Format for ANSWERING REVIEWERS

October 30, 2014

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 13763-review.doc).

Title: Ezetimibe improves hepatic steatosis in relation to autophagy in obese and diabetic rats

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

1 Format has been updated

(1) Font size was changed to 10 as BPG's revision policies for original article.
(2) The information of supported grants in the current study was revised as suggested format.
(3) The revision of abstract has been done according to science editor's suggestion and writing requirements of original articles; no less than 140 words in materials and methods and no less than 150 words in results.
(4) All the abbreviations and acronyms were defined in the text as science editor's suggestion.
(5) The format for accurately writing common units was revised as BPG's revision policies for original article.
(6) Revision of statistical expression has made according to BPG's revision policies for original article.
(7) Comments including background, research frontiers, innovations and breakthroughs, applications, terminology and peer review were included in the text as science editor's suggestion and writing requirements of original articles
(8) Abbreviations used in the table were defined under table as science editor's suggestion.
(9) Figures were decomposable and split as editor's suggestion.

2 Revision has been made according to the suggestions of the reviewer

(1) Reviewed by 02539944

The manuscript by Chang et al. describes the effect of Ezetimibe on hepatic steatosis in a rat model of obesity and type II diabetes. Major conclusion of the manuscript is the induction of autophagy in the liver by application of Ezetimibe and, therefore, a reduction of hepatic steatosis. The idea of Ezetimibe as inducer of autophagy in the liver/hepatocytes is in line with a previous report by a different group earlier this year. There are several points that need to be addressed.

Major points: 1. Figure 2A: The authors need to show that the expression of the housekeeping gene is not changed. With a rather mild elevation of the 3 investigated genes, small changes in housekeeping gene expression could have a major impact.

Response 1: We really appreciate and respect that reviewer points out how important to make sure whether housekeeping gene expression is not changed since it results in different results. We measured two housekeeping genes, actin and GAPDH. In case of actin, ct values were significantly different according to groups. However, GAPDH ct values were not influenced by animal model and ezetimibe administration (LETO control, 19.26 ± 1.42; OLETF control, 18.48 ± 0.32; OLETF Ezetimibe,

19.63 ± 0.30). Therefore, we used GAPDH as housekeeping gene. A following paragraph (a) in the part of RNA analysis, materials and methods was inserted in line 12-14 of page 8.
(a) Inserted paragraph is that 'Expression of each target gene was normalized to housekeeping gene (GAPDH) and expressed as the fold change relative to the control treatment. CT values of GAPDH were not statistically different among groups'.

2. The reason, why these 3 ATG genes were studied and no other autophagy-related genes remains unclear.
Response 2: As reviewer's important point, there are more than 30 ATG genes, which are identified and which of molecular mechanisms of autophagy have been elucidated. Among these genes, we measured 3 ATG genes which function and role have fully been investigated using a method of targeted deletion in animals and cells. According to reviewer's critical question, we included this information in the section of result (page 9, line 16-21) as follows.
Among dentified 30 ATG genes[34], ATG5, ATG6, and ATG7 have been fully demonstrated using method of targeted deletion in animals and cells. In the process of autophagosome formation, ATG5 is conjugated and forms a complex with ATG12 and ATG16[35]. ATG6 and ATG7 are required for autophagy as a part of a lipid kinase complex or by specifically involvement in autophagosome formation[36, 37].
34 Klionsky DJ, Cregg JM, Dunn WA, Jr., Emr SD, Sakai Y, Sandoval IV, Sibirny A, Subramani S, Thumm M, Veenhuis M, Ohsumi Y. A unified nomenclature for yeast autophagy-related genes. Dev Cell 2003; 5: 539-545 [PMID: 14536056 DOI: 10.1016/S1534-5807(03)00296-X]
35 Mizushima N, Yamamoto A, Hatano M, Kobayashi Y, Kabeya Y, Suzuki K, Tokuhisa T, Ohsumi Y, Yoshimori T. Dissection of autophagosome formation using Apg5-deficient mouse embryonic stem cells. J Cell Biol 2001; 152: 657-668 [PMID: 11266458 DOI: 10.1083/jcb.152.4.657]
36 Kametaka S, Okano T, Ohsumi M, Ohsumi Y. Apg14p and Apg6/Vps30p form a protein complex essential for autophagy in the yeast, Saccharomyces cerevisiae. J Biol Chem 1998; 273: 22284-22291 [PMID: 9712845 DOI: 10.1074/jbc.273.35.22284]
37 Ohsumi Y, Mizushima N. Two ubiquitin-like conjugation systems essential for autophagy. Semin Cell Dev Biol 2004; 15: 231-236 [PMID: 15209383 DOI: 10.1016/j.semcdb.2003.12.004]

