Author’s response to reviews

Title: Protective effect of Danggui (Radix Angelicae Sinensis) on angiotensin II-induced apoptosis in H9c2 cardiomyoblast cells

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Author’s response to reviews: see over
Responses for Reviewer 1

Reviewer's report:
This study was designed to investigate the protective effects of Danggui on Ang II-stimulated cardiac apoptosis and the mechanisms. It is an interesting study.

Below are the comments:

Major Compulsory Revisions:
1. In this and previous studies, the authors have testified Danggui protected H9c2 cells from Ang II-induced apoptosis via JNK pathway. To further investigate the importance of JNK pathway in Danggui protected Ang II-induced IGF related cardiac apoptosis, I think you'd better to detect IGF II and IGF IIR expression and PI3K/AKT pathway after JNK inhibitor co-treated with Danggui and Ang II in H9c2 cells.

Our response:
We appreciate the reviewer’s suggestion. We have performed additional experiments and the results are shown in Supplementary Figure S3.

2. In Fig. 6C, in Ang II+Danggui+JNK inhibitor group, activated caspase-3 seemed even much darker than Ang II group, I'm not sure whether it is because the toxic effect of JNK inhibitor or its effect of JNK pathway inhibition. I think you should add a group of JNK inhibitor alone as control.

Our response:
When co-stimulated with Ang II and Danggui, SP600125 inhibited JNK activity (decreased the pro-apoptotic response) and activated p-PI3k and p-Akt (increased the anti-apoptotic effect) (Supplementary Fig S3). SP600125 may play an important role in anti-apoptosis. However, SP600125 alone or treatment with Ang II and Danggui both increased the active caspase-3 expression (Fig. 6C and Supplementary Figure S4). Thus, the mechanism of the JNK inhibitor on Danggui’s protective effect on Ang II-induced apoptosis is needed more study to clarify.

1. All the Western data should be quantified.

Our response:
We have quantified our Western results and the analyses are shown in the Supplementary Figure S2.

Minor Essential Revisions:
4. In Fig. 5B, the gel of cytochrome c showed on the left doesn’t very clear, please provide a representative gel for it.

Our response:
We repeated this many times; however, the same weak results were obtained.

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician

We thank the reviewer for these comments, which have helped us to improve the manuscript.
Responses for Reviewer 2

Reviewer's report:

Major concerns

1. The extract was used. It’s a pharmaceutical preparation which of course consisting of several active and non-active ingredients, which might affect the results. The bioassay guided fraction could be included in the study. The details of the ingredients need to be elaborated and the contents should be identified. What was the percentage of danggui extract in whole formulation.

Our response:

More than 70 compounds, including alkyl phthalides, benzenoids, butylphthalide, coumarins, flavones, organic acids, polysaccharides and trepnes, have been isolated from the root of Angelica sinensis. Among the identified main compounds, Z-ligustilide, phthalides and ferulic acid are known to be its major essential active components (Yi et al, 2009; Chen et al., 2013). We have now included this information in the manuscript.

References:


2. The extract is well known for beneficial effects in heart diseases. Authors undertook this study to understand the mechanism of cardioprotection. However, they used the extract from a pharmaceutical formulation and they could have characterized contents. That would add novelty to this study.

Our response:

The Danggui extract powder used in this study was obtained from Ko Da Pharmaceutical. The company used ferulic acid as the key index active component to perform their batch monitoring test. In the batches we received, the contents of ferulic acid were all similar, at a level of 0.92 mg /g of extract powder (by HPLC analysis with analytical grade ferulic acid as the standard from Fluka).

However, for Traditional Chinese Medicine (TCM), some research studies have proposed that a single active component might have a lesser therapeutic effect, as the combinational effects of the TCM formula is often greater than that of an individual drug or a single component. Therefore, the importance of the individual components for cardioprotection will need further in-depth study.

