Author's response to reviews

Title: The inhibitory effect of Isoliquiritigenin on the proliferation of human arterial smooth muscle cell

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Author’s response to reviews:

Dear editor,

We appreciate editor and reviewers very much for their positive and constructive comments and suggestions on our manuscript entitled “The inhibitory effect of Isoliquiritigenin on the proliferation of human arterial smooth muscle cell” (MS NO.PHAT-D-16-00182). To address the critiques of the reviewers, we revised our manuscript according to the comments. Attached please find the revised version. A point by point response is listed as below.

Reviewer reports:

Reviewer 1:

1. From the cell viability experiments with different dose of ISL the authors might calculate the IC50 of ISL just to strengthen the data.

Response: Thank you very much for your constructive suggestion. IC50 of ISL has been calculated and added in fig.1. (Figures section, figure.1, page 1; Results section, line5, page6)

2. The authors stated that ISL significantly blocked the progression of cell cycle and that the G1/S ratio is significantly increased in ISL treated cells. There is no mention in the figure to the statistical significance of the difference, please add.

Response: Thank you very much. The statistical significance of the difference for figure.2 B has been added (Figures section, figure.2 B, page 1).

3. Please, show a western blotting image more representative of the quantification of Cyclin E. Indeed, if we look at the bands it seems that there is no differences between the control and ISL.
Response: Thank you very much. We have re-provided a representative western blotting image of the quantification of Cyclin E. (Figures section, figure.2C, page 1).

4. The authors should show the autofluorescence (not labelled cells) for both control and ISL treated cells. Regarding the quantification of ROS level, do the authors refer to the relative fluorescence intensity? Please explain what parameter they measured. Moreover I think that "% of control" is a mistake, maybe they meant fold change.

Response: Thank you for your suggestion. The autofluorescence for both control and ISL treated cells have been added (Figures section, figure.3A, page 2). The fluorescence intensity for single cell has been recorded by FACS machine. For statistics, cell mean fluorescence intensity was calculated. Indeed, we made a mistake and have corrected to “fold change” (Figures section, figure3A, page 2)

5. Usually the level of the phosphorylated proteins is quantified on the total proteins. Thus I suggest that the authors quantity p-PI3K on PI3K and p-AKT on AKT.

Response: Thank you for your suggestion. We have quantified p-PI3K on PI3K and p-AKT on AKT. (Figures section, figure4, page 2)

6. In all the experiments of this figure H2O2 treated cells should be added as further control. For the quantification of the western blotting I suggest the same as in Fig. 4.

Response: Thank you very much. H2O2 alone treated cells have been added as further control (Figures section, figure5A,B, page 3). We have quantified p-PI3K on PI3K and p-AKT on AKT (Figures section, figure5B, page 3).

Reviewer 2:

1. In the materials and methods, it is stated that the cells used as a control were untreated cells. Please clarify if the control cell culture media contained the vehicle used to dissolve the ISL or had no treatment.

Response: Thank you very much. Indeed, cells cultured in the medium containing DMSO which were used to dissolve the ISL were used as a control (Materials and Methods, line8-9, page12).

2. A better, more detailed description of the nature of the cell line being used, including if it is immortalized or primary, should be given.

Response: Thank you very much. Primary HASMCs was purchased from YRgene (China) and preserved in our lab (Materials and Methods, line8-9, page12).

3. In the materials and methods, add the purity of ISL that was purchased from Sigma.
Response: Thank you very much. Primary HASMCs was purchased from YRgene (China) and preserved in our lab (Materials and Methods, line3, page12).

4. In the materials and methods, add the purity of ISL that was purchased from Sigma.

Response: Thank you very much. The purity of ISL which was purchased from Sigma is 98% (Materials and Methods, line6, page12).

5. The assay described as cell growth in Figure 1a is an assay of cell number. The axis label in the figure, cell viability, is a better descriptor than cell growth. Please change the reference to this assay in materials and methods and in the results.

Response: Thank you very much. We have changed the reference “cell viability” to CCK-8 assay in materials and methods and in the results (Materials and Methods, line11, 16, page12; Results section, line2-5, page12).

6. Please comment on how the doses of ISL used in this study correlate with levels that might be given in a therapeutic context.

Response: Thank you very much. We had tried our best to look for a formula that can calculate the in vivo dose of medicine according to concentration given in vitro, however, we couldn’t find it. Very pity to say we have no idea about this question.

7. Although it is clear that the ISL caused an inhibition of proliferation, were the cells otherwise healthy? Did they undergo cell death or autophagy upon ISL exposure?

Response: Thank you very much. We have examined cell death by PI staining. Result showed that ratio of PI positive cells in ISL treated HASMCs was just a little higher than that in ISL untreated cells (Figures section, Figure.1A, page 1; Results section, line8-11, page6 ). Therefore, ISL didn’t induced cell death.

8. Another main effect of ISL in other systems is to act as a botanical estrogen, augmenting or interfering with normal hormone signaling. Is this a possibility in the HASMCs? Do these cells have steroid hormone receptors? This should be discussed.

Response: Thank you for this suggestion. We have re-discussed this topic in our manuscript (Discussion, line17-22, page10 and line1, page11 ).