Author's response to reviews

Title: Neuroprotective effects of Liriope platyphylla extract against hydrogen peroxide-induced cytotoxicity in human neuroblastoma SH-SY5Y cells

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

Enclosed is our revised manuscript (Manuscript #4790170321642657) entitled “Neuroprotective effects of *Liriope platyphylla* extract against hydrogen peroxide–induced cytotoxicity in human neuroblastoma SH-SY5Y cells”. Reviewers #1 and #2 raised several specific concerns which we have addressed as follows.

• Reviewer 1.

Park et al. reported the suppression of H$_2$O$_2$-mediated cytotoxicity of LPE in human SH-SY5Y cells. P38 MAPK phosphorylation was repressed by the pre-treatment with LPE in H$_2$O$_2$-treated cells. The results were sound and manuscript is easy to read. However, there are concerns that should be improved.

1. Why did the authors use 100 µM H$_2$O$_2$? How did the authors decide this concentration?

Response: Thank you for your comment. H$_2$O$_2$-induced cell injury *in vitro* model was used to examine the neuroprotective effects of LPE in the SH-SY5Y cells. To determine the final concentration of H$_2$O$_2$, SH-SY5Y cells were treated with H$_2$O$_2$ at concentrations ranging from 10 µM to 500 µM for 24 h (Fig. 1). The cell viability was not affected at 10 or 50 µM H$_2$O$_2$, however, up to this concentration, it was decreased a concentration-dependent manner, with significant cytotoxicity being observed at concentrations > 250 µM. We thought that up to 250 µM was too high to evaluate the neuroprotective effects of LPE because only 20–50% cells were survived in SH-SY5Y cells treated with up to 250 µM. Therefore, we used 100 µM H$_2$O$_2$ (20–30% inhibition) to examine the neuroprotective effects of LPE in SH-SY5Y cells. We have added the
additional sentence into the discussion part.

Figure 1. Effect of H$_2$O$_2$ on the cell viability of SH-SY5Y cells. Cells were seeded in 96-well plates (5×10$^4$ cells/ml) and incubated overnight. Cells were treated with the indicated concentrations of H$_2$O$_2$ for 24 h. 10 – 50 μM H$_2$O$_2$ had no significant effect on SH-SY5Y cell viability. However, H$_2$O$_2$ (100 – 500 μg/ml) decreased cell viability a concentration-dependent manner. The values shown are means ± standard errors (SE; n = 8). *p<0.05, **p<0.01 compared to vehicle.

2. The authors should indicate the method to isolate the protein from cells. Moreover, determination of protein concentration should also be stated.

Response: Thank you for your comment. Whole cell lysates were prepared using RIPA buffer (Millipore Corporation, Billerica, MA, USA) by adding protease inhibitor cocktail and phosphatase inhibitors (Roche Diagnostics, Basel, Schweiz). After washing cells twice with PBS, cells were harvested and collected by centrifugation at 12,000 rpm for 15 min. The pellets were resuspended in RIPA buffer. Protein concentration was determined using the bicinchoninic acid (BCA) assay kit with bovine serum albumin standard. We have added the additional sentence into the methods part.
3. In mitochondria membrane potential assay, how did the authors calculate the Red/Green ration? Please indicate the method to calculate in Materials and Methods part.

Response: Thank you for your comment. To measure the red and green fluorescence intensity ratio, 1024 × 1024 pixels images were collected (n = 8). Red and green fluorescence intensity, respectively, in the individual cells were quantified using FV10i software (Olympus, Tokyo, Japan). We have added the additional sentence into the methods part.

4. In Western blot analysis, please indicate the protocols for measurement of band intensities.

Response: Thank you for your comment. Band intensities were measured using FluorChem™ SP software (Alpha. Innotech, San Leandro, CA, USA). Band intensities were normalized to β-actin or total p38. We have added the additional sentence into the methods part.

5. What amount of proteins did the authors load in each lane?

Response: Thank you for your comment. Proteins from whole cell lysates were loaded 30 µg in each lane. We have already presented in page 8 line 4.

6. LPE suppressed the phosphorylation of p38 in H$_2$O$_2$-treated SH-SY5Y cells. The protective mechanism is still unclear. The authors had better discuss the possible mechanism of LPE-mediated anti-oxidant and anti-apoptotic effects.

