should be short and concise. According to recent studies that would favor future citations to the paper. What is really new in the paper? The DTT effect? The activity of calpain? its inhibition? Some words in the title should be avoided.

Response: We appreciate his/her advice. Now, we have modified the manuscript title as "**Calpain-2 activity promotes aberrant endoplasmic reticulum stress-related apoptosis in hepatocytes"** in the revision.

2. Abstract should be as quantitative as possible for rapid comparison with similar studies. Avoid imprecise terms such as significantly up regulated …but how much? -….."dramatically decrease…..but how much 100%?; 2-fold?. On the other hand, if the effect is not statistically significant no need to mention it because it could be only a tendency not a fact.

Response: We appreciate his/her advice. Now, we have added real values in the revision, including in the abstract of the revision.

3. Globally, the results are not properly described. The authors should first describe in a quantitative manner the data before jump to conclusions. Avoid imprecise and/or qualitative terms such as for example significantly deteriorated…but how much?

Response: We appreciate his/her advice. Now, we have added real values in the revision, including in the abstract of the revision.

4. Figure 1 A. The x-axis should be linear? an equation with the data should be obtained and referred in the legend and use for determination of a precise LD50 and also with a SD

Response: We understood his/her comments. DTT is an inducer of ER stress and its toxicity against BRL-3A cells was near linear in the dose range we used. We did calculated a LD value of about 4 mM and presented the real percentages with SD in the Figure 1. Now, we have calculated the data to achieve

an equation and described in the legend of Figure 1. In addition, the main purpose of this study was to explore the molecular mechanisms by which calpain-2 regulates aberrant ER stress-related apoptosis in hepatocytes. The goal of testing the toxicity of DTT was to find a sub-toxic dose of DTT for our subsequent experiments, together with the dose of DTT in the literature.

5. Some sentences referred that it appear…..even started with apparently, but in fact it DTT it does inhibits cell proliferation. The authors should be more certain of what are describing. Is this experience a control experience to deduce the IC50 for DTT for the following experiments? If so, why the authors used 2 mM instead of 4 mM in the following experiments?

Response: We understood his/her comments. As we stated above, we would like to use a sub-toxic dose of DTT for our subsequent experiments. Because a higher dose of DTT severely decreased the viability of BRL-3A cells, which may cover many molecular processes, we chose 2 mM DTT in following experiments.

6. Why DTT have the observed effect? Does it complexes with essential metals or oxidized essential proteins that imply the observed effects. The explanation should be referred and discuss.

Response: It is well known that DTT can inhibit the formation of disulfide bonds and lead to the accumulation of unfolded proteins in the ER to induce ER stress. Now, we have discussed its functions briefly as " It is well known that DTT can inhibit the formation of disulfide bonds and result in the accumulation of unfolded proteins in the ER, leading to ER stress" in the discussion section of the revision.

7. The figures should be clearly globally improved, as possible, once WJG deserves high quality figures and with rigor would avoid lacking of interest for the data. Legends should be also as complete as possible.

Response: We are sorry for the quality of Figures in the Word file. Actually, we have checked every Figure and ensured all figures with a pixel of 300 dpi, according to the guidelines of your journal. If requested, we would be happy to provide these high quality of figures in TiF files.

8. The increase of calcium concentration should be referred in nM concentrations. Figure 4 is not clear.

Information should be quantitative and taken from the fluorescence experiments and data at the several experimental conditions.

Response: We understood that it was better to measure the levels of intracellular calcium with a unit of nM. However, due to the technical limitations, the Ca^{2+}-sensitive fluorescent Fluo-3 AM dye and fluorescent microscopy can not quantitatively measure the real concentrations of intracellular calcium with a unit, rather than this method offer the relative levels of intracellular calcium between the experimental and control groups of cells, according to the intensity of fluorescent signals. Currently, we are unable to quantitatively measure the concentrations of intracellular calcium, such as using real time imaging of a living single cell with TILLVision. Because we attempted to test whether ER stress induced by DTT could increase the contents of intracellular calcium, relative to that in the control without DTT treatment, we used a fluorescent microscope and Ca^{2+}-sensitive fluorescent Fluo-3 AM dye, which would not be able to achieve a real concentration of intracellular calcium. With this method, we did find that ER stress induced by DTT increased the intensity of fluorescent signals, reflecting increased contents of intracellular calcium in our experimental condition.

