Damian Garcia-Olmo, MD, PhD
Editors-in-chief
World Journal of Gastroenterology

Dear Dr. Garcia-Olmo:

Please find the attached revised version of our manuscript entitled "Caspase-12 mediates carbon tetrachloride induced hepatocyte apoptosis in mice, Hua Liu, Zhe Wang, and Michael J. Nowicki" (World Journal of Gastroenterology, ESPS Manuscript NO: 11127).

We appreciate your and the reviewers' critical comments and thoughtful suggestions. Following these comments and suggestions, we have revised the manuscript. All text changes are in blue in the manuscript. We hope that the revised manuscript will now be acceptable for publication. Please let us know if you and reviewers have any further questions, and we are very willing to revise the manuscript again. Our point-by-point responses to the reviewers' comments/questions are listed following the letter.

With kind regards.
Sincerely yours,

Hua Liu, MD.

Assist professor of Pediatric Gastroenterology.
University of Mississippi Medical Center,
2500 N. State St, Jackson, MS 39216
E-mail: hliu@umc.edu
Phone: 601-984-5978

Reviewer 1:

Main critique:

1. A major pitfall of the paper is the lack of measurement of the ER stress cascade. As the authors point out in their introduction, Pro-caspase-12 is expressed predominantly in the ER and is activated upon ER stress. Yet they show no evidence of ER stress activation upon CCl4 treatment.
   By including some ER stress markers (PERK, CHOP, IRE-1) would significantly strengthen the manuscript and might help to answer how caspase 12 -/- mice are able to attenuate apoptosis following CCl4 treatment, is it by attenuating the ER stress response?

   Response:
   We thank the reviewer for the constructive suggestion. We examined ER stress responses in the CCl4-treated mice using the preserved frozen liver tissue. Western blot results showed that GRP78, CHOP and IRE-1 were up-regulated in the liver tissue from both caspase-12+/+ and 12-/- mice treated with CCl4 (Figure 2 in the revised manuscript). *caspase-12* gene knockout has no effect on the inductions of these proteins suggesting that activation of caspase-12 is a downstream event of ER stress. We have added the new result in the revised manuscript in "Results" and "Discussion".

2. Another area the manuscript is lacking is fully characterizing the extent of liver damage. For instance, the author's only present ALT data to suggest liver damage, other markers of liver damage should be used to fully support this notion.
   Why is aspartate aminotransferase (AST) not measured as both ALT and AST are well established markers of hepatic damage?

   Response:
   Liver function is commonly measured by ALT and AST. Only ALT was measured in the current study because of limited serum sample sizes from the small animal mice. The main purpose of this study is to investigate the apoptotic mechanism in CCl4-treated mice. There are many published studies used ALT measurement only when liver functional study is not the major focus (Transplant Proc. 46(5):1567-72, 2014; Toxicology doi: 10.1016/j.tox.2014.02.008, 2014; J Cell Mol Med. doi: 10.1111/jcmm.12320, 2014…)

3. Authors conclude that attenuating caspase-12 reduces hepatic damage without offering any mechanistic evidence as to how this is achieved.

   Response:
   Caspase-3 is an effector caspase. Once activated, it cleaves key substrates required for normal cellular functions leading to apoptosis. Studies from others and the current study show that caspase12 activates caspase-3 downstream resulting apoptosis. These have been described in second and third paragraphs from the end of the "Disscusion".

4. It has been shown that liver regeneration is closely related to activation of apoptosis and hepatic damage. Activation of the nuclear proliferative marker Ki-67 has also been increasingly implicated in liver regeneration following injury. As such, the manuscript would benefit from assessing proliferation capacity as well.
   If the authors believe that caspase-12 promotes hepatocyte apoptosis through cytochrome release more work is needed to address how mitochondrial function and integrity is affected by both CCl4 treatment and caspase-12 activation.
   Since caspase-12 activation is ER stress mediated, the paper would benefit from looking at the cross talk between the ER and mitochondria following CCl4 treatment, as both organelles have been shown to be dependent on one another.
   Finally, the authors make the conclusion of a positive effect on hepatic integrity by attenuating caspase-12 activation, but in fact no significant analysis is done to really characterize these beneficial effects such as improved mitochondrial integrity.

   Response:
   Previous study has shown that there is a crosstalk between the ER and mitochondria (Oncogene 19: 2286–2295, 2000). In fact, our current study showed the release of cytochrome C and activation caspase-9. However, caspase-12 gene knockout mice significantly reduced caspase-3 activation and provided marked protection against CCl4-induced apoptosis. It appears that mitochondria do not play a key role in CCl4-induced apoptosis in the liver. It will be interesting to examine the mitochondrial function and integrity, and nuclear proliferative marker Ki-67 in our future study, but these are beyond the scope of our current manuscript.

Minor critiques:

1. Figure 5 G: The bottom x-axis of this figure is cut off.

   Response:
   This error has been corrected. Now it is Figure 6 in the revised manuscript.

2. Perhaps, doing some histology like H and E to characterize liver damage. As well as looking at mitochondria integrity through transmission electron microscopy.

