Author’s response to reviews

Title: Sub-chronic treatment with high doses of ascorbic acid reduces lead levels in hen eggs intentionally exposed to a concentrated source of lead: a pilot study

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Author’s response to reviews:

Dear Editor,

BMC Pharmacology and Toxicology

We are very delighted to learn that our paper should become acceptable for publication pending suitable revisions and modifications in the light of the comments raised by the reviewers. Many thanks for providing us with the opportunity to revise our submitted manuscript. Also we would like to thank yourself and the reviewers for the constructive comments which have considerable improved the quality of the manuscript.

We believe that the comments raised are legitimate and consideration worthy. In this revision, we have addressed all of them and the manuscript was revised accordingly.

Please find below a detailed point-by-point account of the revisions made to the manuscript in accordance with the comments raised by the reviewers. All comments were carefully considered and amendments were made as appropriate. For your convenience, the amended text is highlighted in blue font throughout the manuscript.
Hossein Hassanian-Moghaddam (Reviewer 1):

We would like to than Prof. Hossein Hassanian-Moghaddam for his constructive comments on our manuscript. We believe that all comments are legitimate and consideration worthy. Please see below how we addressed all of them and how they impacted the quality of the revised manuscript.

1. In page 3 line 25: I think there is a typing error. "G1 and G1"?

We would like to apologize for this typo. The reviewer is right, G1 and G2. This was corrected. Please see Page 5 Line 26. The amended text was highlighted in blue font for your convenience.

2. Did you make sure that the used plastic bags were themselves lead-free? I know that most of the dark plastic bags contain lead, but still believe you should have determined the lead level in your transparent plastic bags, as well.

We agree with the reviewer that some bags and containers can be a source of lead contamination. In this study, the plastic bags used were soaked in distilled water overnight and water was sampled and analyzed for lead contents. We could not detect lead in our samples. This information has been added to the manuscript. Please see Page 6 Lines 28-30. The amended text was highlighted in blue font for your convenience.

3. This is actually not really my field of expertise but doesn't Triton-X itself affect the numbers of lead analysis? (http://www1.ci.uc.pt/pessoal/brett/docs/105_JEAC.pdf)

In the work of Gouveia-Caridade and Brett cited by the reviewer, electrochemical impedance spectra has been used to follow changes at Nafion-modified carbon film electrodes in the presence of non-ionic surfactants during anodic stripping voltammetry. In this study, lead levels were determined using a using a graphite-furnace atomic absorption spectrophotometric method. In this study, triton-X was used as a surfactant to clean eggs and remove any external contamination. This ensured that lead levels detected represented the lead contents within the body of the matrix analyzed. This has been used in many previous studies, please see (Trampel DW, Imerman PM, Carson TL, Kinker JA, Ensley SM: Lead Contamination of Chicken Eggs and Tissues from a Small Farm Flock. Journal of Veterinary Diagnostic Investigation 2003, 15(5):418-422) and (Singh SK, Kishore N: Volumetric Properties of Amino Acids and Hen-Egg White Lysozyme in Aqueous Triton X-100 at 298.15 K. Journal of Solution Chemistry 2004, 33(11):1411-1427). Please see Page 6 Lines 16-17. The amended text was highlighted in blue font for your convenience.

4. Baseline lead level in egg yolk had been 6. In group treated by vitamin C the 136 µg level had reached to 69 which has been claimed to be insignificantly different from the baseline lead. This seems a bit unrealistic. Are you sure about these results?

We would like to thank the reviewer for pointing out this mistake which we would apologize for. Indeed, there was a significant difference between the two levels p-value &lt; 0.05. The text and the figure were corrected. The text referring to this was removed. Again the statistical analysis
was verified. In this revision, we are using $\mu$g Pb/g in both the text and the figures. Please see Page 8 Lines 3-9. The amended text was highlighted in blue font for your convenience. Again, please see the corrected Figure 1 as well as other revised figures 2-4.

5. As mentioned in the text, similar studies have been performed before (determining the lead level in both shells and yolk and albumen). So, what is the novelty in your work?

We would like to thank the reviewer for raising this comment. The significant increase in lead levels following intentional or environmental exposure of hens to a source of lead is not new and was shown in previous studies. However, the novelty in this work is that this study reports for the first time statistically significant reductions in lead levels in different hen egg parts following sub-chronic treatment with ascorbic acid. Findings from this study bridge a gap existed in earlier experiments. Please see Page 10 Lines 9-12 and Page 11 Lines 6-7. The amended text was highlighted in blue font for your convenience.

6. In discussion, discuss about the possible causes of this effect of vitamin C.

New text was added to the discussion section as the reviewer suggested. Please see Page 11 Lines 11-15. The amended text was highlighted in blue font for your convenience.