9. Discussion should be more assertive and concise and eventually be divided in sections with titles highlighting the major results.

Response: We understood his/her comments. Now, we have modified the discussion section by adding necessary statements and information. However, we do not prefer to segment the discussion section because combination of different layers of information we discussed together may make more sense and help readers in understanding the story.

10. For instance, Ca-ATPase inhibitors are referred but the role of this P-type ATPase in the ER stress in not mention or referred. Does DTT affect ion pumps? If so, the calcium concentration will increase, stress induced and concomitantly cell death.

Response: These questions were interesting. Now, we have discussed the role of DTT in the revision as stated above. Because the ER holds the major pool of calcium in most cells, ER stress can cause calcium efflux from the ER to increase in the levels of intracellular free calcium. We did find that DTT treatment increased the contents of intracellular free calcium. However, we did not analyze whether DTT could modulate Ca^{2+}-ATPase expression and function, which are not available in the literature. Hence, whether

DTT can modulate Ca^{2+}-ATPase expression and function as well as potential mechanism remain to be further explored in the future studies.

11. In conclusion section, partial conclusions first and then global conclusions would also favor the take home message of the paper. Still, it not clear what is new in the paper.

Response: We appreciate his/her advice. We have modified the conclusion section by highlighting the new findings from this study as "The results indicated that enhanced calpain-2 activity promoted aberrant ER stress-mediated apoptosis of hepatocytes" and subsequently, we concluded that the process and significance of our findings as "Our data suggest that ER stress may induce calcium release from the ER and lead to the recruitment and activation of calpain-2 in the ER, where, calpain-2 activates caspase-12 and caspase-3, and triggers apoptosis in hepatocytes. Therefore, ER stress may be a new therapeutic target and our findings may provide new evidence to demonstrate the importance of calcium-dependent calpain-2 in caspase-12 activation and ER stress-related apoptosis in hepatocytes" in the revision.

Replies to the Reviewer 2

Specific Comments

1. The introduction and the discussion are very truncated and should be expanded.

Response: We appreciate his/her advice. Now, we have added some information to emphasize the rationale and tested measures in the introduction and discussed the significance and novelty of our findings more in the discussion of the revision.

2. There is no explanation about the cell model used and why it was chosen. Were the cells confluent when treated?

Response: We chose rat non-tumor hepatocyte BRL-3A cell line, instead of human hepatocytes as a cell model because the caspase-12 gene in most human hepatocyte lines is mutated and these human cell lines may be not sensitive to ER stress-related apoptosis. We have specified that "When the cells reached at 80% confluency, the cells were treated with DTT, an inhibitor of disulfide bond formation, to induce ER stress" in the method section of the revision.

3. GRP78, ATF4, CHOP and PERK expression was evaluated by Western blotting, however their relationship to liver cells specifically and liver function is not explained or justified. These proteins need to be described and their role in hepatocyte detailed.

Response: We appreciate his/her advice. Now, we have introduced these measures in the introduction section as "During the process of ER stress, the ER chaperon GRP78 changes its binding from ER transmembrane protein PERK to unfolded/misfolded proteins to releases PERK [10]. The released PERK is subjected to self-phosphorylation, which promotes the expression of transcription factors of ATF4 and CHOP in cells [11]. The up-regulated ATF4 and CHOP can enter the nucleus to regulate the transcription of related genes [12]" in the revision. These measures have been widely used for the evaluation of ER stress in many types of cells. However, how these proteins regulate the function of hepatocytes remains controversial, dependent on varying models. As we used them as markers of ER stress, we do not like to speculate much to avoid potential misleading.

