Peer Review File

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Reviewer A

In this manuscript, the authors (Zhang et al.,) sought to reveal the mechanism by which Sen Yuan Dan exerts protective effects again ischemia-reperfusion damage on cardiac myocytes. The authors employed H9C2 cells culture system and clearly showed that SYD has protective effects against ischemia-reperfusion injury. In addition, the authors also showed that SYD treatment restored decreased mitochondrial content caused by hypoxia-reoxygenation. The results are intriguing and show the effect of SYD. However, there are some critical points to be addressed.

Major points

1. The authors show that SYD restored mitochondrial content (Fig. 3 and 4). In addition to the existing data, the authors need to show mitochondrial DNA copy numbers and Oxphos complex activity assay to support the data.

Response: The results of the present study provide preliminary evidence that SYD can restore mitochondrial content. We will conduct more in-depth studies in future experiments, including experimental testing of mitochondrial DNA copy number and Oxphos complex activity, and your suggestion provides a good methodological reference for our research.

Reviewer B

In this study entitled "Protective effects of Shen Yuan Dan on myocardial ischemia-reperfusion injury via the regulation of mitochondrial quality control", authors showed that SYD protects H9c2 myocytes through mitochondrial quality control and their protection after stimulated injury. This manuscript is interesting, but lots of serious issues are addressed.

Major comments

1. In this experiment, authors cultured H9c2 in serum-free low-glucose medium for 12 h in 5% CO2 incubator, then incubated in 10% FBS high-glucose medium for 6 h in 5% CO2 incubator. Is this model hypoxia-reoxygenation? In this culture condition, O2 concentration would not be reduced, so it is not hypoxia. In addition, in usual low-glucose medium include 5 mg/dL glucose, which is usual concentration of blood glucose. In

addition, title of this manuscript is “ischemia-reperfusion injury”. This title is far from this experiment.

Response: We have modified our text as advised (see Page 5, line 165-170).

2. In Method section of MTT assay, when authors add SYD in cultured cells? Were H9c2 cells incubated for 24 h with SYD?

Response: After cells incubating for 24 h, SYD was added into each well. Then, cells were incubated with SYD for another 4 h.

3. In Method section of Experimental grouping and cell processing, cells in control group were incubated in 1% FBS DMEM medium for 12 h (L175). Low glucose DMEM? High glucose DMEM? Similar expressions were also included in group III and IV. In addition, DMEM represent Dulbecco's Modified Eagle's Medium. Therefore, DMEM medium is Dulbecco's Modified Eagle's Medium medium (L159, L175 etc.).

Response: We have modified our text as advised (see Page 6, line 193-199).

4. In Method section of JC-1 staining, authors would culture cells using dish or flask, how adherent cultured cells were mediated to 1x106 floating cells/mL for FACS analysis.

Response: We have modified our text (see Page 6, line 203).

5. In Methods section of ATP, SOD, and MDA, authors measured protein levels of samples. Methods of measuring protein level are needed.

Response: We have modified our text as advised (see Page 6, line 212-261 and Page 7 Line 266-267).

6. In Method section of Statistical analysis, what method was used as post-hoc analysis of one-way ANOVA?

Response: We have modified our text as advised (see Page 8, line 311).

7. In this experiment authors use 3 methyladenine. However, explanation about the role of this compound was not found. Perhaps, 3MA was used as an inhibitor of autophagy. Authors should clearly mention about 3MA.

Response: We have modified our text as advised (see Page 5, line 149-150).

8. In results section of 1st paragraph, results of MTT assay and determination of SYD dose were included. However, the title of this paragraph is different from definition of SYD dose. So, this reviewer recommends that results from definition of suitable dose of SYD and effect of SYD on mitochondrial membrane potential are divided. In addition, in Figure 1a there is no differences among all groups. So, how authors decided the suitable dose of SYD?

Response: As figure 1 A depicted, when the concentration of SYD is 100 μg/mL, the cell inhibition rate is positive. Therefore, we chose 100 μg/mL SYD for subsequent experiments.

9. In Results section of SOD and MDA, authors wrote that SOD activity was markedly reduced by H/R stimulation (L264). However, SOD activity was tended to be reduced but not significant in H/R group.

Response: We have modified our text as advised (see Page 9, line 338).

