Author's response to reviews

Title: Astaxanthin mitigates cobalt cytotoxicity in the MG-63 cells by modulating the oxidative stress

Authors:

Dahe Li (shanghaildh@hotmail.com)

Wenwen Tong (tww944057350@sina.com)

Denghui Liu (liudh997@163.com)

Yuming Zou (zouym0771@163.com)

Chen Zhang (zhangchenshanghai@163.com)

weidong xu (weidongxu2016@yeah.net)

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Author’s response to reviews:

Reviewer reports:

Reviewer 1: The authors have used cell culture model to demonstrate the beneficial effects of ASX in cell culture model. The findings may be too preliminary to be convinced about any beneficial effects of ASX in the treatment of metal-on-metal bearing toxicity as seen after major joint replacement surgery

Abstract: Concise and to-the-point

Introduction: 1) There are grammatical errors that can be fixed. For example: "caused the generation metal particles and ions"- may be replaced by "caused the generation of metal particles and ions"; Another one: "small amount of cobalt ion in the human body is essential to good health"; 'to' may be changed by 'for'. "Cobalt ion was found at MoM patients` whole blood or serum": "at" may be changed by "in"

Response: Thank you. The above-mentioned grammatical errors were fixed. And the whole manuscript was respected carefully for other possible errors. All corrections were highlighted in red in Introduction section, line 59-75, page 3..

2) Have Cr and Co in parenthesis when cobalt and chromium terms are first introduced.
Response: Thanks. The ‘(Co)’ and ‘(Cr)’ have been added where they were first introduced (Introduction section, line 64, page 3).

3) Line 40: Mention briefly the full form of CSF and RANKL

Response: Thank you. The full form of CSF and RANKL, colony stimulating factor and Receptor Activator for Nuclear Factor-κB Ligand were added after their abbreviations (Introduction section, line 73-74, page 3).

4) The authors have treated MG-63 cell line with ASX. As a rationale, the authors just mentioned about the anti-inflammatory, antioxidant property observed in one study [24]. This is not sufficient to justify the rationale for using ASX in the study. The authors need to add more research evidence of the utility of ASX as anti-inflammatory, antioxidant and immune-modulant.

Response: Thanks. More references have been added to the mentioned part and highlighted in red (Introduction section, line 76-93, page 3-4)

Methods: 1) Give the full form of ATCC

Response: Thanks. The full form of ATCC, American Type Culture Collection, has been added in parenthesis after its abbreviation (Method section, line 107, page 4) and to the List of abbreviation.

2) Add another paragraph describing MG-63 treatment conditions. What are the doses of Co and ASX? How long was the treatment etc.

Response: Thank you. A paragraph describing MG-63 treatment conditions was added. (Method section, line 110-113, page 4)

Results: 1) Co-induced significant (50%) cytotoxicity was observed with a Co conc. of 200μM. Is that conc. physiological?

Response: Thanks. Actually, the 200μM Co concentration is not supposed to be a physiological one. According to Witzleb et al.[1], patients who underwent metal-on-metal hip articulation could have a Co concentration no more than 4.28 μg/L at 2 years after surgery, which is far lower than 200μM (given the molecular weight of Co is 58.93. However, the cell line that we used, MG-63 cells, is more cancerous, which is presumed to be more tolerant to Co toxicity. To investigate the protective effect of astaxanthin against Co toxicity, we employed a concentration at which Co induced half of the cell death.

2) Fig. 1B: Indicate the conc. unit of ASX in the figure.

Response: Thanks. The concentration unit (nM) of ASX has been added to fig. 1B.
3) Fig. 1D: Western blots figures are not very convincing. The bands for cas-3 don't show too much of a difference between 1, 5 and nm of ASX. Similar comment for fig. 2A. Also please indicate how many times the experiments were repeated.

Response: Thanks. The Group 1 was the control cells that was treated neither by Co (II) nor astaxanthin. Hence, there was indeed no difference between Group 1 and Group 5 or Group 6. So was for fig. 2A. Sorry about the possible inconvenience brought by our illustration.

4) Did the authors use any known antioxidant or a chelating agent to compare the results vs. ASX?

Response: Thanks. And sorry, the authors did not used known antioxidant agent as a control. Nevertheless, other researchers have reported that ASX is a more potent antioxidant than vitamin C, vitamin A etc [2].