4. The apoptosis assay is not well explained and the figures showing PI and Annexin are too small (Figs 1 and 6). There is no explanation about the distribution of cells in the different quadrants. Please explain what the axes and quadrants stand for in those figures.

Response: We understand his/her comments. Now, we have clarified that each quadrant of cells in the Figure 1 legend as "The percentages of mechanically damaged (B1 quadrant), healthy (B3), early (B4) and late apoptotic and necrotic (B3) cells were analyzed by flow cytometry" in the revision. In addition, we have specified "The percentages of apoptotic cells in individual groups were quantified by flow cytometry" in the method section.

5. Fig 1A shows cell viability. At 2mM concentration 50% of the cells are dead. Where are those cells on panel C, they should be PI positive and annexin positive.

Response: We understood his/her comments. As we shown in Figure 1A, treatment with 2 mM DTT reduced the cell viability about 30%, relative to the control without DTT treatment while the same treatment induced about 7% of cells undergoing apoptosis. It is well known that the process of apoptosis is dynamic and many apoptotic cells continually undergo necrosis and other types of cell death. Second, while the MTT assay tested the cell viability the apoptosis assay quantifies the percentages of cells at

apoptosis status. Accordingly, it is not surprising to detect higher frequency of cells with impaired cell viability than that of real apoptotic cells in our experiments.

6. Fig 6 (all panels) shows more late apoptosis/necrosis (PI positive and annexin positive) in upper part of quadrant B3 than Fig 1, although silencing calpain should be protective. Because the experiment and figures are not well explained it is difficult for this reviewer to evaluate the validity of this experiment.

Response: As we explained the flow cytometry analysis of apoptosis assay above, in figures 1 and 6, the B3 quadrant represents healthy cells, the B1 quadrant represents mechanically damaged cells, the B4 quadrant represents the early apoptotic cells and the B2 quadrant represents the late apoptotic and necrotic cells. We used the same methods for flow cytometry and analysis of apoptotic cells with the same gating strategies in Figure 1 and 6. We do not think that the late apoptotic and necrotic cells in the figure 6 are more than those in figure 1 (all B2 quadrats were 0%). The cells in the upper part of quadrat B3 may reflect some cells with mild injury (very low PI stained, but negative for Annexin-V). According to the gating strategies, these cells remained in the healthy quadrat and the number of these cells between Figure 1 and 6 was similar.

7. The initiation of apoptosis by DTT and the concentrations used should be justified from citations in the literature.

Response: We appreciate his/her advice. As stated above, we have specified why we chose the sub-toxic dose of 2 mM DTT for subsequent experiments in the discussion section of the revision and added one reference in the method section.

8. Fig 4 shows labeling with the Ca2+-sensitive fluorescent dye, Fluo-3 AM. The conclusion from this experiment is that the level of intracellular Ca is increasing. This is erroneous. What is shown in that figure is the number of cells positive to Fluo3-AM as compared to the total number of cells. It does not indicate that the level of Ca in an individual cell is increasing. The number of Fluo3 positive cells should be compared to the total number of cells in the field which seems different in all figures. A better way to demonstrate that Ca is increasing in a single cell is to follow live the level of Ca immediately after addition of DTT. Please explain what AM stands for. Ca in the Fig 4 seems mostly extracellular. Since confocal microscopy was used why not use a higher magnification? Was the dye rinsed off before

measurement?

Response: We understood his/her comments. First, the Fluo-3 AM is the name of Ca^{2+}-sensitive fluorescent dye and can penetrate the cell membrane. Intracellularly, Fluo-3 AM can be cleaved by the esterase to form Fluo-3, which binds to Ca^{2+}, resulting in a strong fluorescent signal that reflects intracellular calcium contents. We have added a relevant reference to support this result.

