Author’s response to reviews

Title: Diosgenin ameliorates palmitic acid-induced lipid accumulation via AMPK/ACC/CPT-1A and SREBP-1c/FAS signaling pathways in LO2 cells

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Author’s response to reviews:

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Dear editors and reviewers:

Thanks for your letter and comments concerning our manuscript entitled “Diosgenin ameliorates palmitic acid-induced lipid accumulation via AMPK/ACC/CPT-1A and SREBP-1c/FAS signaling pathways in LO2 cells”. Those comments are all valuable and very helpful for revising and improving our paper, as well as the important guiding to our researches. We have studied comments carefully and have made corrections which we hope to meet with approval. Revised portion are marked in red in the paper. The main corrections in the paper and the responds to the reviewer’s comments are as following:
Responds to Reviewer #1

Major comments:

1. Introduction does not reflect the importance of AMPK/ACC/CPT-1A and SREBP-1c/FAS signaling pathways in regulating lipid accumulation. Hence, the objectives of this study are obscured. Please revise the introduction accordingly.

   We are very pleased to accept your professional suggestion. The studies about AMPK/ACC/CPT-1A and SREBP-1c/FAS signaling pathways in regulating lipid accumulation have added to the introduction section (line 99, page 3). We have pointed out the importance of these pathways in lipid metabolism according to your suggestion.

2. Please revise the following sentence written under 'Cell culture, viability assay and treatment' sub-heading and mention the seeding density. Because, seeding density is very important to reproduce your work.

   "LO2 cells were cultured in 6 well-culture plates and allowed to grow overnight to 70% confluence."

   We are very sorry for our negligence of the detail in cell culture. Related contents have added to the methods section (line 167, page 6).

3. Please write down the name of the animals used to raise the primary and secondary antibodies. Specify the fluorescent conjugates used to label the secondary antibody.

   We have re-written this part according to the Reviewer’s suggestion. The animals used to raise antibodies and the specific fluorescent conjugates used to label the secondary antibodies were added to the methods section (line 142, page 5).

4. Please change the 'Immunofluorescence Analysis' sub-heading to 'Detection of reactive oxygen species and mitochondrial membrane potential', as it is a simple fluorescent technique rather than an immunofluorescence.

   We have made correction according to the Reviewer’s suggestion. The sub-heading “Immunofluorescence Analysis” was corrected as “Detection of reactive oxygen species and mitochondrial membrane potential”. (methods section, line 224, page 7).
5. In this study the cells were divided into the following groups

A) control group (incubated in DMEM containing 10% FBS)
B) model group (PA at a selective concentration for 24 h)
C) DSG groups (PA + different concentrations of DSG for 24 h)
D) AMPK activator group (PA + A-769662 for 24 h)
E) AMPK inhibitor group (PA + DSG + Compound C for 24 h).

Data were analyzed using one-way ANOVA and post-hoc. However, presentation of the results is not comprehensive. In some results, it is obvious that one group has significant differences with multiple groups, while the presentation of the results is not reflecting that. Please revise the figures to make them more comprehensive and communicative. Moreover, in the text, instead of writing 'significantly different' or 'distinct increase/decrease' only add the original 'p values' in brackets as well.

Thank you for reminding us to check the figures to make this research more scientific and rigorous. As Reviewer suggested, we have changed the figures to make it more comprehensive and communicative by using more intuitive comparison and comparing different DSG groups. The comparison between one group and another group was designed to explain the objectives of this study, therefore we didn’t compare each of the groups with statistical significance. Moreover, the original “p values” was added to brackets behind the “significantly different” or “distinct increase/decrease”. We would be glad to respond to any further questions and comments that you may have.

6. In discussion, DSG received little attention. Based on the results, further explanation is needed on the effect of DSG in regulating AMPK/ACC/CPT-1A pathway. Discussion requires major revision.

Thanks a lot for pointing out the weakness of this research. We have re-written the discussion section mainly by three fields: oxidative stress/mitochondrial dysfunction, fatty acid β-oxidation and lipid synthesis. In this section, we discussed the correlation between these pathological/physiological processes and hepatic lipid accumulation. The regulation of DSG on AMPK/ACC/CPT-1A and SREBP-1c/FAS pathways were associated with its impact on oxidative stress, β-oxidation and lipid synthesis, which was used to explained the anti-NAFLD effects in our work. Then, more discussions focused on DSG were added. We divided the DSG-related contents into three parts as mentioned above. Based on AMPK/ACC/CPT-1A and SREBP-1c/FAS pathways, the effect and potential mechanism of DSG in each part were investigated and added.
7. Please revise the conclusion to align with the objectives.