Yongxia Liu (Reviewer 2):

We would like to thank Dr. Yongxia Liu for his constructive comments on our manuscript. We believe that his comments and remarks have significantly improved the quality of the revised manuscript.

1. Language needs to be revised.

We would like to thank the reviewer for this remark. The manuscript has undergone careful revision to improve the language, flow and clarity. We think that the manuscript reads better now.

Abstract

Page 2

2. Line 10 and 11: The unit used "mg/kg/day" was confused, it means 200 mg/kg body weight or feedstuff?

Information was added to clarify this ambiguity. We apologize for this ambiguity in the earlier version of this manuscript. This is now expressed as mg/kg body weight/day. Please see the revised abstract as well as the revised results sections. The amended text was highlighted in blue font for your convenience.
3. Line 16 and 18: The p value in line 16 was in italic, but line 18 was not.

The p value was made italic to be consistently in the same style throughout the manuscript. Please see the revised abstract and results sections. We also revised the legends of the Figures for this as well. The amended text was highlighted in blue font for your convenience.

4. Line 29: Key words need to be limited to six.

Key words are now limited to six as the reviewer suggested. Please see the revised key words.

Methods

Page 5

5. Line 14-21: Did you know the lead level in the adult egg laying hens before experiments, have you detected it? Did you know the hens were healthy or not?

All hens used in the study were clinically normal mixed-breed adult egg laying hens. They were observed by a licensed veterinarian for signs of toxicity and ensured their welfare throughout the different stages of the study. Eggs laid during the acclimatization period were collected and analyzed for lead levels. Baseline lead levels are provided in the text and in the figures. Please see Page 5 Lines 7-9 and Lines 21-22. The amended text was highlighted in blue font for your convenience.

Page 6

6. Line 12-16: Can you ensure that there was no lead loss in the preparation process?

In this study, calibration curves were built using similar matrices. As lead levels were determined from the calibration curves built in the same matrices (eggshell, egg-albumen (white) and egg-yolk (yellow)), we can ensure that any loss of lead during the analysis was accounted for. Please see the Page 7 Line 1. The amended text was highlighted in blue font for your convenience.

Results

7. Is it equal on the egg production among each hen? If not, this problem will affect the results or not? It means that whether your statistics were performed on the same basis.

The hens laid almost equal number of eggs during the study period. Statistical analysis was performed using Analysis of Variance (ANOVA) with Bonferroni multiple comparisons. Please see Page 7 Lines 6-7. The amended text was highlighted in blue font for your convenience.
8. The figures' resolution ratio need to be improved.

Figures are provided in TIFF format with a resolution of 600 dpi.

Phitsanu Tulayakul (Reviewer 3):

We would like to thank Dr. Phitsanu Tulayakul for his quality comments on our manuscript. Below you can find a detailed account of how we addressed all his comments.

1. In Page 5, Line 48, G1 and G1 must be corrected to G1 and G2, right?

We would like to apologize for this typo. The reviewer is right, G1 and G2. This was corrected. Please see Page 5 Line 26. The amended text was highlighted in blue font for your convenience.

2. Please added in your material and methods that why the dose of lead acetate was used at 200 mg/kg/day in this study? This level is met the LD50 of the lead acetate or else so please give more information and rationale for using this level and route of exposure via food or drinking water?

The decision to use lead acetate dose of 200 mg/kg body weight/day was added in the methods section as the reviewer suggested. Please see Page 5 Line 28-29. The amended text was highlighted in blue font for your convenience.

3. Please giving more information about the rationale of using 500 mg/kg/day of ascorbic acid in this study? Please put it in material and method.

The decision to use ascorbic acid dose of 500 mg/kg body weight/day was added in the methods section as the reviewer suggested. Please see Page 6 Line 5-7. The amended text was highlighted in blue font for your convenience.

4. In Page 6, Line 34, Please indicate the analytical procedure about what number of standard method used in this study or please cites the method used in this part.

More details on the analytical method was added as the reviewer suggested. Please see Page 6 Lines 25-30. The amended text was highlighted in blue font for your convenience.

5. In the flowchart the Hens number should be 18 as explained in material and methods or correct it to the similar value.

We would like to apologize for this typo. The reviewer is right, the number was 18 and not 28. This was corrected. Please see the corrected flowchart.
6. I think the significant different should have only 2 levels of * = 0.05 and ** = 0.01 which it is enough to explain the evidence in your study and the samples size of this study was limited. Anyway it depends the author and the criteria of the journal.

We agree with the reviewer that the two levels are sufficient. We amended the text and figures accordingly. Please see Page 7 Line 8. The amended text was highlighted in blue font for your convenience. Again, please see the amended legends of the figures 1-4.

Once again, we would like to thank yourself and the reviewers for the constructive comments and hope to hear from you soon.

Kind regards,

Ramzi Shawahna