Author's response to reviews

Title: Baicalein, an active component of Scutellaria baicalensis Georgi, prevents lysophosphatidylcholine-induced cardiac injury by reducing reactive oxygen species production, calcium overload and apoptosis via MAPK pathways

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Author's response to reviews: see over
Dear Editor,

We respectfully submit the revised manuscript MS: 1411007975113343, entitled “Baicalein, an active component of Scutellaria baicalensis Georgi, prevents lysophosphatidylcholine-induced cardiac injury by reducing reactive oxygen species production, calcium overload and apoptosis via MAPK pathways”, for your consideration for publication in the “BMC Complementary and Alternative Medicine”.

We deeply appreciate the editor’s expertise comments to improve this manuscript. All comments have been responded point by point and additional experiments have been performed as suggested. In addition, to improve the language with better flow, the manuscript has been edited by a native English speaker.

We thank you and the reviewers in advance for your time and consideration. We look forward to hearing from you again.

Sincerely,

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Revised MS: 1411007975113343, entitled “Baicalein, an active component of Scutellaria baicalensis Georgi, prevents lysophosphatidylcholine-induced cardiac injury by reducing reactive oxygen species production, calcium overload and apoptosis via MAPK pathways”

We greatly appreciate the comments of the editor and reviewers on our study and the suggestions to improve the manuscript. We are glad to know that the manuscript is considered acceptable for publication after revision. As suggested, the manuscript has been extensively revised and the experiments suggested by the editor and reviewers were carried out. We hope that this revised manuscript will be acceptable for publication in the *BMC Complementary and Alternative Medicine*.

Below please find the responses based on the specific comments:

**Reply to Reviewer #1’s comments:**

Comment 1: In the paper, design of the experiment is unreasonable. Such as: The dosage of baicalein is different.

Comment 1.1. This paper inferred Baicalein pretreatment of H9c2 cells attenuated lysoPC-induced cell death in a concentration-dependent manner. In observation of “Effects of baicalein on pro-apoptotic proteins”; “Inhibitory effects of baicalein on lysoPC-induced expression of caspase-3 and caspase-9” why the author choose pretreated with BE as 0.1 to 10 µM?

**Response:** In this study, we found that baicalein (1 to 10 µM) attenuated lysoPC-induced cytotoxicity as shown by the MTT assay (Figure 1b). In our preliminary study, we also found that pretreatment of baicalein (10 µM) showed significantly preventive effects on lysoPC-induced expression of caspase-3 and caspase-9. Therefore, we decided to choose 0.1 µM as the low dose and 10 µM as the high dose, so that dose-dependent response can be observed.

Comment 1.2. In the experiments of “Inhibitory effects of baicalein and catalase (100 µM) on lysoPC-induced ROS production in H9c2 cells” “Effects of baicalein on lysoPC-induced Ca^{2+} responses”, why the author choosed catalase? What is the aim to observe the effect of catalase? In observation of “Effects of baicalein on lysoPC-induced Ca^{2+} responses”, why not measure the [Ca^{2+}]i of untreated H9c2 cells? It needed the level of [Ca^{2+}]i of untreated H9c2 cells as control.

**Response:**
1. Our previous study also found that lysoPC-induced ROS production in rat VSMCs, and ROS has been suggested as a mediator of increased calcium overload (Hsu JH et al., Atherosclerosis 2011;217:379-386). Since catalases are ubiquitous enzymes that may degrade hydrogen peroxide, an important ROS, we used catalase in our study to show that baicalein may inhibit calcium overload through ROS reduction.

2. In Figure 3b, we showed the increased amount of $[\text{Ca}^{2+}]_i$ after lysoPC stimulation with and without baicalein. In fact, in Figure 3a, the baseline level of $[\text{Ca}^{2+}]_i$ before lysoPC stimulation in control group represented the untreated $[\text{Ca}^{2+}]_i$ of H9c2 cells. The mean level of baseline $[\text{Ca}^{2+}]_i$ of control group is 106 nM ---- as shown in Fig 3a.

Comment 1.3. In the part “Inhibitory effects of baicalein and catalase (100 µM) on lysoPC-induced ROS production in H9c2 cells”, Why the author chose 50 µM lysoPC, 0-5 µM baicalein?