   Response:
   In the current study, apoptotic cells were quantified by a modified TUNEL protocol, and were controlled by morphological study (more details in “Methods” and “Results”). These examinations were done by Dr. Steven A. Bigler. He was a pathologist and the chairman of Department of Pathology in our institution. Dr. Bigler left the institution and went to private practice last year. His laboratory was closed. We can no longer trace the tissue blocks and tissue slides.

3. Discussion should elaborate more on the clinical significance of the findings with regards to illness symptoms in humans exposed to CCl4 and the implications of inhibiting caspase-12 clinically for patients.

   Response:
   We have added more clinical information about CCl_4 toxicity in the last paragraph of the "Discussion".
   "The primary effects of carbon tetrachloride in humans are on the liver, kidneys, and central nervous system. Human symptoms of acute (short-term) inhalation and oral exposures to carbon tetrachloride include headache, weakness, lethargy, nausea, and vomiting. Acute exposures to higher levels and chronic (long-term) inhalation or oral exposure to carbon tetrachloride produces liver and kidney damage in humans".

Reviewer 2:

1. the KO mouse was purchased but nevertheless it should be described. Is it a general KO or cell-type specific? Can any literature be cited where this mouse has been characterized?

   Response:
   They are general caspase-12 gene knockout mice. This information has been added in "Methods" and a reference regarding the development of the mice has also been provided.

2. the amount of CCl4 applied to the mice is given as "µl/kg BW". Instead of "µl" the actual amount (e.g. "µg") should be given. Whether 300 µl is indeed a small amount (as stated by the authors) cannot be judged

   Response:
   In most of the publications, administration of CCl_4 is described as µl or ml/kg BW. We have calculated the dose from ul to ug and added this information in the "Methods".

3. in the methods description for determination of lipid peroxidation it is only stated "as described". A brief summary of the method must be given anyway. Was MDA measured in whole liver lysates?

   Response:
   A brief summary of the method was added to the "Methods".

4. the main experiments were only performed at one time-point (24h after CCl4). This does not allow to conclude how the complete process of recovery was affected. At which time would apoptosis and ALT reach normal levels again and was this different in the KO-animal? Like this it cannot be stated that lack of Caspase 12 indeed reduces damage. It seems so at the 24h time-point but how about later times? May be the whole response is only delayed in the KO mice but not really inhibited?

Response:
The *caspase-12* knockout mice were initially developed by Dr. Yuan Junying's laboratory at Harvard University. Yuan et al demonstrated that the *caspase-12-/-* mice significantly decreased renal tubular apoptosis induced by ER stress inducer tunicamycin at day 4 (Nature 403:98-103, 2000). Based on this observation, we did an initial pilot experiment and examined CCl4-induced hepatocyte apoptosis at day 1, 4 and 7. The result showed that hepatocyte apoptosis in wild-type mice started to decrease at day 4 and significantly reduced at day 7, while the apoptosis in *caspase-12-/-* mice remained insignificant during the course of the experiment. Our intention of the study is to examine the acute phase of injury. Previous study showed that CCl4 induced hepatic apoptosis as early as 6h (Am J Pathol 1998; 153:515-525). Thus we did the pilot study in the current manuscript to determine the starting peak of CCl4-induced hepatocyte apoptosis. The result showed day 1 (24h) as the starting peak, thus we chose this time point for the majority of experiments in the study.
I have done Medline and Pubmed searches for the studies using *caspase-12-/-* mice. No study has examined apoptosis beyond 4 day in different model/tissue injury.

5. fig. 1C: should also be a bar diagram sice the individual data are not obtained from the same animal at each time-point. The symbols are too small as well. "MDA" should be explained in the legend. –

   Response:
   As the reviewer recommended, we changed Figure 1C to bar graph and MDA is explained in legend.

6. it is stated several times that lipid peroxidation started to increase after 6h. This is not correct, as shown in fig. 1C at 6h all groups show similar levels and are not increased yet. At 12h there is a clear increase but when this started was not investigated (as stated above: a curve is not the correct presentation here). –

   Response:
   We mentioned ROS generation at 6h in the "Results" for explanation old Figure 2 by mistake. This error has been corrected. We also mentioned ROS generation at 6h in "Discussion". However, this is an observation by other investigators in their publication.

7. Fig. 5C: What kind of samples were investigated here? Whole liver lysates or isolated cells? If the Cytochrome-C was measured in whole cell (or liver) lysates it should also include the mitochondria, right? This means that the statement "cytosolic Cyto-C" would not be correct..

   Response:
   We thank the reviewer to point out this. We forgot to describe the sample that we used for the cytochrome C study. For measurement of cytochrome C release, soluble cell factions of the liver cells were used for western blot analysis. We have added this information in the revised manuscript in the last section of "Results".

8. Fig. 5F is not mentioned in the legend. Is the actin shown the control to all other figures (A-E)? (see next page)

Response:
"Fig 5F" (now Figure 6F) is a representative blot for actin as control. For densitometric analyses, the values of density for caspases and cytochrome C were corrected by loading control actin for each individual sample. We have added these in Figure 6 legend.

Reviewer 3:

The manuscript provides novel information. It is well written. The design, materials and methods are satisfactory. The results support the conclusions drawn. I didn't find mistakes which are obvious. I am of the opinion that the paper may be accepted for publication by WJG.

Response:
We appreciate the reviewer's comments.