Author’s response to reviews

Title: Identification of novel candidate indicators for assessing zinc status during pregnancy in mice from microarray data

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Author's response to reviews:

Dear editors and reviewers,

Thanks for your letter and the comments for our manuscript entitled “Identification of novel candidate indicators for assessing zinc status during pregnancy in mice from microarray data”. Those comments are all valuable and helpful for improving our paper, as well as the important guiding significance to our researches. Our manuscript has been checked and revised carefully according to reviewers’ comments and suggestions. The point-by-point responses to the reviewers’ comments were listed as below. We would like to re-submit this revised manuscript to “BMC Pharmacology & Toxicology” and hope it is acceptable for publication in the journal. Please do not hesitate to contact us with additional questions or concerns.

Reviewer reports:

Marina Galvez-Peralta, PharmD, PhD, FCP (Reviewer 1): The authors summarize the microarray data of female mice exposed to low Zn diet. Although the reviewer appreciate the effort on that direction, the manuscript on its actual form lack of significant information:

1. Length of zinc deficient diet on the female mice. Were they exposed prior gestation, during whole gestation? The methods section is too superficial.

Response: Thanks for your valuable comments. The length of zinc deficient diet on the female mice was from 6 weeks prior to gestation to the conclusion of experiments, which has been added into our manuscript.

2. What else was on the mice diet? It is know how heavy metals (Cadmium) or other metals could compete for Zn absorption.
Response: Thanks for your constructive comments. The nutrients in mouse diet were largely unknown because the microarray data were downloaded from a public database, and the presence of other metals such as cadmium in the diet that could compete with Zn absorption was unknown. These descriptions have been added as a limitation of our study.

3. The introduction is very weak. Authors mention ZIP1 and very brief effects of Zn deficiency. There are 14 ZIP transporters and so ZnT transporters. Many more deficiencies associated with Zn deficiency than the ones listed.

Response: Thank you very much. Your suggestions are valuable for improving our manuscript. We have cited other references to support, as follows: Severe zinc deficiency in pregnancy can result in increased fetal loss and high rates of congenital malformations in several organs of surviving fetuses [3, 4]. Previous studies have shown that changes in expression of several zinc transporters influence zinc homeostasis and metabolism and subsequently change zinc status [11-14]. Moreover, dysregulation of other molecules, such as cytokines [15], ProSAP/Shank family members [16], antioxidant enzymes and heat shock proteins [17] were associated with zinc status.

4. It is hard to extrapolate mice to human data, since the genetic origin of the placenta differs between mice and humans (mainly from the fetus vs. mainly from the mother).

Response: Thanks for your valuable comments. We have emphasized that this study aimed to identify potential zinc status indicators and to clarify the mechanisms underlying zinc deficiency-induced organ damage and mortality in mice. In conclusion, CXCR2, ANXA1, and CCR3 as well as olfactory receptor-related genes (proteins) may be used as biomarkers to assess zinc status in mice. Given your kindest suggestions, we have considered it as a limitation of our study, as follows: it is hard to extrapolate mouse data to human biochemistry, as the genetic origin of the placenta differs between mice and humans. Further experiments using human samples will provide stronger evidence for clinical guidance.

Beibei Huang, Ph.D. (Reviewer 2): In this paper, the authors identified potential biomarkers for zinc deficiency during pregnancy on the placenta. Via WGCNA method the authors analyzed Gene Ontology Biological Process and KEGG pathway of genes, and constructed protein-protein interaction network to predict the transcription factor and miRNAs regulating DEGs.

The manuscript fits the scope of Pharmacology and Toxicology.

Page 6 Why not MAS method? RMA method is more complicated, consistent for the two results after background adjustment? Why did authors use WGCNA to analyze differentially expressed genes? The creators of WGCNA specifically said that the results of differential expression analysis should not be used as input for WGCNA. It seems better to maybe do only differential
expression or only WGCNA. There might be a good reason to differential analysis first, it's just that they didn’t specify.

