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February 22nd, 2018

Dr. Ze-Mao Gong
Science Editor
World Journal of Gastroenterology

Dear Dr. Gong,

We are re-submitting our revised manuscript entitled “Mitochondrial dysfunction with associated antioxidant capacity define NASH disease severity in two distinct mouse models” for consideration for publication in *World Journal of Gastroenterology*.

We thank the three reviewers and the editor for their critical comments and have incorporated their suggestions into a revised version of the paper that we think is improved from the original submission. Specific changes and rebuttals to the reviewer’s comments can be found in the following section. We hope you find the revisions appropriate and the manuscript now acceptable for publication.

Sincerely,

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Specific rebuttal:

Reviewer #1: I have read with great interest the study by Boland et al. and consider it to be a significant contribution to the field of NAFLD study. I have no significant comments regarding the manuscript, different from the other reviewers, and consider it fit for publication once they resolve the issues already raised. I'd like to commend the authors for their diligence and expect further developments of their work.

RESPONSE: We thank the reviewer very much for their kind review.

Reviewer #2: 1) Authors must increase the numer of mice in experimental groups since 5 or 6 mice are not sufficient for a correct statistical analysis;

RESPONSE: At this time we are not able to repeat these studies with higher n/group, however we do believe we adequately powered to detect significant differences between groups for the majority of the endpoints studies. For the *ob/ob* model, with the existing n/group of 6 for *ob/ob* LFD and 10 for *ob/ob* AMLN we have 99.7% power to detect a difference of 20% change in liver lipid assuming the same standard deviations. Actual difference between these groups was 25%. Likewise, for liver collagen quantitation we are powered to 99.3% to detect the reported difference between LFD and AMLN diet in the *ob/ob* model. In the FATZO model, for liver fat we are powered at 71.6% to detect a 20% difference in liver fat with current n/group and error. The actual difference reported was 32% change, for which the power calculation is 100%. For hepatic collagen, we are less well-powered, only 31% predicted statistical power to detect a difference between the two groups. However, the standard deviations were not different between groups, a one-way ANOVA was statistically significant, and Tukey's post hoc test revealed statistically significant differences between groups. We are comfortable that our conclusions are valid based on the current n/group and do not propose to repeat FATZO mouse experiments with a larger n/group that are already statistically sound.

2) Authors should provide new and better images of type I collagen IHC: in the session of result they state that "hepatic fibrosis assessed by quantfication of type 1 collagen stained area was significantly greater in ob/ob LFD vs. lean livers…"page 12, but the relative figure ", panel E do not allow trhe reader to appreciate this difference. The staining of collagen type 1 is the same for ob/ob LFD and lean mice;

RESPONSE: The collagen increase in *ob/ob* LFD mice vs. lean mice is subtle, certainly on IHC sections and images the differences are hard to see, in line with the general view that mice on low fat diets do not generally develop liver fibrosis within the timeframes of our analysis. We have inserted a new *ob/ob* LFD Col1a1 IHC image in Figure 2E that better reflects the difference compared to lean controls.

3) In figure 4D both immunofluorescence that graph do not show a significative difference between the number of mitochondria, also in relation with the panel C in which they showed the mitochondria area.

RESPONSE: Figure 4D representative images show increased numbers (but not length or size) of mitochondria per hepatocyte, and multiple hepatocytes were quantified to statistically prove that *ob/ob* AMLN hepatocytes have more mitochondria. We agree at first glance this increase is not visually striking because C57BL6J and *ob/ob* LFD hepatocytes have more fused (longer) mitochondria compared to *ob/ob* AMLN hepatocytes, which are nearly all fragmented, thus the overall amount of green fluorescence looks equal among all three images. Further, based on quantification shown in Figure 4E we demonstrate that *ob/ob* AMLN hepatocytes have increased numbers of mitochondria, but these are *smaller* than mitochondria in C57Bl6J and *ob/ob* LFD hepatocytes. The mitochondrial quantification from electron microscopy analysis in Figure 4C further supports the finding that *ob/ob* AMLN hepatocytes have smaller mitochondria, and thus the overall mitochondrial area is reduced compared to C57BL6J and *ob/ob* LFD hepatocytes.

Which method has been used to quantify mithocondrial lenght and the number for total cytoplasmatic area? The same observation for FATZO mice (figure 9).