Second, it is true that there are some cells with obvious fluorescent signals while others did not show the green signals. However, the Fluo-3 AM-based fluorescent microscopy or flow cytometry analysis of intracellular calcium contents have been widely used and the intensity of fluorescent signals reflects the intracellular calcium contents[23]. Because the goal of our experiments was to determine whether DTT-induced ER stress increased intracellular calcium contents, relative to that in the control cells, we used the relative intensity of fluorescent signals to show intracellular calcium contents in Figure 4B.

Furthermore, as stated above, we do not have specific instrument for real-time imaging the real concentrations of intracellular calcium in a living single cell so that we chose the current method. If available in the future, we will test the real concentrations of intracellular calcium. We did not take high magnification images because we tried to capture more cells in one image. In the future, we will take higher magnification images to analyze it.

Finally, we have specified that the cells were washed twice using 1.0 ml HBSS before measurement in the method section of the revision.

9. Fig 5. It is very difficult to conclude that the dye is in the ER. Since confocal microscopy is available a higher magnification should be used.

Response: It is true that we would be very difficult to observe the ER without specific method because the ER is an organelle and can only be observed by transmission electron microscopy. In this study, we analyzed calpain-2 location by immunofluorescent microscopy after stained the cells with fluorescent anti-calpain-2 and ER-track Red, which is a specific red fluorescent dye for marking the ER. This method has been used by several groups of researchers[25, 26]. We found that some anti-calpain-2 stained signals were overlapped with ER-track red signals, indicating that calpain-2 accumulated in the ER. We think that these data are credible. We did not use a laser confocal microscope to take images because the instrument was out of service when we performed the experiments.

Additional Minor Comments:

1. Please expand the description of your Methods.

Response: We appreciate his/her advice. Now, we have added necessary information in the method section. Because these experimental procedures have been widely used we decided to add a reference to reduce unnecessary repeating in the manuscript.

2. N-succinyl-Leu-Leu-Val-Tyr-AMC is cited without explanation or definition. Please describe.

Response: N-succinyl-Leu-Leu-Val-Tyr-AMC is a fluorescence substrate of calpain. Now, we have specified it in the revision as "We measured cellular calpain enzymatic activity using a fluorescent substrate *N*-succinyl-Leu-Leu-Val-Tyr-AMC, as described previously [24].

3. This sentence should be corrected "The ER stress-related apoptosis is independent of mitochondria and death receptors, rather than is mediated by activating caspase-12 [30-32]. Activated caspase-12"

Response: We have changed the sentence to "The ER stress-related apoptosis is independent of mitochondria and death receptors, and has been thought to be mediated by activating caspase-12, an ER-anchored caspase' in the revision.

Replies to the Reviewer 3

Specific Comments

1. The methodology is not clear. Authors have not mentioned what group of rats were selected and how the BRL-3A cells were harvested and treated with varying doses of dithiothreitol.

Response: In this study, we did not use any animal. In addition, we used 2.0 mM DTT to treat BRL-3A cells for 0, 6, 12 and 24 h in most experiments. We have modified the method section by adding necessary information in the revised manuscript.

2. A few grammatical corrections are noted in the returned manuscript. They may be incorporated in the revised paper.

Response: We are sorry for our carelessness. Now, we have carefully checked every sentence in the revision to eliminate/reduce any potential syntax error. This revised manuscript has been proofread by

two native English biologists from *Medjaden,* a professional publication service company. We think that this manuscript is easily understood in terms of a scientific story and its language writing.

Replies to the Reviewer 4

Specific Comments

1. What is the Number of plates in each expremens.

Response: As we stated, all experiments were performed in triplicate from three separate experiments.

2. The discussion is deficient manysentences without references need more in depth discussion of the results compared to other studies

Response: We appreciate his/her advice. Now, we have modified the discussion section by centering on the novelty, significance of our findings and the similarity and difference of our findings with others reported in the revision.