Author’s response to reviews

Title: Chlorogenic Acid Protects PC12 Cells Against Corticosterone-induced Neurotoxicity Related to Inhibition of Autophagy and Apoptosis

Authors:

Xiaowen Shi (xiaowenshi1990@163.com)
Nian Zhou (kyky654321@163.com)
Jieyi Cheng (13611738323@163.com)
Xunlong Shi (xunlongshi@fudan.edu.cn)
Hai Huang (hhai3552@sina.cn)
Mingmei Zhou (zhoumm368@163.com)
Haiyan Zhu (haiyanzhu@fudan.edu.cn)

Version: 1 Date: 12 Aug 2019

Author’s response to reviews:

Dear Dr. Graeme Sills,

Thank you for taking your valuable time to read the revised manuscript. We have addressed the comments raised by the reviewers, and the amendments are highlighted in the manuscript in revised mode. Below this letter is our explanation on revision according to the comments of the reviewers.

We hope that the revised version of the manuscript is now acceptable for publication in your journal.

I look forward to hearing from you soon.

With best wishes,

Yours sincerely,

Mingmei Zhou, MD, PhD, Associate Professor
Corresponding author
Thank you for taking your valuable time to read the revised manuscript. The following is our explanation on revision according to the comments of the reviewers.

To Reviewer 1#, Dr. Haroon Khan:

1. Introduction is too short.

Thank you for your suggestions. We have revised this part of the text in the “Introduction”, added relevant reports on the anti-depression study of chlorogenic acid, and related research on autophagy and neuronal apoptosis intervention is an important part of regulating the pathophysiology of depression in the main text, lines 612-656, and 889-934.

2. Results are written but can be improved.

Thanks to the reviewer's suggestion, in the results, we added a description of the changes in cell morphology after administration, and revised the title of Section 3.4. It is embodied in the manuscript 1634-1667, 2045, and 2267-2278.

3. Discussion need improvement as previous studies are not effectively addressed

Thank you for your valuable suggestion. In the “Discussion”, we increased the discussion of apoptosis and autophagy interactions and their potential role in depression and modified the relationship between CGA neuroprotection and apoptotic autophagy, and optimized the conclusions. It is embodied in lines 229-2321, 2354, 2410, and 2686-2810 of the manuscript.

To Reviewer 2#, Dr. David Moranta:

Major points.

1. Title of the manuscript should be changed since it has not been demonstrated that inhibition of autophagy is responsible of CGA neuroprotection. Both events are produced by CGA but it has not been demonstrated a causal link. Additionally, in the same way, last paragraph from discussion should be changed since authors has not demonstrated that AKT/mTOR is responsible of neuroprotective effect of CGA on CORT exposure.

Thank you for your valuable attention and suggestion. The title has been changed to “Chlorogenic Acid Protects PC12 Cells Against Corticosterone-induced Neurotoxicity Related to Inhibition of Autophagy and Apoptosis”.

2. Explanation of the densitometric analysis of western-blot should be extended. Authors should specify whether they detect D.O.I. from membranes and number of replicates used to compare between groups. Additionally, housekeeping proteins are used in these analyses, but author do not explain if this analysis is from the same membrane. Authors must include information about re-blotting protocol if they do or explain how they obtain housekeeping protein images. Finally, author use sometimes tubulin as housekeeping (load control) and in other times GADPH… authors must explain the reason and justify it. Why figure 1B is not densitometric quantified?

Thanks for the attention. In Fig. 1, we supplemented the grayscale analysis of Fig. 1CB by adding Fig. 1D, which is the calculated mean of 3 experiments. The target gene and housekeeping genes in the displayed WB map are the same membrane source data, without washing and re-hybridizing. The protein transfer process is performed on the same membrane, but when the antibody is incubated, the corresponding bands are tailored according to the molecular weight, and then antibody incubation and subsequent color development are performed separately. Regarding the question of internal reference selection, this experiment has no special design in its selection. Both GADPH and Tublin are commonly used internal reference genes in WB, and antibody types with more stocks in the laboratory are often selected as internal reference genes.

3. Reference 14 should be better included in materials and methods sections (section 2.3 cell viability). Authors should include a brief description about how cell viability is detected and represented (how % cell viability rate is calculated from absorbance is calculated). Authors refer to these results sometime as cell proliferation and others as cell survival or viability… they are not the same and authors should be consistent with the process they refer.

Thanks for the comments, we have made the corresponding changes, and reference 14 has been moved to Section 2.3. In this experiment, we examined the activity of mitochondrial succinate dehydrogenase in living cells after 24 hours of administration by MTT assay to reflect the effect of the drug on cell proliferation activity. The living cells reduced MTT to formazan. The formazan was dissolved in dimethyl sulfoxide and its absorbance was measured at a wavelength of 570 nm. The ratio of the absorbance of each of the administered groups to the control group reflected the change in the number of living cells.