RESPONSE: We have edited the methods section to include more detail on mitochondrial quantitation. The section now states "confocal images were viewed in a blinded fashion using 3Dmod software on a Wacom Cintiq 22HD art tablet (Vancouver, WA, USA). Mitochondrial length and number per total cytoplasmic area were quantified from ≥ 15 images per group (N ≥ 3 biological replicates) via manual tracing of cell boundaries, nuclei, lipid droplets and mitochondria. Total cytoplasmic area was calculated as area within the cell boundary minus the nuclei and lipid droplet areas." We have also added an additional citation highlighting the use of this method to quantify organelles from electron microscopy micrographs: Boland, B.B., C. Brown, Jr., C. Alarcon, D. Demozay, J.S. Grimsby, and C.J. Rhodes, *beta-Cell Control of Insulin Production During Starvation-Refeeding in Male Rats.* Endocrinology, 2018. **159**(2): p. 895-906.

4) Since mithocondria are related to oxidative stress, it could be interesting to analyze the role and the quantification of glutathione peroxidase and its activity.

RESPONSE: We have included additional data showing *Gpx1* mRNA is signficantly increased in FATZO AMLN livers compared to C57BL6J controls, but unchanged in all other groups (Figure 10A).

Reviewer #3: 1.- If nonalcolic steatohepatitis (NASH) is a disorder included within the Non-alcoholic fatty liver disease (NAFLD) the following sentence is not clear: " Hepatic mitochondrial function and oxidative stress in metabolically-relevant, pre-clinical models of NAFLD vs. NASH have not been fully assessed." maybe the authors would be write "two preclinical models of NAFLD, simple steatosis vs NASH" or "less severe vs more severe preclinical models of NAFLD"

RESPONSE: We thank the reviewer for this suggestion and have edited the sentence to state "Hepatic mitochondrial function and oxidative stress in metabolically-relevant, pre-clinical models of simple fatty liver vs. NASH have not been fully assessed."

2.- Why is the NASH core between Ob / ob and FAzto different?

RESPONSE: We have refined the NASH scores to include the same components for both the *ob/ob* and FATZO animals, described in the methods as follows: "The following parameters were graded to generate the overall NASH score: macrovesicular steatosis (0=<5%, 1=5-33%, 2=34-66%, 3=>66%); ballooning degeneration (0=absent, 1=present); lobular inflammation (0=no foci, 1=rare foci, 2=occasional foci, 3=frequent foci); biliary hyperplasia (0=none, 1=mild, 2=prominent); CD68 immunoreactivity (0=normal, 1=minimal increased, 2= more than minimal increase)."

3.- Brand and Nichols (ref 22) do not describe leak control ratio (LCR) LCR . It is Koliaki C et al in Cell Metabolism 21, 5: 739-746 4.-Brand and Nicholls describe Sate3 adp plus 1 µM oligomycin as state 4o not state 3o

RESPONSE: We have corrected this reference error in the manuscript, which now cites Koliaki C et al, Cell Metabolism 21, 5: 739-746.

5.-Non fasting levels of glucose and insulin really is not the best indicative for the diabete indicator. In fact SD of Terminal non-fasting plasma insulin levels is too higher

RESPONSE: We do not claim that any animals in this manuscript are diabetic, rather we refer to them as hyperinsulinemic as demonstrated by data in Figure 1C-D and Figure 6C-D.

6.- he author do not show the mouse serum levels of the leptin.

RESPONSE: *ob/ob* mice do not produce leptin, and the leptin levels in FATZO mice have previously been reported in reference 14, Droz, B.A., B.L. Sneed, C.V. Jackson, K.M. Zimmerman, M.D. Michael, P.J. Emmerson, T. Coskun, and R.G. Peterson, *Correlation of disease severity with body weight and high fat diet in the FATZO/Pco mouse.* PLoS One, 2017. **12**(6): p. e0179808.

7.- Figure 4D selected by the authors does not reflect the differences shown in the graph "mitochondrial number by cytoplasmic area". What methodology/software have you used to quantify Mitochondrial length and number per total cytoplasmic area?

RESPONSE: Please refer to our response to point 3 of Reviewer #2.

8.- "Maximal mitochondrial respiratory capacity was significantly increased in ob/ob LFD compared to lean hepatocytes (+45%, $p< 0.05$; Figure 3B)" is not Figure 3B

RESPONSE: Thank you for pointing out this error. We have corrected this statement in the paper to reflect the proper figure, Figure 5B.

9.- Why do not the authors perform the electron microscopy studies in mouse FAZTO?

RESPONSE: We demonstrated in Figure 4 that quantification of HSP60 stained primary hepatocytes can be used to analyze mitochondrial number and size, and the values obtained via this method recapitulate those obtained via analysis of electron microscopy micrographs.

GPx enzyme is responsible for the detoxification of H2 O2 (when it is present in low concentration but the authors are not considered the GPX.

RESPONSE: Please refer to our response to point 4 of Reviewer #2.