Peer Review File

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Reviewer A:
In this manuscript, the authors found that Diabetes induces hepatocyte pyroptosis by promoting oxidative stress-mediated NLRP3 inflammasome activation during liver ischaemia and reperfusion injury.

Major critiques:
1. Survival curve should be calculated.
Response: Thank you for your suggestions. There are no mice dead from this 70% partial liver IR model.

2. Detailed information about C57BL/KsJ-leprdb/leprdb should be provided.
Response: Thank you for your great suggestions and comments. We have added the information about db/db mice in the "Results" section (see line 183-185) as following: Db/db mice at 8 weeks of age displayed obesity and had an increased body weight compared with C57BL/6 mice (50.3±1.9 g *vs*. 27.1±1.1 g). Blood glucose was higher in db/db than C57BL/6 mice (420±37 mg/dl *vs*. 98±9 mg/dl).

3. Both qRT - PCR analysis and ELISA test of IL-1β, IL-6, TNFa should be added.
Response: We have added the qRT - PCR analysis and ELISA test of IL-1β, IL-6, TNFa in Fig.2B (see line 204-205).And increased mRNA levels of IL-1β, TNFa, IL-6 and IL-18 and serum levels of IL-1β, TNFa and IL-6 were found in db/db mice after liver IR injury.

4. Caspase-3/7/8/9 activities should be tested.
Response: We have added the WB of caspase-3 and cleaved caspase-3/7/8/9 in Fig.2A and no significant differences were observed between db/db and control mice (see line 200-204).

5. Both procaspase-1 and cleaved caspase-1 should be detected by WB in one image.
Response: We have added the WB of procaspase-1 in Fig.2A and Fig.5C.

6. What is the Clinicopathological characteristics of patients with liver ischaemia and reperfusion injury according to Diabetes.
Response: Indeed, the perioperative hyperglycemia/diabetes result in a poor organ function and increase the rate of liver graft rejection in patients post liver transplantation, which could be improved by intensive insulin treatment (PMID: 29289983; 27788805; 30390037). However, we did not analyze the impact of hyperglycemia/diabetes on liver IR injury in humans.
Thank you for your comments. We have added the above discussion in the manuscript (see line 296-300).

7. Poor writing and experiments design. The results of this experiment are not reliable. The author needs to provide all the original data including WB image.
Response: We have revised the manuscript carefully and provided the original data including WB images. Also, the manuscript was edited for proper English language, grammar, punctuation, spelling, and overall style by one or more of the highly qualified native English speaking editors at AJE.

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Diabetes induces the pyroptosis of hepatocyte by promoting oxidative stress-mediated NLRP3 inflammasome activation during liver ischemia and reperfusion injury

prepared by the authors

Chengyu Shi, Qi Wang, Zhuqing Rao, Yong Shi, Song Wei, Hao Wang, Xu Lu,Ping Wang, Ling Lu, Haoming Zhou, Feng Cheng

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Reviewer B:
The present study aimed to the role of oxidative stress and hepatocellular pyroptosis in liver IR injury in diabetic mice and they found diabetes induces hepatocyte pyroptosis during liver IR injury. It is lack of novelty and the underline mechanism has not yet been explored. Several comments are listed as follow and perhaps can help to improve the present study.

1. There are some many publications i.e (PMID: 31700865; 31625631) study about diabetes and liver injury and pyroptosis, which showed the important role of pyroptosis in diabetes liver injury, maybe the author should carefully review the publications and design more experiments to improve the current study.
Response: Thank you for your great suggestions and comments. We have added some experiments and discussion about that (see line 284-292). In a previous study, we demonstrated that hyperglycemia aggravates acute liver injury by promoting NLRP3 inflammasome activation in liver-resident macrophages. NLRP3 inflammasome inhibition protected the liver from liver damage, inflammation and steatosis of experimental steatohepatitis with diabetes. Although increasing evidence indicates that NLRP3 inflammasome activation plays an important role in liver IR injury in the setting of hyperglycemia/diabetes, the precise effects of NLRP3 regulation by hyperglycemia/diabetes on hepatocellular pyroptosis remain largely unclear.