3. What are the active components of this herb, do the authors can relate the activity to active components.

Our response: As mentioned above Z-ligustilide, phthalides and ferulic acid are known to be its major essential active components. Z-ligustilide (Feng et al, 2012) and polysaccharides (Jiang et al. 2009; Zhang et al., 2010) from the Danggui extract have been shown to have anti-apoptosis or antioxidant effects, and both ferulic acid and Z-ligustilide have been shown to have the effect of memory
amelioration (Lin et al, 2012).


4. How do the authors comment that same herb has been shown effective in inducing apoptosis in cancer and provide Chemopreventive and anticancer activity. Whereas, in heart the extract is showing antiapoptotic effect. Do the apoptosis modulation is site dependent.

**Our response:**
There are several active components in *Danggui*, and therefore the same herb has different applications in different types of cells. As different organs may have different regulation networks in the cells, this may lead to the same herb showing different results. For example, Indirubin-3′-monoxime, a derivative of indirubin, which is one of the active constituents of *Danggui*, can induce apoptosis and tumor cell death (Perabo et al, 2006). On the other hand, Z-ligustilide and Polysaccharides have different effects as described above.


5. Abstract should be edited to be crystal clear in putting all things together in a meaningful approach.

**Our response:** We appreciate the reviewer’s suggestion and have revised the Abstract to make it clearer.

6. The introduction should be revised to put objectives and background in a clear way and the interpretation i.e. discussion and conclusion should be balanced and supported by the data, rather speculative and being overwhelming?

**Our response:** We appreciate the reviewer’s suggestion and have revised the Introduction, Discussion and Conclusion. We have also performed more experiments as suggested by another reviewer. The results are described in the manuscript or included in the supplementary data.
7. Novelty is a major concern, considering the available knowledge, from below references. And


**Our response:**

Thanks for providing these relevant references. We have now integrated the findings of these studies into our Introduction and Discussion sections.

Minor comments

2. cardiac apoptotic effects in cardiomyoblast cells, DELETE ‘CARDIAC’.

**Our response:** We have deleted this from the manuscript.

3. and rescue effects on Ang II-stimulated H9c2 cardiomyoblast cells, DELETE ‘RESCUE’, replace this rescue effect with better alternate

**Our response:** We have deleted this from the manuscript.

3. Angiotensin II (Ang II) is a risk factor for cardiovascular diseases, REPHRASE IT

**Our response:** We have rephrased this sentence as “Angiotensin II (Ang II) has been shown to play important roles in mediating cardiovascular diseases”.

4. This study revealed the mechanism of Danggui as a potential cardiac-protective and therapeutic traditional Chinese medicine, REPHRASE IT

**Our response:** We have rephrased this sentence as “Our results suggest that Danggui has a potential cardiac-protective effect that may be used for the prevention of cardiac apoptosis”. 
5. What authors mean by, RESCUE EFFECT

Our response:
Both pre-treatment and post-treatment with *Danggui* attenuated the damaging effects of Ang II. We now understand that this might not sound clear, and have deleted it and just kept “has a protective effect” in the text.

6. In addition, IGF-II and IGF-II receptor (IGF-IIR) have been shown to be associated with the development of heart attack [4-7]., rephrase this sentence

Our response:
We have rephrased this sentence as “In addition, IGF-II and IGF-II receptor (IGF-IIR) have been shown to be associated with the development of cardiac hypertrophy [3-6].”

7. Danggui extract powder was provided by Ko Da Pharmaceutical (Taoyuan, Taiwan). So it mean there is a conflict of interest.

Our response:
We have now added “Danggui extract powder was provided by Ko Da Pharmaceutical. The company had no role in this study.” in the Competing interests section.

8. the solution was centrifuged and sterilized by filtering through a 0.2 µm syringe filter and stored at -20°C. Why it was stored at -20, this is so unstable.

Our response:
Our description was incorrect in the Methods section. We did keep the solution at -80°C. We have now corrected this in the text.

9. What concentration was used, 50, 250 or 100 it’s not very clear in throughout the paper.

Our response:
We have revised this in the Methods and Discussion sections: treatment with *Danggui* extract (0, 50, 100, 250, 500, 1000 µg/ml) 1 hr before or after Ang II treatment.