Response: In a previous study, Takahashi M et al. used 50 µM lysoPC to induce apoptosis in human endothelial cells (Atherosclerosis 2002;161:387–394). Similarly, in our preliminary study, we also found that 50 µM lysoPC induced significantly increase the ROS production. That is the reason we chose 50 µM lysoPC to perform our study. We also appreciate the opinion about the concentration of baicalein. In the revised manuscript, the concentration of baicalein has been changed to 0-10 µM to be consistent with other experiments.

Comment 2. Figure 4 did not show the pictures of all groups.

Response: We have revised this figure and showed the pictures of all groups in Figure 4.

Comment 3. Phosphorylation of ERK1/2 can protect apoptosis of cardiomyocytes. The paper showed that baicalein inhibited activations of all three MAPK induced by lysoPC. Please explain the mechanism.

Response: In general, p38 and JNK mediate the apoptosis while ERK1/2 can promote cell growth. In our study baicalein inhibited activations of all three MAPK induced by lysoPC, we speculate that the effect on ERK by baicalein was offset by effects on JNK and p38, resulting in net effects of preserving cell survival. Even though it is generally believed that phosphorylation of ERK1/2 can protect apoptosis of cardiomyocytes. However, there is some evidence suggesting that ERK1/2 also contributes to cell death of cardiomyocytes. For example, activation of ERK1/2 has a role in Bcl-2 family-mediated cell apoptosis caused by doxorubicin in cardiomyocytes (Liu

Reply to Reviewer #2’s comments:
Major concerns
1. MAP kinases were determined by immunoblot only. Activity should be measured in addition.
   Response: We agree with the reviewer’s suggestion. Additional experiments of MAP kinases activity have been done and added to the Result section, in page 11, and in Figure 7.

2. All experiments were conducted in cell lines. Selected studies should be done in primary cardiac cells to determine whether they have similar responses. For example, Yu et al (ref #13) showed no decrease in cell viability with 10 µM lysoPC, whereas viability of H9c2 cells is approximately 50% with 10 µM lysoPC. The majority of experiments are carried out using 10 or 50 µM lysoPC.
   Response: We appreciate the reviewer for this important point. Yu et al (ref #13) showed that in adult rat cardiomyocytes, 10 µM lysoPC had no significant effect on the cell viability when these cells treated for “5 min”. In the present study, treatment of H9c2 cells by lysoPC with the same concentration (10µM) resulted in a decreased significantly of cell viability (about 50%), but with the treatment duration of “24 h”. In fact, in our preliminary study, we found that when neonatal rat cardiomyocytes were treated with lysoPC (10 µM) for 24 h, the cell viability was also decreased to 44.7%, which was similar to our results in H9c2 cell lines (figure below). Even so, we agree this point as our study limitation, which has been added in the paragraph of Discussion (page 14).

Figure. Protective effects of baikalein on lysophosphatidylcholine (lysoPC)-induced cell death of neonatal rat cardiomyocytes. Cardiomyocytes were treated with lysoPC for 24 h in the
presence and absence of baicalein (0.1 to 10 µM). Values represent mean ± S.E.M, n = 9. Control: H9c2 cells were placed in serum-free medium. **P < 0.01, versus control group; ***P < 0.01 versus cells exposed to lysoPC alone.

3. This is an extremely high concentration to apply to cells. Circulating concentrations are normally 100 uM and increase 2-3 fold with ischemia. Circulating LPC is approximately 99% protein bound. What is the concentration of free lyoPC in ischemic areas? Would a concentration as high as 50 uM have a direct adverse effect on the lipid bilayer properties of the cell? Previous studies with lysoPC in primary cardiac myocytes typically use 1-10 µM.

**Response:** We agree with the reviewer about this point. Indeed, a previous report indicates that the physiological concentrations of lysoPC in the human peripheral venous blood are 55.3±8.6 µM, but under ischemia, lysoPC concentration are increased to 178±18.0 µM in the human coronary sinus (Am J Cardiol, 1990;66:695-698). Yu et al showed that 10 to 20 µM free lysoPC may be present in the ischemic tissue (ref #13). We agree that high concentration as 50 µM may have a direct adverse effect on the lipid bilayer properties of the cell. However, as shown in some previous publications, it is not uncommon to use high concentration to induce cell injury for the study purpose. For example, Liao CK et al showed that 30 µM lysoPC destroyed the structure and function of gap junctions in rat primary cardiomyocytes (Toxicology 2013;314:11–21). Maeda S et al reported that 50 µM lysoPC induced expression of Na⁺/Ca²⁺ exchanger type 1 (NCX1) mRNA (J Pharmacol Sci 2009;109:565–572). Golfman LS et al demonstrated that 100 µM lysoPC induced arachidonic acid release and calcium overload in H9c2 cells (J. Lipid Res. 1999. 40: 1818–1826.).