3. LC3 is not a simple marker of autophagosome formation. It can also indicate a block of autolysosome formation. Therefore, it cannot be used as sole indicator of autophagy induction. The authors calculate the ratio of LC3-II and LC3-I, however the Western Blot in Figure 2B has a poor quality. Statistical analysis based on n=2 are not allowed.
Response 3: LC3 is known to exist on autophagsomes and serves as a widely used marker for auophagosomes, to date (EMBO J. 2000;19:5720–5728, Mol Biol Cell. 2004;15:1101–1111, Cell. 2010;140(3):313-26). As reviewer's point, however, LC-3 is also involved in autolysosome formation. Therefore, to monitor autophagy and modulate autophagic activity, numerous and precise methods as follows need to be considered; a) Monitoring autophagic activity by the measurement of autophagosome accumulation or suppression of steps in the downstream pathway of autophagosome formation such as via the AMP-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR) pathway, b) monitoring the number of autophagosomes by the use of electron microscopy and fluorescence microscopy, c) monitoring autophagic flux using LC3 turnover assay with chemical inhibitors, measuring degradation of LC3 and its selective substrate, p62, and d) gene modification were suggested. As reviewer's important comment, we also think LC3 expression is not enough to be used as a sole indicator of autophagy induction. This limitation was included in the section of discussion (page 11, line 30-33; page 12, line 1-4). Thank you for your valuable point.

Response 4: We really appreciate that reviewer suggested a representative blot needs to be changed. According to reviewer's suggestion, a blot with good quality was replaced in Figure 2B. Thank you so much.

Response 5: We are sorry that the way how to show our results was unclear and confusing. In our animal study, there were 3 LETO control rats, 5 OLETF control rats, and 6 OLETF ezetimibe-administrated rats.

4. Besides increasing the number of tested rats, the authors need to consider at least 1 additional method to convincingly show the induction of autophagy by Ezetimibe in vivo.
Response 6: Increasing number of animal and addition of conclusive method to measure autophagy induction is important to verify clearly our hypothesis as reviewer's critical view. We described these limitations of our study in the discussion section (page 11, line 30-37; page 11, line 1-4).

5. How does the suggested effect look like, when only LC3-II is analyzed by Western Blot rather than calculating the ratio of LC3-II/I?
Response 7: Your question is very reasonable, since we also had deeply taken into consideration how to quantify and show LC3 protein abundance. According to many studies as follows, the ratio of LC3-II to LC3-I has been used; a) Gastroenterology. 2012;142(3):644-653.e3; b) Methods Enzymol. 2009;452:199-213; c) Exp Gerontol. 2010;45(2):138-48; d) J Cell Biochem. 2010;111(6):1426-36. However, the amount of LC3-II may be appropriate to compare between samples, rather than comparison of LC3-I and LC3-II, or summation of LC3-I and LC3-II for ratio determinations as reviewer's comment or a paper (Autophagy. 2007;3(6):542-5). When LC3-II was quantified as reviewer's suggestion, LC3-II expression was same as the ratio between LC3-I and LC-II.

6. In Figure 3C there is no obvious change in LC3-II levels among the groups. Instead the authors again calculate the ratio of LC3-II/I. Looking at the band intensities, the ratio of LC3-II/ in controls ("Con") should be higher than calculated by the authors, since the band of LC3-I is weaker compared to, e.g. "Ez". It appears, there is a mistake in the quantification.
Response 8: A representative used in the original article was taken from different study by mistake and confounding. We rectified this mistake and a proper representative blot was placed in Figure 3C. We would like to express our deep sense of gratitude to the reviewer for his/her meticulous review and comment. Thank you.

7. It is a far-fetched interpretation when the authors call the application of PA in cell culture as "induction of hepatic steatosis".
Response 9: Hepatic steatosis is characterized by fat accumulation within hepatocytes. To increase the level of TG in hepatocytes, we used 0.5 mmol/L PA which significantly increased TG concentrations by 71% compared to control (Figure 3A).

8. For autophagic flux experiments the degradation of p62 should be investigated.
Response 10: To demonstrate the effect of ezetimibe on autophagy, we also agree that to measure p62 expression, an indicator of autophagy degradation would be desirable. Unfortunately, there was no leftover sample *in vivo* study. *In vitro* hepatocytes, additional western blot experiment could be done and added in the revised text. As shown in Figure 3C and D, ezetimibe-incubated hepatocytes in the presence with PA significantly increased LC3 protein level and decreased p62 protein abundance, compared to PA treated cells. This result was illustrated in the line 1-2, page 10.
Except LC3-II delivery to lysosome, to be measure p62 expression level in the presence and absence of autophagy inhibitor may be important for an indication of autophagic flux, however it was not possible in the present study due to lack of sample. Still, numerous studies illustrate autophagic flux using only the amount of LC3-II in autophagic flux experiment. Please refer to a) J Biol Chem. 2013;288(22):15947-58, b) J Pharmacol Exp Ther. 2011;339(2):487-98, c) PLoS One. 2011;6(9):e25269, d) Cell. 2010;140(3):313-26, and e) J Cell Biochem. 2010;15;111(6):1426-36. Our results showing the combination of PA and ezetimibe in the presence of autophagy inhibitor increased the ratio of LC3-II and LC-I suggested that ezetimibe affects autophagic flux. To demonstrate changes in p62 level together with LC3-II abundance would be more desirable to know the precise mechanism.

