Point-by-point response to reviewers' comments concerning manuscript No. 19417

July 23, 2015

Dear Dr. Fang-Fang Ji,

I thank you and the reviewers of ***World Journal of Gastroenterology*** for taking the time to review manuscript No. 19417.

We revised manuscript as you and the reviewers' recommended. The correction in an annotated version are the points you and the reviewers have indicated. We agree with you and the reviewers in all points except those I have mentioned.

Thank you and the reviewers again for considering our manuscript to be published in the ***World Journal of Gastroenterology***. We look forward to receiving your answer soon.

Reviewer 1

The MS of Lee HJ et al entitled "Peroxisome proliferator-activated receptor-delta agonist ameliorated inflammasome activation in nonalcoholic fatty liver disease", is about a hot topic of research and potentially applicable to the treatment of NAFLD a growing health problem with no well-proven pharmacological intervention though the use of PPAR-γ agonist has been explored before in similar models. However major concerns arise. If well conducted, the MS is promising.

In the introduction, a more comprehensive discussion about the potential use of other drugs i.e. PPARs agonists or others in similar rodent models of NAFLD should be presented. Some had effects on proinflammatory cytokines and some agonists of PPAR-δ had also been explored, i.e. Carabelli J, et al. High fat diet-induced liver

steatosis promotes an increase in liver mitochondrial biogenesis in response to hypoxia. J Cell Mol Med. 2011;15:1329-1338 and Rosselli MS, et al. Losartan reduces liver expression of plasminogen activator inhibitor-1 (PAI-1) in a high fat-induced rat nonalcoholic fatty liver disease model. Atherosclerosis. 2009;206:119-126 .

Response:

Thank you for your comment. We have cited the recommended papers in the reference list and modified the text in the Introduction section (2nd paragraph) as follows.

"Regarding the pathogenesis of NAFLD[5, 6], hepatic steatosis sensitizing the liver and making it more prone to additional insults. Factors such as increased oxidative stress, pro-inflammatory cytokines and impaired adenosine triphosphate (ATP) production[7, 8] could trigger necroinflammation and lead to the progression of steatohepatitis, Although many kind of drugs such as thiazolidinediones, vitamin E, losartan, and silybin have been evaluated in several studies, few pharmacological treatments can be recommended at present[9-11]."

In methods, the use of GAPDH as a control for loading in Real time PCR should be justified. A panel of other housekeeping genes has to be explored and proven that GAPDH is the more stable by using a software such as GeNorm. Statistical analysis seems not to be appropriate because many of variable are ratios or percentages, which probably are not normally distributed. A log transformation of variables should be done or other non-parametric test should be applied. This can make some differences to be not significant.

Response:

Thank you for your comment. We completely understand and agree with your opinion. As you pointed out, housekeeping genes like β-actin and GAPDH, most commonly used, may be inappropriate as internal references because of their variability in certain experimental conditions. Also, our sample is very small (4~5 per group), so the statistical limitations were inevitable. In future research, we will try to overcome these limitations.

In results, some contradictions are evident, In table 2, the food consumption in the HFD is lower (which has biological sense) but in the text authors affirm just the opposite. Results for transaminases should be expressed as mean ± SD as described in statistical section and not just as media(?).

Response:

Thank you for your comment. The food consumption is greater in the HFD group compared with the HFD + LPS, and HFD + LPS + GW501516 groups (not control group). We modified the text in the Results section (1st paragraph) as follows.

“The body weight and BMI of the HFD group were significantly higher compared with those of control mice (all $P < 0.05$) (Table 2). The food intake was greater in the HFD group compared with the HFD + LPS and HFD + LPS + GW501516 groups…”

“…Serum AST and ALT levels were significantly increased in the HFD group compared with the control group (AST, 123.8 ± 30.54 *vs.* 52.6 ± 10.33 IU/L; ALT,

136.6 ± 69.43 *vs.* 23.4 ± 4.04 IU/L, all $P < 0.05$). GW treatment in HFD+LPS group significantly reduced serum AST and ALT compared with HFD (AST, 45.67 ± 11.11 *vs.* 123.8 ± 30.54 IU/L; ALT, 20.5 ± 6.12 *vs.* 136.6 ± 69.43 IU/L, all $P < 0.05$)…"

Minor comments: Language needs minor polishing. The resolution of figures is poor.

