

## ANSWERING REVIEWERS

January 28, 2014



Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 8723-edited.doc).

**Title: Proteomic analysis of liver mitochondria from rats with nonalcoholic steatohepatitis**

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**Name of Journal:** *World Journal of Gastroenterology*

**ESPS Manuscript NO:** 8723

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

### **To reviewer 1:**

(1) The work presents interesting findings of mitochondrial proteins identified to be altered in a rat NASH model. The paper is weakened by the fact that only one protein was validated by immunoblotting, and by the lack of functional information on the effects of these protein alterations.

**A:** Thank you for your suggestion. This is the weakness of this study and we admitted and wrote in the section of Discussion “Thirdly, of the 24 identified proteins, only Hadha was verified and confirmation of the other 23 proteins and their functions are needed in future study”.

(2) The hepatic index is not a standard measure and should be described.

**A:** Thank you for your suggestion. We described the hepatic index in the section of method.

(3) It is not clear what the data in Table 1 are intended to show. Presumably it is that NASH was induced in the high fat diet-fed mice. Serum TG and cholesterol are not evidence of NASH, liver TG content is needed.

**A:** Thank you for your suggestion. NASH is defined as hepatic steatosis and inflammation. Here, serum TG and cholesterol are the evidence of steatosis while HAI index reflected inflammation level. We now added TG content to the table.

(4) Were the protein fractions determined to be pure preps of isolated mitochondrial proteins?

**A:** Thank you for your suggestions. According to current well acknowledged method in mitochondrial protein purification, the protein fractions are determined as pure preps of isolated mitochondrial proteins.

(5) Hadha levels are shown only for a single liver from each group. Multiple samples should be examined for Hadha expression. Additional proteins should be validated by Westerns.

**A:** Thank you for your suggestions. The data on Hadha level is preliminary. Our result was in contrast with the result of decreased Hadha gene level in NAFLD human subjects as described by kohjima et al (Int J Mol Med. 2007 Sep;20(3):351-8). Nevertheless, our result is on protein level while their result is on mRNA level, which indicated the potential existence of post transcriptional regulation such as miRNA regulation and methylation. Besides, it is our next step to investigate both gene and protein expression of Hadha in larger animal and patient samples in the future. Now, we admitted this weakness in the section of discussion.

## **To reviewer 2**

(1) The authors reported the analysis of the proteome of liver mitochondria from NASH rat model. By using the two-dimensional electrophoresis combined with matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry, they found 24 dys-regulated proteins with more than 1,5 fold difference between control and NASH rats. They verified the down-regulation of Hadha in livers of NASH animals by western blot. I believe that some points should be clarified and discussed. The authors should evaluate the purity of isolated mitochondria, for example by western blot using an antibody against cytochrome C or VDAC.

**A:** Thank you for your suggestion. Generally, using our method is able to get purified mitochondria but it is better if we can use western blot by an antibody against cytochrome C or VDAC. We will adopt this method in future study.

(2) The authors showed that Hadha expression decreases in their experimental model, but there is a publication by Kohjima and colleagues (Int J Mol Med. 2007 Sep;20(3):351-8) where they demonstrated an increased expression of Hadha in NAFLD human patients. Li

and colleagues should discuss and clarify this aspect.

**A:** Thank you for your suggestions. The data on Hadha level is preliminary. Our result was in contrast with the result of decreased Hadha gene level in NAFLD human subjects as described by kohjima et al (Int J Mol Med. 2007 Sep;20(3):351-8). Nevertheless, our result is on protein level while their result is on mRNA level, which indicated the potential existence of post transcriptional regulation such as miRNA regulation and methylation. Besides, it is our next step to investigate both gene and protein expression of Hadha in larger animal and patient samples in the future. Now, we admitted this weakness in the section of discussion.

(3) The authors verified only one protein of the 24 found. They should confirm other proteins expressed only in mitochondria and involved in novel mechanisms for NASH progression, for example Agmatinase and 3-mercaptopyruvate sulfurtransferase.

**A:** Thank you for your suggestion. This is the weakness of this study and we admitted and wrote in the section of Discussion “Thirdly, of the 24 identified proteins, only Hadha was verified and confirmation of the other 23 proteins and their functions are needed in future study”. We will consider verify those proteins only expressed in mitochondria in future study, such as Agmatinase and 3-mercaptopyruvate sulfurtransferase, as you recommended.

(4) In the graph of Figure 3, the authors showed the significant decrease of Hadha expression but without illustrating the standard deviation.

**A:** Thank you for your suggestion. We now added the standard deviation in figure 3

### **To Reviewer 3**

(1) The article consists on the proteomic analysis of liver mitochondria in Sprague-Dawley rats with non-alcoholic steatohepatitis and controls. It consists in a interesting study, with well-drawn sections, where the abstract clearly reflects the research performed. About material and methods, regarding to the NASH rat model chosen, it would be advisable to explain the sample size election in order to obtain statistical significant differences.

**A:** Thank you for your suggestion. According to our experience, 12 rat of each group is enough for sample selection, we add evidence in the section of method.

(2) Control group was fed with a basic diet, but it was not itemized the caloric composition adjusted or the fat and protein percentage in order to compare them with control group.

**A:** Thank you for your suggestion. The composition of fat rich diet was described in our previous study as mentioned in the section of method. We will do our utmost to calculate and itemize the caloric composition of fat and protein percentage in future study.

(3) Furthermore, liver sections were stained with hematoxylin-eosin, but reticulin or Masson's Trichrome were not performed as part of the routine procedure.

**A:** Thank you for your suggestion. reticulin or Masson's Trichrome staining was usually used for liver fibrosis detection. We may integrate those staining in future study.

(4) Besides, it was only calculated the histological activation index, but NAFLD Activity Score is the most used scoring system in non-alcoholic fatty liver disease.

**A:** Thank you for your suggestion. NAS is the most used scoring system in NAFLD. However, it includes aspect in fibrosis. In this study, we didn't do staining to show liver fibrosis, so we can't evaluate NAFLD with NAS, which will be incorporated in next study.

(5) In Table 1 it could be interesting to show different markers between NASH and control livers, although they don't show statistical significant differences, as GGT, bilirubin, platelets

**A:** Thank you for your suggestion. As you mentioned, there is no significant differences in GGT, bilirubin, bile acid and other markers. To avoid redundant space occupancy, we did not show these negative results in Tables.

(6) Regarding to Figure 2, it is recommended to show control and NASH gel separately, not in the same image.

**A:** Thank you for your suggestion. We can't separate different expressed proteins from control and NASH gel by eye. To increase the readability, we used the fusion image with circle expressing significantly dys-regulated proteins.

3 References and typesetting were corrected

4 Language has been polished by Native English speaker with certificate

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

Sincerely yours,

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