2. Quality of pathological images need to be improved in figure 1,2,3 and 5.

Response: We have remade and scanned the pathological image to improve the quality in figure 1,2,3 and 5.

3. There are many advanced detection methods to detect ROS release, please improve the methods to make the study more credible.
Response: Thank you for your great suggestions. We have added the ROS detect by DHR fluorescent probe in Fig.4C (see line 227-229). And the results show the increasing levels of ROS in the db/db mouse groups.

4. The author also detected apoptosis, and from the H&E images, there were necroptosis area, please explain the three different types of cell death apoptosis, necroptosis and pyroptosis how to act during liver ischemia reperfusion.
Response: We have added the analysis of caspase-3/7/8/9 activation in Fig.2A, and no significant differences were observed (see line 202-204). In fact, the precise type of cell death during liver IR injury remains uncertain. A recent study demonstrated that necroptosis inhibition had no significant effects on liver IR injury (28957350). Interestingly, both kupffer cell necroptosis and pyroptosis were found post liver IR by our and other group (28289160, 30634142). Here, we found that diabetes promoted hepatocyte pyroptosis as indicated by liver pathological examination and NLRP3 activation.

5. From the figure 1B, the injury score of diabetes mice in sham group seem significantly compared with those in sham group, maybe metabolic factors act as more important role during diabetes liver injury. The author should detect the metabolic signaling according to your data.
Response: Thank you for your comments. Indeed, as reported by others, serum AST and ALT levels were significantly higher in db/db diabetic mice than non-diabetics, even in the absence of hepatic IR (15217400). Similarly, increased serum levels of AST/ALT and higher Suzuki scores were observed in the sham group of db/db mice as compared with the sham control (C57BL/6) mice. After IR, the db/db mice showed much more severe liver injury as indicated by dramatically higher levels of AST/ALT and Suzuki scores, which could be abrogated by NLRP3 inhibition. These results suggested us that diabetes aggravated liver IR injury by promoting hepatocellular pyroptosis. However, we did not measure the metabolic signaling, which may also contribute to the acute liver injury in diabetic mice.

Reviewer C:
This research was to determine the role of oxidative stress and hepatocellular pyroptosis in liver IR injury in diabetic mice, the advices were as follow:

1. NLRP3 plays an important role in inflammation reaction at the transcription level, the EMSA should use to detect the activation of NF-κB;
Response: Thank you for your great suggestions. Critical roles of NF-κB signaling in inducing the transcription of NLRP3 have been reported (PMID: 30315268,

28151474). In addition, various studies have also shown the role of ROS in regulating NF-κB activation (27124102, 26120027).
Consistent with the findings by others (31464308), we analyzed the activation of NF-κB by western blot and found that increased NF-κB activation in db/db diabetic mice (Fig.2D) (see line 206-207). However, whether ROS could regulate NF-κB/NLRP3 signaling was not determined in the present study, which would be interested for further studies.

2. Insulin resistance is closely associated to inflammatory reaction. The effect of intervention on insulin resistance should be evaluated，such as hyperinsulinemic euglycemic clamp technique；
Response: The detrimental role of insulin resistance in promoting inflammatory reaction and aggravating liver IR injury has been reported (27565076). Interestingly, ROS could regulate both cellular injury/death and inflammation, and crosstalk between insulin resistance and ROS has been observed in many studies (32192190, 28232636). Thus, it would also be interesting to determine the role of insulin resistance in modulating inflammatory response during liver IR in diabetic mice. However, in the present study, we just focused on the liver parenchymal cell death but not the inflammation post-IR, and the insulin resistance was not assessed and intervened.

3. Masson and Oil Red O Staining should be carry on to evaluate histopathology；
Response: Thank you for your great suggestions. We have added the Masson and Oil Red O Staining in Fig.1D (see line 191-194). The fat accumulation and fibrosis levels in liver tissue of db/db mice were significantly higher than those of control mice, but IR injury did not affect it.

4. The language style should be re-edited；
Response: We have revised the manuscript carefully and polished the spelling and grammar in the manuscript. Also, the manuscript was edited for proper English language, grammar, punctuation, spelling, and overall style by one or more of the highly qualified native English speaking editors at AJE.


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