Response:

Thank you for your comment. As per your suggestion, the manuscript was proofread by a native English speaker. And the resolution of figures was adjusted as indicated.

Reviewer 2

Introduction section: about mechanisms involved in the pathogenesis of NAFLD, recently a multi-steps hypothesis was been reported in literature (Abenavoli et al. World J Gastroenterol. 2014 Dec 7;20(45):16831-40). Many studies reported the efficacy of milk thistle and its components in the treatment of NAFLD (Loguercio et al. Free Radic Biol Med. 2012 May 1;52(9):1658-65; Abenavoli et al. Expert Rev Gastroenterol Hepatol. 2015 Apr;9(4):519-27).

Response:

Thank you for your comment. We have cited the recommended papers in the reference list and modified the text in the Introduction section (1st and 2nd paragraph) as follows.

"…Certain portion of NAFLD eventually progressed to liver cirrhosis and hepatocellular carcinoma [1, 3]. Recently, the two-hit hypothesis was been reported in

the literature[4], but the precise mechanism involved in the development and progression of NAFLD is not entirely understood."

"Regarding the pathogenesis of NAFLD[5, 6], hepatic steatosis sensitizing the liver and making it more prone to additional insults. Factors such as increased oxidative stress, pro-inflammatory cytokines and impaired adenosine triphosphate (ATP) production[7, 8] could trigger necroinflammation and lead to the progression of steatohepatitis, Although many kind of drugs such as thiazolidinediones, vitamin E, losartan, and silybin have been evaluated in several studies, few pharmacological treatments can be recommended at present[9-11]."

Methods section: NAFLD is a liver disease with cytonecrotic and cholestatic components. Why GGT was not dosed in this study?

Response:

Thank you for your comment. We completely understand your opinion. Unfortunately, we did not measure GGT level due to lack of sample volume and economic problem. In the next experiment, we will measure GGT level as well as AST or ALT level reflecting your suggestion.

Discussion section: the pro-inflammatory profile during NAFLD, can improve by the administration of probiotics? What is the idea of the Author about it?

Response:

Thank you for your comment. As you mentioned, we absolutely think that probiotics may be an emerging therapeutic strategy to treat NAFLD. As we noted in the

Discussion, bacterial endotoxins may play a key role in the pathogenesis of NASH, and accumulating evidence has shown that probiotics administration decrease serum endotoxin levels in patients with various diseases. If the opportunity arises, we would like to prove the effects of probiotics in NAFLD/NASH model.

Reviewer 3

Authors studied effects of PPAR-δ activator; GW501516 on inflammasome pathway in rat NASH model (high fat diet, LPS/PA) and HepG2 cells culture. they shown that the beneficial role in rat liver was through increased NLRP3-10 in HepG2 cells. My comment: * the beneficial effect on rat was minimal, since glucose tolerance and insulin resistance did not developed. there seems to be a problem with HFD + LPS, since their data is better than controls at table 2.

Response:

Thank you for your comment. We completely understand your opinion and agree with it. As you pointed out, we failed to make the typical NASH model in the *in vivo* study. We expected that the chronic exposure of low-dose LPS would lead to make steatohepatitis in mice fed an HFD. As we noted in the Discussion, to overcome this limitation, future studies using endotoxins as HFD-enhancing factors in the murine NASH model are warranted.

*the resolution of all figures are not good, so cannot be read.

Response:

Thank you for your comment. The resolution of figures was adjusted as indicated.

Reviewer 4

The manuscript by Lee et al. describes the therapeutic effect of an PPAR delta agonist in an in vitro and in vivo models of NAFLD. Although the issue is interesting and relevant to human therapeutics, the manuscript has several shortcomings that should be properly addressed by the authors before further consideration. . Careful English editing of the manuscript should be performed.

Response:

Thank you for your comment. As per your suggestion, the manuscript was proofread by a native English speaker.

As there is no data regarding the in vivo effect of GW on inflammasome expression or activation, the conclusion provided in the abstract in not valid.

Response:

Thank you for your comment. We completely understand and agree with your opinion. In this study, GW treatment inhibited overexpression of caspase-1 and IL-1β in the mice. Although we did not measure the each inflammasome component *in vivo* animal model, we demonstrated that IL-1β and caspase, final pathway of inflammasome activation, were significantly activated. To overcome this limitation, future studies using large number of animals are warranted.

Absolute liver weight or related to body length should be provided. As there are

significant changes in body weight across the different treatment groups, relating organ weight to body weight is misleading.