4. What is the mechanism by which lysoPC induces ROS production?

**Response:** Recently studies suggest that NADH/NADPH oxidase is an important source of lysoPC-induced ROS. Yamakawa et al. suggested that ROS generated through NADH/NADPH oxidase are essential for the growth-promoting signals activated by lysoPC in VSMCs (Arterioscler Thromb Vasc Biol 2002;22:752–8.). Similarly, our recent report also found that lysoPC upregulated expressions of NAD(P)H oxidase subunits, Nox1 and Rac1 in VSMC (Hsu JH et al., Atherosclerosis 2011;217:379-386).

5. Figure 1a-is there a significant decrease in cell viability? Add significance symbols

**Response:** As suggested, we have added the significance symbols in the Figure 1a.

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6. Figure 6—changes in caspase activity are minimal. They may be statistically significant but are they biologically significant?

**Response:** We have changed the figure so that the difference between caspase-3 and caspase-9 activity can be shown more clearly.

7. Figure 7a & c-p-ERK signal between control and 10 uM lysoPC are clearly different in the immunoblot (with similar total ERK signal), but similar in the densitometry data, which has small SE bars.

**Response:** Because the data are given as the percentage of c-p-ERK signal from untreated cells, which were set at 100%. Therefore, we showed the small SE bars in the control group in Figure 7.

**Minor concerns**

8. In the Background section, the authors state “lysoPC……..is the main mediator of cardiac dysfunction” and refer to a manuscript from 1998. It is overreaching to make this statement.

**Response:** We agree with the reviewer’s comment and have amended this sentence. Lysophosphatidylcholine (lysoPC) -- and may cause deleterious effects on cardiac function during cardiac ischemia.

9. In the Discussion, the authors state that “lysoPC can accumulate up to 100-200 uM in…..membranes of injured cardiomyocytes”. This is incorrect, based on the references cited.

**Response:** This sentence has been deleted. We have revised the sentence as “It has been reported that the concentration of lysoPC in myocardium may increase in the setting of cardiac ischemia”.

**Reply to Reviewer #3’s comments:**

In this study, the authors have examined the cardioprotection effect of baicalein on lysoPC-induced cytotoxicity in a rat ventricular myocardial cell line H9c2. They showed that baicalein attenuated lysoPC-induced H9c2 cell death in a concentration-dependent manner, the mechanism is not only associated with reduced ROS production and Ca2+ influx, but also related to inhibit the expression and activity of caspase-3 and caspase-9, as well as phosphorylation of ERK1/2, JNK, and p38. In general, the study is appropriately designed for addressing the questions that authors intend to ask.
Minor Comments:

1. One issue that I’m interested in is why the authors chose rat embryonic cardiomyocytes H9c2, not mouse or human cardiomyocytes in the study. Present conclusions may be set the scope of study in the rat.

   **Response:** For technical reasons, the H9c2 cell line instead of cardiomyocytes was selected as a model for this study because it is less labor-consuming to culture these cells and we believe it has similar features of cardiomyocytes. However, we agree it is a limitation of our study and this point has been added in the discussion (page 14).

2. Statistical differences should be marked in Fig 1a.

   **Response:** As suggested by the reviewer, we have marked the statistical differences in the Figure 1a.

3. It’s better to add the results of BE 10 µM in ROS testing assay. In addition, why the author used 10 µM lysoPC in pathway studies but not 50 µM lysoPC (used in cell viability assay) should be explained in the text.

   **Response:** As suggestion, we have added the data of baicalein 10 µM as shown in Figure 2. The reason why we did not use the high concentration of 50 µM lysoPC in the pathway study is because it could cause significant decrease of cell viability, so that we couldn’t collect enough attached cells to perform these experiments. **This has been clarified in the paragraph of study limitation in Discussion Section (page 14).**