As the Reviewer suggested, we have revised the conclusion part in abstract and conclusion sections (line 54, page 2 and line 445, page 14) in the revised manuscript. In conclusion section, we emphasized the importance of oxidative stress, fatty acid β-oxidation and lipid synthesis in intracellular lipid accumulation, and the anti-NAFLD effect of DSG might be mediated by AMPK/ACC/CPT-1A and SREBP-1c/FAS pathways.

Minor comment:

1. Please avoid abbreviation for the terms that are being used first time in the text.

We feel sincerely apologized for our negligence about the detail in the abbreviation. We checked the paper and corrected the mistakes. For example, DSG was used first time in the abstract section (line 38, page 2).

Responds to Reviewer #2

(1) There are evidences of several poorly formed English languages which needs through revision.

We are very sorry for our incorrect English writing. We have checked and revised the manuscript to improve the English writing.

(2) I am not very much convinced with the controls used. For example use compound C, it is a partial blocker of AMPK. The author may have used some genetic blocker like siRNA.

Thanks a lot for your suggestion. The reasons why we used Compound C in this study as followed:

(i). Compound C is widely used in cell-based, biochemical and in vivo assays as a well-known selective AMPK inhibitor[1]. Numerous studies have shown that Compound C has a potent inhibitory effect on AMPK activity[2-6].

(ii). In many pharmacological researches, the inhibition of AMPK pathway was usually mediated by the administration of Compound C[1, 4, 7-9]. Notably, many previous studies focused on DSG and its analog applied Compound C in the AMPK loss-of-function experiments[7, 8, 10], which have an impact on our study. Therefore, we chose Compound C as a pharmacological blocker used in our study.
(iii). There is no doubt that siRNA is an excellent tool to inhibit AMPK activity. Since its discovery, siRNA has been widely recognized for its utility as a genetic blocker[11]. Lots of studies on AMPK pathway applied siRNA to inhibit AMPK activity[12-15]. We will be very pleased to use RNA interference technique in the further investigations.

(3) It was not clear from the manuscript if the treatment of diosgenin and the inhibitors were together or they were treated one after the other. This need to be mentioned clearly since it will impact the data.

We are sorry that this detail was not clear in the original manuscript. I have revised this part in methods section (line 173, page 6). In the inhibitor group, cells were pre-incubated with Compound C for 5h to inhibit the activation of AMPK in advance, and then treated with PA and DSG for another 24h.

(4) AMPK is known to inhibit ACC, also the authors shows the same in Fig. 10. But in the data (fig. 9) diosgenin was found to increase the level of p-ACC. This about a bit confusing since diosgenin is also known to increase AMPK (Fig. 10).

I feel sincerely apologized for our unclear expression. The phosphorylation of ACC by AMPK leads to the inactivation of ACC (inhibitory phosphorylation). Therefore, the ratio of p-ACC/ACC is negatively related to the ACC activity. We have corrected the text and Fig. 10 to make it more rigorous (line 99, page 3).

(5) Was there any dose dependent effect of diosgenin? Apparently it seems not. Then what was the rationality of using all the three doses in all experiments?

It is really true as Reviewer suggested that we didn’t observe the obvious dose dependent effect of DSG. The reasons why we used three doses in all experiments as followed:

(i). Whether DSG works in a dose dependent manner is one of the objectives of our research. The maximum nontoxic concentration of DSG was 10μM according to CCK-8 assay. Therefore, another two lower gradient concentrations were chosen to explore this goal. In many experiments, 0.1μM DSG did not exert a weaker effect than 1μM DSG, and both of them were significantly different from 10μM DSG. These findings showed a trend of dose-effect in DSG. So, we reserved all the three doses in this work.

(ii). In our study, the concentration range of DSG was 0.1-10μM. The results can provide a dose basis for further researches.
(6) Discussion portion needs to be more focused and with additional referencing.

We are very pleased to accept your suggestion. Briefly, the discussion section was re-written based on oxidative stress/mitochondrial dysfunction, fatty acid β-oxidation and lipid synthesis, which were crucial in liver lipid metabolism. In this part, we discussed the regulation of DSG on AMPK/ACC/CPT-1A and SREBP-1c/FAS pathways to explain its impact on oxidative stress, β-oxidation and lipid synthesis. New references were added as Reviewer’s suggestion.

We appreciate for Editors/Reviewers’ warm work earnestly, and hope that the corrections will meet with approval. We look forward to hearing from you regarding our submission. It is our pleasure to respond to any further questions and comments that you may have.

Once again, thanks very much for your comments and suggestions.

Sincerely.

Fuer Lu, M.D. Ph.D.

References:


