

## **Answering Reviewers**

June18, 2015

Dear Editor,

Thanks for your letter and the comments from the reviewers about our manuscript. We have carefully checked this manuscript and revised it according to your kind advice and reviewer's valuable comments. Please find enclosed the edited manuscript in Word format (file name: 17556-Answering reviewers.docx). If you have any question about this manuscript, please don't hesitate to let me know.

**Manuscript title:** RAR  $\alpha$  promotes autophagy to alleviate liver injury caused by ischemia and reperfusion through the Foxo3/p-Akt/Foxo1 pathway

**Revised manuscript title:** RAR  $\alpha$  promotes autophagy to alleviate liver ischemia and reperfusion injury

**Author:** Chen Zhong, Li-Yong Pu, Ming-Ming Fang, Zhen Gu, Jian-Hua Rao and Xue-Hao Wang

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

**ESPS Manuscript NO:** 17556

The manuscript has been improved according to the suggestions of reviewers:

**1. Format has been updated.**

**2. Revision has been made according to the suggestion of three reviewers.**

**Response to Reviewer No.00058872**

**Authors state that ATRA enhanced the expression of the anti-apoptotic protein Bcl-2 in their model. Well, the serum Bcl-2 is the mirror of enhanced expression in vivo, as evidenced in NAFLD patients, where the imbalance of apoptosis -antiapoptosis plays a key role. Authors, in order to support their data, and expand this model also to a more common clinical setting, obesity-related NAFLd, are invited to quote this paper, i.e., Serum Bcl-2 concentrations in overweight-obese subjects with nonalcoholic fatty liver disease. World J Gastroenterol. 2011 Dec 28; 17(48): 5280-8. It is better evaluate SD not SEM. It is useful to state the number of animals, in text, and elsewhere.**

**Answer:** Thanks for your advice, and I read the paper in detail. (1) The first problem was about Bcl-2. Firstly, the result was confirmed in NAFLD, and, the mechanism of this was different from liver I/R injury. Secondly, in liver I/R injury, Bcl-2 in tissue can better reflect the apoptosis than in serum, and this opinion is accepted by most articles<sup>[1-3]</sup>. But the mentioned finding was interesting. We will test separately and compare with the level of bcl-2 in serum and in tissue in the next experiment, and then get our own conclusion. (2) The other question was about SEM. We had revised in the article. We have tried our best to revise the manuscript according to your comments and the places of changes were marked by red words. If there is something wrong, please point it out.

- [1] **Ke B**, Shen XD, Zhang Y, Ji H, Gao F, Yue S, Kamo N, Zhai Y, Yamamoto M, Busuttil RW, Kupiec-Weglinski JW. KEAP1-NRF2 complex in ischemia-induced hepatocellular damage of mouse liver transplants. *J Hepatol* 2013; **59**:1200-1207 [PMID: 23867319 doi: 10.1016/j.jhep.2013.07.016]
- [2] **Wang PX**, Zhang R, Huang L, Zhu LH, Jiang DS, Chen HZ, Zhang Y, Tian S, Zhang XF, Zhang XD, Liu DP, Li H. Interferon regulatory factor 9 is a key mediator of hepatic ischemia/reperfusion injury. *J Hepatol* 2015; **62**:111-120 [PMID: 25152205 doi: 10.1016/j.jhep.2014.08.022].
- [3] **Huang J**, Yue S, Ke B, Zhu J, Shen XD, Zhai Y, Yamamoto M, Busuttil RW, Kupiec-Weglinski JW. Nuclear factor erythroid 2-related factor 2 regulates toll-like receptor 4 innate responses in mouse liver ischemia-reperfusion injury through Akt-forkhead box protein O1 signaling network. *Transplantation* 2014; **98**:721-728 [PMID: 25171655 doi: 10.1097/TP.0000000000000316].

#### **Response to Reviewer No.00004520**

**In this paper it is reported that the administration of all-trans retinoic acid (ATRA) to mice submitted to ischemia/reperfusion (IR) protects from liver injury by promoting autophagy, and this effect depends on Foxo3/p-Akt/Foxo1 signaling. This is a work written very badly, in bad English, which contains some weaknesses and whose results are poorly commented.**

**(1) The presence of several grammar and syntax mistakes renders the paper poorly understandable.**

**Answer:** Before submitting, this manuscript was edited for English language usage, grammar, spelling and punctuation by the native English-speaking editors at NPG Language Editing. Following your advice, we sent this article to NPG Language Editing for re-editing the whole article. Meanwhile, the professor Bibo Ke of UCLA was invited to check this paper furtherly for us. If there has any mistakes, please point it out without any hesitation.

**(2) The authors write that “as a substrate, p62 is reduced with the upregulation of autophagy” and cite reference 20. It is unusual to write “upregulation of autophagy”, it is preferable “enhanced autophagy”. In reference 20 it is demonstrated that proteotoxic stress “induces p62 phosphorylation (not reduction) at its ubiquitin-association (UBA) domain that regulates its binding to ubiquitinated proteins”. P62 is an adaptor protein (not a substrate), which has an ubiquitin-binding domain and a LC3-II binding domain. P62 can associate with ALFY and PI3P and this aggregate mediates autophagic degradation. Moreover, p62-LC3 complex plays a role in the recognition of autophagosomes (see for instance Redox Biology 2015;4:242-259; Age (Dordr). 2014;36:9626). The authors should explain the apparent discrepancy between the increase in p62 level they observed and the decrease in LC3-II during IR. The levels of the complex p62-LC3-II should be determined.**

**Answer:** It's a very good question. Because the relation between LC3 and p62 was not clarified in many articles. I read the mentioned articles. It is the fact that p62 was a bridge between LC3 and ubiquitinated substrates<sup>[1]</sup>, incorporated into the completed

autophagosome, and then degraded in autolysosomes<sup>[2]</sup>. However, it means that autophagic degradation was activated when the expression of LC3II was increased but p62 was decreased<sup>[3-5]</sup>. Because most authors thought that p62 was the substrate of the autophagy. In addition, in most autophagy-related articles, the levels of the complex p62-LC3-II were not detected. Hence, we also took the same measure to detect the autophagy.

[1] **Björkøy G**, Lamark T, Brech A, Outzen H, Perander M, Overvatn A, Stenmark H, Johansen T. p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. *J Cell Biol* 2005; **171**:603-614 [PMID: 16286508].

[2] **Klionsky DJ**, Abeliovich H, Agostinis P, Agrawal DK, Aliev G, Askew DS, Baba M, Baehrecke EH, Bahr BA, Ballabio A, Bamber BA, Bassham DC, et al. Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. *Autophagy* 2008; **4**:151-175 [PMID: 18188003].

[3] **Yang L**, Li P, Fu S, Calay ES, Hotamisligil GS. Defective hepatic autophagy in obesity promotes ER stress and causes insulin resistance. *Cell Metab* 2010; **11**:467-478 [PMID: 20519119 doi: 10.1016/j.cmet.2010.04.005].

[4] **Liu A**, Fang H, Dahmen U, Dirsch O. Chronic lithium treatment protects against liver ischemia/reperfusion injury in rats. *Liver Transpl* 2013; **19**:762-772 [PMID: 23696274 doi: 10.1002/lt.23666].

[5] **Liu A**, Fang H, Wei W, Dirsch O, Dahmen U. Ischemic preconditioning protects against liver ischemia/reperfusion injury via heme oxygenase-1-mediated autophagy.

*Crit Care Med* 2014; **42**:e762-771 [PMID: 25402296 doi: 10.1097/CCM.0000000000000659].

**(3) FOXO3A and pAKT induce the leakage of nuclear FOXO1 (see for instance ref. 17). The authors state that they observed that “the increased levels of Akt phosphorylation diminish nuclear Foxo1 accumulation”. This is not true because Foxo1 has not been determined in isolated nuclei, nor immunohistochemical evidence of the nuclear Foxo1 levels has been presented.**

**Answer:** Thanks for your kind reminding. I was too careless to find this mistake. It's the fact that the protein was extracted from nuclei, and used to detect the level of Foxo1. I was so sorry that because of my negligence made you confused. Hence, we had checked carefully the whole paper again.

**(4) As concerns Caspase 3 cleavage, it is preferable to include in the figures the immunoblots showing the un-cleaved (35 kDa) Caspase 3 and the cleaved protein bands (17-19 kDa).**

**Answer:** It was usually that cleaved caspase3 had two bands(17-19KD) in western blot. But in our experiment, it had a single band. This finding made us so confused, then we searched some related papers in PubMed to support my result. Finally, there were some articles that cleaved caspase3 also had one band<sup>[1-2]</sup>. We speculated that the two bands of cleaved caspase3 was hardly to separate in some cases.

[1] **Wang PX**, Zhang R, Huang L, Zhu LH, Jiang DS, Chen HZ, Zhang Y, Tian S, Zhang XF, Zhang XD, Liu DP, Li H. Interferon regulatory factor 9 is a key

mediator of hepatic ischemia/reperfusion injury. *J Hepatol* 2015; **62**:111-120 [PMID: 25152205 doi: 10.1016/j.jhep.2014.08.022].

[2] **Huang J**, Yue S, Ke B, Zhu J, Shen XD, Zhai Y, Yamamoto M, Busuttil RW, Kupiec-Weglinski JW. Nuclear factor erythroid 2-related factor 2 regulates toll-like receptor 4 innate responses in mouse liver ischemia-reperfusion injury through Akt-forkhead box protein O1 signaling network. *Transplantation* 2014; **98**:721-728 [PMID: 25171655 doi: 10.1097/TP.0000000000000316].