10. In Results section and Figure 1b, JC-1 staining is an indicator of mitochondrial membrane potential. Red fluorescence indicates preservation of MMP and green fluorescence indicates loss of MMP. However, in Figure 1b levels of green fluorescence (FL1) are almost similar among all groups. Red fluorescence, but not any changes of green fluorescence, is only decreased in HR group. These phenomena are very curious.

Response: In figure 1B, the horizontal coordinate JC1-1 represents the green fluorescence channel and the vertical coordinate JC1-2 represents the red fluorescence channel. In experiment of flow cytometry, different coordinates represent different fluorescence. Compared with the Control group, the green fluorescence in H/R group was evidently increased. Compared with H/R group, the green fluorescence was slightly decreased in H/R+3MA and H/R+SYD group.

11. In Results section and Figure 2a, ATP levels in cultured cells are almost none after H/R stimulation. In this experiment, HR stimuli is low glucose and FBS free stimulation followed by high glucose and 10%FBS condition, did this stimulation loss almost all ATP? If so, in this condition almost all cells would fall into cell death. Authors should assess several indicators of cell death.

Response: We have modified our figure.

12. In Results section and Figure 3a, reviewer could not find green fluorescence and mitochondrial morphology. Authors should show clear fluorescence image with higher magnification. In addition, authors wrote that degree of mitochondrial networking was remarkably diminished and decreased number of mitochondria in H/R group and SYD strengthened the degree of mitochondrial networking (L283-289). Mitotracker green is mitochondrial indicator, however, Mitotracker green is MMP-dependent mitochondrial indicator, so MMP-lost mitochondria are not stained. So, number of mitochondria and networking of mitochondria would not be assessed by this probe. Authors should use MMP-independent mitochondrial probe and or other assay of mitochondrial content.

Response: The Mito Tracker Green FM (MTG) used in this experiment is a durable mitochondria-specific, green fluorescent probe that only requires a mild covalent sulfhydryl group to bind to mitochondrial proteins and can be taken up and accumulated by living cells. Compared to the traditional mitochondrial fluorescent probe TMRM, its ability to localize to mitochondria is not affected by mitochondrial membrane potential and its fluorescence intensity remains unchanged after mitochondrial depolarization. Therefore, it can be used to assess the number and dynamic changes of mitochondria.

13. In discussion, authors wrote “following exposure to stress, mitochondrial fission is activated” (L358-359). In this process Drp-1 and Fis 1 would have critical roles. However, in this model all of the mRNA levels of fusion and fission proteins are reduced. In this condition, how fission was induced, and mitochondrial networking was destroyed in H/R?

Response: We have modified our text as advised (see Page 10, line 371-377; Page 11, Line 416-417) and the figure has been revised.

14. Authors should discuss the mechanism of SYD on mitochondrial quality control factors and SOD. Is somewhat component in SYD could access promoter elements of quality control factors?

Response: We have modified our text as advised (see Page 12, line 470-471). The question of whether there is component in SYD that can access promoter elements of quality control factors will be investigated in the future.

15. In Discussion part, authors wrote that activation of PINK1/Parkin pathway by NBP could attenuate MIRI. On the other hand, in this experiment SYD suppressed PINK1/Parkin and attenuate MIRI. Please discuss this discrepancy.

Response: We have modified our text as advised (see Page 13, line 504-505).

Minor comments

1. In Method section (L181), "100 ug/mL" is incorrect and "100 μg/mL" is perhaps correct.

Response: We have modified our text as advised (see Page 6, line 198).

2. In Method section (L202), "3000 ℃" is incorrect.

Response: We have modified our text as advised (see Page 7, line 265).

3. MMP represent mitochondrial membrane potential. So, mitochondrial MMP is incorrect (L391-392).

Response: We have modified our text as advised (see Page 10, line 400).

4. In Figure 1 authors use "a" "b" "c" (small letter), on the other hand in figure legends authors wrote "A" "B" "C". In Figure 2, 4, 5, authors shod use "a" "b".

Response: We have modified our figures as advised.

5. In Figure 3A scale bar should be included in representative photos. Sample size of this figure 3B is lacking.

Response: We have modified the figure as advised and the sample size has been added in the figure legend (see Page 22, line 765).