Response: Thank you for your comment. Previous papers reported that H$_2$O$_2$ had cytotoxic effects in various in vitro model by a mechanism involving pro-apoptotic factors (Bax, caspases, PARP) activation and intracellular signaling pathway [1-7].
Especially, MAPK signaling pathway has been suggested as an important mechanism in oxidative stress-mediated neurodegenerative diseases [8, 9]. To address the possible mechanism of LPE-mediated neuroprotective effects, we determined whether LPE inhibited the activation of MAPK signaling pathway (ERK, JNK, p38) in H$_2$O$_2$-treated SH-SY5Y cells. As showed our result, H$_2$O$_2$ only activated p38 not ERK and JNK. In addition, p38 inhibitor blocked the cell loss in H$_2$O$_2$-treated SH-SY5Y cells; therefore, H$_2$O$_2$ required p38 activation for the induction of cytotoxicity. Our results demonstrated that LPE protected the cell growth against H$_2$O$_2$-induced cytotoxicity through inhibition of p38 phosphorylation. In addition, LPE only treatment did not affect the level of phosphorylated p38 in SH-SY5Y cells (Fig. 2). Therefore, we conjectured that LPE has neuroprotective effects by inhibition of p38 phosphorylation in a background of stressful state by H$_2$O$_2$-induced oxidative stress and apoptotic/necrotic cell death. We have changed to this paragraph into the discussion part.

![Figure 2. LPE did not affect phosphorylated p38 in SH-SY5Y cells.](image-url)
• Reviewer 2.

In this paper Park et al investigated the effects of Liriope platyphylla extract (LPE) on H$_2$O$_2$-induced cytotoxicity in SH-SY5Y cells. They reported that LPE pretreatment attenuated apoptotic cell death, intracellular oxidative stress and mitochondrial dysfunction induced by H$_2$O$_2$. In addition they found that p38 activation was involved in the neuroprotective effects of LPE. In general, the experiments are well conducted and the manuscript is well organized. Here are suggestions to further improve the manuscript before being accepted for publication.

This manuscript needs minor essential revisions.

1. Figure 3 and Figure 5A only show the representative images. It is suggested to add the quantitative figures with statistical analysis.

Response: Thank you for your comment. As the reviewer suggested, we have quantified results and added the quantitative graph in the Figure 3 and 5A.

![Figure 3](image)

**Figure 3.** Quantitative data showed that percentage of healthy, early apoptotic, late apoptotic, and necrotic cells according to treatment. Values are reported as means ± SE
Figure 4. Quantification of band densities for phosphorylated p38 / total p38 were measured. Data are expressed as means±SE (n=3). **p < 0.01, compared to vehicle without H$_2$O$_2$, ##p < 0.01, compared to vehicle with H$_2$O$_2$.

2. There are some minor errors:

(1) Page 8 line 12: “(Santa Cruz Biotechnology))”

Response: Thank you for your comment. We have deleted the repeated parentheses.

(2) Page 12 line 13: “while LPE pretreatment” lacks the concentration of LPE. Figure 1B shows that only 50 µg/ml LEP significantly increased the cell viability.

Response: Thank you for your comment. We have added the concentration of LPE in the sentence as below.
(3) Page 13 line 5: “however, LPE pretreatment” is short of the concentrations of LEP.

Response: Thank you for your comment. We have added the concentration of LPE in the sentence as below.

(4) Page 14 line 15: “early apoptosis (Annexin-V/PI, lower left quadrant)”

Response: Thanks for your correction. We have revised our manuscript.

(5) Page 17 line 13: “the phosphatidylinositol 3-kinase (PI3K/Akt)”

Response: Thanks for your correction. We have revised our manuscript.

(6) Page 18 line 11: “RA” lacks its full name.

Response: Thanks for your correction. We have spelled out in page 6 line 18.

(7) Page 23-26: Reference 3 and 4 lack stopping page, Ref. 8 is short of pages, Ref. 32 and 33 have not used the abbreviation of journal’s name.

Response: As the reviewer commented, we have revised the references.

(8) Page 29-31: Please carefully check the punctuation marks in Figure Legends.

Response: Thank you for your comment. We have checked punctuation marks again.
We thank the referees for the valuable comments, and you for your efficient handling of the manuscript.

Best regards,

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