**(5) The evidence of autophagy in liver subjected to IR +/- ATRA must be confirmed by current methods of autophagy determination such as, for instance, by ultrastructural observations and/or degradation of long-lived proteins.**

**Answer:** Autophagosome detected in liver by TEM was the directly evidence, as the limitation of lab condition, this purpose did not come true. In many related studies, although autophagosome was not provided, LC3 was usually to stand for autophagy<sup>[1-4]</sup>. In our paper, we also detected the level of LC3 by the western blotting to acquire the intense of autophagy.

[1] **Zhu J**, Lu T, Yue S, Shen X, Gao F, Busuttil RW, Kupiec-Weglinski JW, Xia Q, Zhai Y. Rapamycin protection of livers from ischemia and reperfusion injury is dependent on both autophagy induction and mammalian target of rapamycin complex 2-Akt activation. *Transplantation* 2015; **99**:48-55 [PMID: 25340604 doi: 10.1097/TP.0000000000000476].

[2] **Rickenbacher A**, Jang JH, Limani P, Ungethüm U, Lehmann K, Oberkofler CE, Weber A, Graf R, Humar B, Clavien PA. Fasting protects liver from ischemic

injury through Sirt1-mediated downregulation of circulating HMGB1 in mice. *J Hepatol* 2014; **61**:301-308 [PMID: 24751831 doi: 10.1016/j.jhep.2014.04.010].

[3] **Huang H**, Kang R, Wang J, Luo G, Yang W, Zhao Z. Hepatitis C virus inhibits AKT-tuberosus sclerosis complex (TSC), the mechanistic target of rapamycin (MTOR) pathway, through endoplasmic reticulum stress to induce autophagy. *Autophagy* 2013; **9**:175-195 [PMID: 23169238 doi: 10.4161/auto.22791].

[4] **Liu A**, Fang H, Dahmen U, Dirsch O. Chronic lithium treatment protects against liver ischemia/reperfusion injury in rats. *Liver Transpl* 2013; **19**:762-772 [PMID:23696274 doi: 10.1002/lt.23666].

#### **Response to Reviewer No.00012156**

**Wang et al. described that the role of autophagy and the relationship between ATRA and autophagy in liver IR injury. The authors reported that ATRA pretreatment alleviated liver IR injury by inducing autophagy and it may involves RAR $\alpha$  activity. To demonstrate the mechanism of RAR $\alpha$ , authors used LE540 to inhibit RAR $\alpha$  during ROS-inducing cell damage in vitro and indicated that RAR $\alpha$  activation enhanced Foxo3a and p-Akt expression and decreased Foxo1 level. They concluded that ATRA activates RAR $\alpha$  to protect the liver from IR injury by upregulating the Foxo3a/p-Akt/Foxo1 pathway to promote autophagy. The contents are interested but logic is not incomplete.**

**(1) Autophagy is well known to be occurred by a small stress, i.e., fasting. Is there any relation between the fact of suppression and the autophagy? The**



**explanation and/or the results which support the relation were necessary.**

**Answer:** This is a good question. It is fact that starvation can induce autophagy. Before the experiment, we had thought how to deal with this problem. Mice should undergo the operation, so fasting cannot be avoided. We though the autophagy inducing by fasting had little effect on the result. There were two reasons to explain. Firstly, the control group was same with the drug group on fasting time and operation. This design can weaken the effect of autophagy on liver I/R injury. In addition, there was reported that fasting for one day can protect liver I/R injury and this protective role was related with autophagy<sup>[1]</sup>. In our paper, the intension of autophagy that was activated by the fasting time ( $\leq 12h$ ) was so weak that did not influence the result. Hence, the results were credible.

[1] **Rickenbacher A**, Jang JH, Limani P, Ungethüm U, Lehmann K, Oberkofler CE, Weber A, Graf R, Humar B, Clavien PA. Fasting protects liver from ischemic injury through Sirt1-mediated downregulation of circulating HMGB1 in mice. *J Hepatol* 2014; **61**:301-308 [PMID: 24751831 doi: 10.1016/j.jhep.2014.04.010].

**(2) There were no results and/or discussion of the effect and functional change of ATRA on stellate cells, because the receptor for ATRA locate on the stellate cells. For clarifying the mechanism of ATRA functional changes of ATRA should be examined.**

**Answer:** Thank you for your advice. Hepatic stellate cell (HSC) is our research target, because it is mainly responsible for the synthesis and storage of ATRA and has ATRA receptor. As the intial of study, however, we firstly focused on the hepatocytes that

was the victim in liver I/R injury. Besides, ATRA reduced the damage of hepatocytes and had a protective role in liver I/R injury. In the next research, we will study the relationship between ATRA and HSC in liver I/R injury.

### **3. References and typesetting were corrected.**

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

Sincerely yours

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