Minor points:

1. What was the vehicle to apply Ezetimibe in rats?

Response11: Same volume of PBS was used.

2. In Table 2 the liver tissue weight is measured in "%"? Footnotes are lacking to explain superscript letters.

Response 12: Thank you so much. Liver tissue weight was expressed as % of body weight. Unit of liver weight was revised in table 2.

3. The letters in Figure 3A are not explained.

Response 13: As following reviewer's comment, we explained the meaning of letters in figure legend.

<Reviewed by 02903629>

1. First, the significant deficiency for this manuscript is sample size. The authors do not introduce the theory-guided, especially for the LETO control group (just 3 rats). Obviously, the conclusion is quite unpersuasive. Additional experiments should be conducted to confirm that difference between two groups is statistically significant but not sampling error. It cannot be accepted before more data is added.

Response 1: As reviewer's point of small sample size, number of 3 rats in one group could not be enough to estimate the statistical difference between groups. In the present study, LETO rats showed the smallest SE among groups. In addition, the purpose of using LETO rats was just to compare to OLETF rats showing hepatomegaly, obese, and diabetes. However, small number in our animal study is the limitation of the present study which described in the discussion section (page 10, line 25-27; page 11, line 30-33; page 12, line 1-4). In addition, statistical difference was re-evaluated compared between OLETF control and OLETF ezetimibe using student's *t* test and table 2 was revised after statistical analysis (page 19).

2. In Discussion section, deeper discussion should be added. The author just give a quick glance. Many factors is not clearly illustrated. Such as how does autophagy regulate hepatocyte lipid metabolism and hepatocellular injury and death? There are potential beneficial effects of a therapeutic increase in hepatocyte autophagic function: 1) Decrease triglyceride and cholesterol accumulation. 2) Improve insulin signaling. 3) Prevent cellular injury from oxidative stress. 4) Block TNF and Fas death receptor-mediated liver injury. 5) Reduce endoplasmic reticulum stress and the resultant cellular damage and insulin resistance. 6) Prevent hepatocellular carcinoma development.

Response 2: As reviewer's valuable suggestion, a paragraph regarding the influence of autophagy on hepatocyte lipid metabolism and hepatocellular injury and death was included in the discussion (page 11, line 5-17) with following references.

45 Singh R, Kaushik S, Wang Y, Xiang Y, Novak I, Komatsu M, Tanaka K, Cuervo AM, Czaja MJ. Autophagy regulates lipid metabolism. Nature 2009; 458: 1131-1135 [PMID: 19339967 DOI: 10.1038/nature07976]

46 Wang Y, Singh R, Xiang Y, Czaja MJ. Macroautophagy and chaperone-mediated autophagy are required for hepatocyte resistance to oxidant stress. Hepatology 2010; 52: 266-277 [PMID: 20578144 DOI: 10.1002/hep.23645]

47 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]

48 Komatsu M, Waguri S, Ueno T, Iwata J, Murata S, Tanida I, Ezaki J, Mizushima N, Ohsumi Y, Uchiyama Y, Kominami E, Tanaka K, Chiba T. Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. J Cell Biol 2005; 169: 425-434 [PMID: 15866887 DOI: 10.1083/jcb.200412022]

49 Mathew R, Karp CM, Beaudoin B, Vuong N, Chen G, Chen HY, Bray K, Reddy A, Bhanot G,

Gelinas C, Dipaola RS, Karantza-Wadsworth V, White E. Autophagy suppresses tumorigenesis through elimination of p62. Cell 2009; 137: 1062-1075 [PMID: 19524509 DOI: 10.1016/j.cell.2009.03.048]

3. In Table 2 some difference such as insulin resistance are mentioned. But the author do not explain this difference in the discussion.
Response 3: With the gratitude of reviewer's point, impact of ezetimibe on insulin resistance was included in the discussion (page 10, line 29-32).

3. The authors want to prove that Ezetimibe improves hepatic steatosis in relation to autophagy in obese and diabetic rats. Some fundamental serum index such as LDL-C and HDL-C is not shown.
Response 4: Unfortunately, we couldn't conduct an additional experiment to measure serum concentrations of LCL-C and HDL-C due to lack of blood. However, changes in serum and liver concentrations of TG, FFA, and TC by ezetimibe administration indicate the favorable effect of ezetimibe on magnitude of hepatic fat accumulation.

4. In Table 2, superscript letters a, b, and c is not clear, and they should be noted under the Table.
Response 5: We revised and added correct information about superscript letter (page 19), as reviewers comment. Thank you

5. Four Figure legends are so long, and they should be compressed.
Response: Thank you for your comment. We made legends short (page 24) and the part of methods was revised (page 8, line 3-4) as suggested.

6. Why the author just select male rats? Please explain this.
Response: We appreciate the reviewer's comment pointing out an issue of bi-gender animal research. We discussed this as the limitation in the current study and suggested another animal study with female animals for future following study (page 11, line 34-37).

3 References and typesetting were corrected
(1) Revision for pubMed citation numbers and DOI citation has been done according to writing requirements of original articles.
(2) Font size was changed to 10 as BPG's revision policies for original article.

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

Sincerely yours,

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