4. In Figure 1 and 2, the scale or magnification used to obtain the images is not indicated. Additionally, authors should explain better the morphology variation detected. What do they see and how they analyze this morphology variation? Authors should comment/discuss about the fact that LC3II is induced after 24h exposure to CORT… Have authors analysed time-course effect also for cell viability or morphology change? Do these effects correspond with LC3II phenotype
observed? And a similar question for fig 3D… do authors have test a time-course for CGA effect on CORT LC3II induction?

Thanks for the attention. According to your suggestion, the scales in Figures 1 and 2 have been added. A description of the morphology of cell damage has been complemented by the "MethodsResults" of the text. High concentrations of CORT can lead to cell irregular shape, poor adhesion, and even shedimpaired cell proliferation, cell gaps, and even cell shedding, cell aggregation, reflecting the toxicity of the drug. In the results, F1CB, DC showed an increase in the LC3-II / LC3-I ratio with time, which is related to the degree of cellular damage. It was confirmed in the model that significant cell damage and autophagy induction occurred in the conditions of intervention of PC12 cells at 400 μM CORT for 24 hours. With further reference to the literature [1514, 3128], we selected this concentration as the modeled concentration based on this in the later experiments.

5. In figure 2D, changes in PARP density is observed for 25 and 50 microg/ml of CGA… is this consistent? Have authors quantified this? Which is the significance of that effect? Similar commentary for figure 5F for changes in PARP density

Thanks for the attention. C-PARP is considered to be a marker of apoptosis. In F2D, both CGA 25 and 50 μg/ml showed a consistent trend of inhibiting C-PARP expression, and the corresponding quantitative data showed in F2E, which showed that CGA had protective effect on CORT-induced cell damage with dose-effect relationship. In F5F, the effects of CGA and autophagy inhibitor CQ on apoptosis were mainly compared. Quantitative analysis can be found in F5H. The results also show that both autophagy inhibitor and 25 μg/ml of CGA have anti-apoptotic activity, which is presumed to be associated with autophagy.

6. Densitometric analysis from figure 3D do not reflect exactly WB from figure 3C… at 25 and 50 microg dose LC3II seem to increase. Similar comment for figure 4A… CORT effect alone on AKT seems not to be so low in WB as in densitometric analysis.

Thanks for the comments. Figures 3D, 3C, and 4A are representative graphs in each set of experiments, while grayscale data analysis is an average analysis of three replicates, and there are cases that the graphs and data are not completely coincident.

7. Title from section 3.4 must be changed since it has not been demonstrated that AKT/mTOR is responsible for effect on autophagia of CGA.
Thanks for your suggestion, it has been revised to “CORT/CGA treatment regulated the AKT/mTOR signaling pathway in PC12 cells”.

8. Author should explain the reason why they use 25 microg of CGA in Figure 5.

Thanks for the valuable suggestions of the reviewer. In the previous experiment, we tested 4 concentrations, and the results showed that CGA at 25 and 50 ug/ml could exert neuroprotective effects on CORT-induced nerve injury, which was significantly increased in cell viability compared with the model group, and the amount of apoptotic protein C-PARP significantly reduced, and the conversion rate of LC3I to LC3II was significantly reduced. However, the in vitro activity test of traditional Chinese medicine often has Hook Effect. Therefore, we chose a medium dose of 25μg/ml with relatively strong activity, and compared with autophagy inhibitors 3MA and CQ on autophagy and apoptosis effects, to speculate the roles of CGA in autophagy and apoptosis in the model, and their relationships.

9. Authors should include the results or 3-MA and CQ effects on PC12 cells death or apoptosis; and last sentence from results section is a speculation not a result. This should be moved to the discussion.

Thanks for your suggestion, and I have moved the relevant sentence of lines 217 and 218 in the original text to the section of “Discussion”.

Minor Points

1. Authors should discuss the reason why 100 microG/ml of CGA induce toxicity. Have authors checked 100CGA with CORT? There is a synergic effect in citotoxicity?

Thank you for your valuable suggestions. Drugs have a lot of toxicity to cells, and may be involved in cell proliferation, metabolism, and competitive binding of certain active receptors. In our study, we initially evaluated the effects of drugs on cell viability through the MTT method, and did not further and systematically study the synergistic effects of the two drugs. We regret to admit that this is a shortcoming in our research work and needs to be improved in the future work.

2. There are some recent publications that could be included in the manuscript discussion, between other things regarding brain affects from systemic administration of CGA since they
suggest the possibility of their use to treat depression (i.e. Yan et al. J. Biosci 2018; Kumar et al. Xenobiota 2019 and Naunyn 2019, etc.).

We are very grateful to the reviewers for the valuable recommends. This is a good complement to our research, and I have added these advances to the discussion section.

3. The last word from introduction is not correct (autophagy).

Thanks for the suggestions, and it has been revised.

4. Authors should avoid repeating the experiment protocol several times in the legends of the figures.

Thanks for your attention. We have made some deletion in the text.