10. Danggui is commonly used to treat various gynaecological conditions, but is also recommended to help prevent and treat some forms of cardiovascular disease [19]. It need to be rephrased.

Our response:
We have revised this sentence to be “*Danggui* is not only commonly used to treat various gynecological conditions, but recently it has also been shown to prevent doxorubicin-induced chronic cardiotoxicity and reduce myocardial injury in animal models [18, 19].”
11. Drug toxicity of Danggui extract on H9c2 cardiomyoblast cells, rephrase it.

**Our response:**

We have moved some details of the experimental information to the Methods section and revised this section.

12. WW Kuo, CJ Liu, LM Chen, CH Wu, CH Chu, JY Liu, MC Lu, JA Lin, SD Lee and CY Huang [22], follow a standard format

**Our response:**

We have reformatted the reference.

13. The title should be edited

**Our response:**

The title has been changed to “Protective effect of Danggui (Radix Angelicae Sinensis) on angiotensin II-induced apoptosis in H9c2 cardiomyoblast cells”.

14. The whole manuscript must edited for English improvement, several redundant sentences required edits and improvement and more of scientific writing

**Our response:**

The manuscript has now been edited through to improve the English and proofread by a native English speaker.

15. The extract preparation and its fractionation should be detailed and the components need to be characterized.

**Our response:**

Traditionally, extracts are prepared by boiling in water for 1-2 h and extracting twice. After filtration, the extraction solution is lyophilized to prepare extract powder. However, we were not able to obtain the detailed protocol from Ko Da Pharmaceutical regarding the preparation processes or find out whether the solvent extraction method was used or not. The company did provide information with regards to the ferulic acid content, an index component they use for quality control.

Discretionary Revisions Otherwise, the manuscript is interesting and add knowledge. However, characterising the components could add more value. Considering the complimentary and alternative medicine, I am in favour after the major revision done.

We thank the reviewer for these comments, which have helped us to improve the manuscript.
Response to Reviewer 3
- Minor Essential Revisions

The authors described their study using Danggui extracts to restore the apoptotic effects of AngII on H9c2 cells. They found that Danggui extracts could protect H9c2 cells from AngII-induced apoptosis by inhibiting caspase 3 and caspase 9 activation, stabilizing the mitochondrial membrane potential. Inhibiting the PI3K pathway could block Danggui’s effect. The study is well performed and presented. I have only two comments for the authors.

1. In figure 1, pre-treatment could improve the cell viability at the concentration over 250 µg/ml; while the post-treatment showed similar effect at the concentration of 50 µg/ml. In subsequent experiments, the concentrations authors used are at 50 or 100 µg/ml either pre- or post-treatment. It seems that both pre- or post-treatment at 50 µg/ml showed similar effects, which is inconsistent with the cell viability assay. Authors should discuss this.

Our response:
In Figure 1, the post-treatment showed higher effect at the concentration of 50 µg/ml than that of pre-treatment. After we quantified our Western results (Supplementary Figure S2), we found post-treatment with 50 µg/ml Danggui extract showed significant decreased the active caspase-3 expressions than pre-treatment with the same dose as shown in the representative Western result in Figure 4 and Suppl. Figure S2. There was also a similar trend for caspase-9, although the changes between pre- or post-treatment was not significant different. In addition, post-treatment with 50 µg/ml Danggui extract also shown increased the p-Bad, p-PI3K and p-Akt expressions than pre-treatment. The Western blot data were consistent with the cell viability assay (Fig. 1).

Our previous description probably was not clear. We have revised this in the manuscript now.

2. From this study, the most impotent findings are Danggui can inhibit AngII-induced apoptosis and JNK inhibitor can block Danggui’s effect, which are shown in figure 7. While in this figure all other steps are not proven in this study. Therefore, I suggest to delete this figure.

Our response:
We have revised Figure 7 and obtained more data from additional experiments as suggested by another reviewer. Only the steps that are relevant to this study are shown.

We thank the reviewer for these comments, which have helped us to improve the manuscript.