Discussion: Too concise. The authors need to discuss about the molecular pathways related to metal toxicity with a focus on JNK, Bcl, NGkB etc.

Response: Thanks. More discussion about the molecular pathways has been added and highlighted in red (Discussion section, line 251-259, page 9-10)

Reviewer 2: I believe the manuscript is lacking important Introduction (background) and Discussion information. Please consider adding more on cobalt and its toxicity, what is the standard of care for cobalt toxicity, and typical cobalt blood levels seen in patients experiencing/not experiencing adverse effects of MoM hip replacement. Also consider adding additional information on ASX including its pharmacokinetics (if known) to help the reader understand why you are proposing ASX to help ameliorate cobalt toxicity (ie, can it be taken orally, is it absorbed well in the GI, etc.). In the Discussion, please draw the conclusions for the reader including how the concentrations of cobalt used in the studies correlate to patients with MoM hip replacements and how the doses of ASX chosen are relevant to human populations (can these ASX concentrations be achieved without side effects in humans). Please remove discussion statements from the Results section and include in the Discussion section.

Response: Thanks for your comments. Information about ASX’s pharmacokinetics has been added to the discussion section (Discussion section, line 239-242, page 9).

Please add the IC50 for cobalt in the cell line used.

Response: Thanks. The IC50 for cobalt in the MG-63 cell line is 200μM as indicated in figure 1A.

Pages of manuscript were not numbered and line identification is repeated from 1-65 on each page. This makes it difficult to site specific comments relating to specific sentences. Please correct.
Response: Thanks. The line number has been set to be continuous, and page numbers were also added.

Please review entire manuscript for the correct use of the English language.

Response: Thanks for your comment. The manuscript has been thoroughly reviewed and edited with highlighting in red.

Reviewer 3: The authors reported cobalt toxicity in MG-63 cells, and got that Astaxanthin could mitigate cobalt toxicity. The subject and components of this paper is in the content of the journal. There are still some concerns especially methodology should be considered.

(1) The section of "Introduction" should be improved, which is not a clear rationale of the whole paper. It is expected to include the role of ASX on Co toxicology. It would help the readers better understand the innovation points of this paper.

Response: Thanks for your comment. The introduction has been improved. And the current rationale is: 1) the clinical significance for cobalt toxicity intervention, 2) the potential of ASX in anti-oxidation, and 3) what we are to investigate in the current research.

(2) Please provide the exact methods of exposure experiment, which are the most important protocol in this study. You should add the details including exposure ways, concentrations, times, etc., step by step? And explain why you chose these doses of Co and ASX.

Response: Thanks. And sorry for our carelessness. Half a paragraph has been added to the manuscript and highlight in red (Method section, line 110-113, page 4).

(3) Wondering have you did QA/QC for the toxicity experiment? If so, please add analytical QA/QC methods and results.

Response: Thanks. And the authors have not conducted QA/QC for the toxicity experiment. Because all our experiments were conducted ex vivo in MG-63 cells. Further researches would be done for the exploration of ASX application as an antidote against Co toxicity. The current study is to propose a possible therapy for Co toxicity.

(4) Are there joint effects of Co and ASX as a chemical? Any medication is also a poison. Some in vitro experiments or references on the inhibiting effect should be added in the paper? At least the single ASX treatment on cells should be supplied.

Response: Thanks. MTT experiment showed that single ASX treatment could enhance the cell viability of MG-63 cells. The figure was added as supplementary figure 1A.

Figure 1 Astaxanthin treatment significantly elevated the MG-63 cell viability.
(5) The authors need to provide strong evidences of the effect of JNK response. Please discuss the possible toxicology/therapy mechanism on ROS and JNK pathway.

Response: Thanks. The current research employed a siRNA and an inhibitor to downregulate the JNK expression. It was found that JNK downregulation did not significantly alter the expression the ROS-related expression of HO-1. Hence, the antidote effect of ASX against cobalt toxicity might be involved with molecular signaling conduction other than JNK pathway. Nevertheless, the conclusion that the protective effect of ASX was JNK-independent is still premature. Therefore, we altered our conclusion and title to ‘Astaxanthin mitigates cobalt cytotoxicity in the MG-63 cells by modulating the oxidative stress’.

(6) Language is not clear and must be revised. The paper should be looked and edited carefully by an English native speaker before being finally submitted.

Response: Thanks. The manuscript has been edited by an English native speaker. Extensive edition has been conducted and highlighted in red.

Reference