Response:

Thank you for your comment. We assessed the liver weight to body length (g/cm) in all groups. There were no significant statistical differences among four groups. We added following comments in the Results section (1st paragraph) of the paper and modified the Table 2.

"…The proportion of liver weight to body weight or body length was similar among the four groups."

Table 2. Clinical and biochemical characteristics

	Control (*n* = 5)	HFD (*n* = 5)	HFD + LPS (*n* = 5)	HFD + LPS + GW501516 (*n* = 6)
Food/week (g)	20.78 ± 1.41	17.33 ± 0.82[a]	14.08 ± 1.28[a,b]	13.92 ± 1.23[a,b]
BW (g)	26.70 ± 0.69	41.04 ± 4.69[a]	30.98 ± 3.86[b]	29.87 ± 3.31[b]
BMI (g/cm^2)	0.33 ± 0.02	0.41 ± 0.04[a]	0.36 ± 0.02[b]	0.35 ± 0.02[b]
Liver/BW (%)	3.50 ± 0.30	3.04 ± 0.46	2.77 ± 0.27	3.08 ± 0.94
Liver/BL (g/cm)	0.10 ± 0.01	0.13 ± 0.03	0.09 ± 0.01	0.10 ± 0.03

AST (IU/L)	52.6 ± 10.33	123.8 ± 30.54[a]	62.0 ± 13.71[b]	45.67 ± 11.11[b]
ALT (IU/L)	23.4 ± 4.04	136.6 ± 69.43[a]	49.6 ± 31.09[b]	20.5 ± 6.12[b]
TG (mg/dL)	97.8 ± 19.33	69.6 ± 21.55	65.2 ± 13.16	79.2 ± 23.82
TC (mg/dL)	86.6 ± 10.92	118.4 ± 37.16	116.0 ± 9.43	135.3 ± 34.09[a]

Data are presented as mean ± SD. *P*-values are presented as: [a] $P < 0.05$ versus control; [b] $P < 0.05$ versus HFD. BW, body weight; BMI, body mass index; BL, body length; AST, aspartate aminotransferase; ALT, alanine aminotransferase, TG, triglyceride; TC, total cholesterol; SD, standard deviation; HFD, high fat diet.

Authors should properly address the surprising effect of LPS, a pro-inflammatory factor, administration to HF fed animals regarding ALT and AST. Besides, the proper group to compare HFD+LPS+GW values is HFD+LPS, no the HFD group.

Response:

Thank you for your comment. We completely understand your opinion and agree with it. As you pointed out, we failed to demonstrate the effect of LPS in mice. We expected that the chronic exposure of low-dose LPS would lead to make steatohepatitis in mice fed an HFD. As we noted in the Discussion, to overcome this limitation, future studies using endotoxins as HFD-enhancing factors in the murine NASH model are warranted. Also, we used a one-way ANOVA to compare four groups, not only two groups as you mentioned.

The sentence “PA and LPS together, but not alone, elicited the mRNA expression of NLRP3, NRLP6, and NLRP10” is not correct for NLRP10. It is misleading to state that some value “tends to be increased” when the difference is not at all statistically significant (MDA values).

Response:

Thank you for your comment. We have modified the text in the Results section as follows.

“…PA and LPS together (delete “but not alone”) elicited the mRNA expression of NLRP3, NRLP6, and NLRP10 (all $P < 0.05$) (Fig. 3)…”

“…In the *in vivo* study, levels of MDA, a product of lipid peroxidation, in liver homogenates, there was no statistically significant difference in four groups (Fig. 6A) ...”

Data presented in Figure 6 does not show at all that AMPK activity is significantly increased. In fact, WB points to an increased expression of AMPK protein in all groups except control.

Response:

Thank you for your comment. As your comment, p-APMK expression is increased in all groups except control (figure 6B, upper panel). When we corrected the loading control using total AMPK, p-APMK expression was increased 1.6 folds after GW treatment compared with control, as indicated in densitometric measurement (figure

6B, lower panel).

Figure 7 is missing .

Response:

The Figure 7 exists in the last page of the manuscript.

Discussion is too lengthy.

Response: I absolutely agree with you. As you suggested, we modified the discussion section as follows.

Thank you for your consideration of our paper.

I hope the revised manuscript will better meet the requirements for publication in ***World Journal of Gastroenterology.***

Sincerely,

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