Response: Thanks for your valuable comments. The microarray data GSE97112 was generated based on the platform of Affymetrix Mouse Gene 2.1 ST Array. The raw data was read using the R 3.4.0 extension package oligo [19] (Version 1.44.0, http://www.bioconductor.org/packages/release/bioc/html/oligo.html). This package did not include the background correction function of MAS method, we thus selected RMA method. In addition, the focus of differential analysis is the difference of disease phenotype and the WGCNA was used to identify gene sets that were significantly associated with zinc deficiency from DEGs. Therefore, the identified genes were differential expressed and co-expressed. If we do WGCNA directly from the beginning, it is possible that the identified genes will not be differential expressed between phenotypes, which I think is meaningless. The reason why WGCNA is done after differential analysis is to further explore the differentially expressed genes between different phenotypes from the similarity of expression patterns. It may be more meaningful to conduct subsequent analysis based on the co-expressed genes.

Page 7 What are these weight values? correlation of genes? gene co-expression similarity measure defined here?

Response: Thanks for your constructive comments. We are so sorry for the inappropriate description. The power weight values have been soft-thresholding power value, which indicated that the weighted value of the correlation coefficient.

Page 8 In gene ontology enrichment analysis, many tools can be used. Most tools are based on SUPER GEOMETRY DISTRIBUTION, what about DAVID? Can you explain it? In Table 2, the sample size enough to calculate P?

Response: Thanks for your valuable comments. DAVID is also based on hypergeometric distribution, which has been added into our manuscript. For example, among all the genes in mice, 7720 genes were enriched in all KEGG pathways, and 1080 genes were enriched in mmu04740:Olfactory transduction. In this study, 12 genes out of 14 genes in module 1 (Table 2) were included in the gene sets of KEGG pathways, but all enriched in mmu04740:Olfactory transduction. Using DAVID, p value is calculated using EASE Score based on Fisher’s exact test. Because 12/12 is more than random chance comparing to the mouse background of 1080/7720, p value is significant. Therefore, the calculation of P value is not only dependent on the size of gene set we input, but also related to the total background genes and the number of genes in a certain pathway. I think the number of these genes is enough to calculate P value.

Besides, why did authors 1) do PPI network construction, 2) predict TFs and miRNAs regulating DEGs, and 3) do therapeutic drug prediction? I assume that it’s to identify genetic pathways that might be affected by zinc deficiency, but they did not clearly state their reasons.
Response: Thanks for your constructive comments. We have explained the reason for these analyses, as follows:

PPI network is available for identification of cellular functions of proteins in various organisms [27], facilitating to identification of key proteins associated with zinc deficiency.

TFs and miRNAs can play important regulatory roles in gene expression. To better understand the regulatory mechanism affected by zinc deficiency, TFs and miRNAs that could regulate key genes/proteins associated with zinc deficiency were predicted.

The Drug-Gene Interaction database (DGIdb) database [33] (http://www.dgidb.org/) was used to predict genes targeted by the therapeutic drug, which will provide a new perspective for designing effective targeted drugs for prevention of zinc deficiency.

Is there a way to externally validate that the three genes they found are actually predictive of/connected with zinc deficiency? Maybe use another dataset for validation? Because in my own experience, chemokines and chemokine receptors, which CXCR2 and CCR3 are, are often differentially expressed between any two biological states, so it might not actually be all that informative.

Response: Thanks for your valuable comments. Your suggestions are valuable for improving our manuscript. However, we did not find the appropriate dataset for validation. We have considered it as a limitation of our study, as follows: Moreover, we did not perform experiments or analyze another appropriate dataset to validate the differential expression of key genes associated with zinc deficiency.

Why do they suggest pharmacological targeting of these genes? I would think that it would be better to just eat foods containing a lot of zinc (meat, beans) rather than using medicines, which could have side effects (especially to the fetus.)

Response: Thanks for your special comments. Your suggestions are constructive. We are very agreed with you and have added it into the discussion of our study, as follows: In addition, therapeutic drug prediction indicated that CXCR2 and ANXA1 may be targets of drugs, suggesting these two genes may be implicated as therapeutic targets to reduce risk of zinc deficiency. Given the side effects of many drugs, especially to the fetus, the best option is to consume dietary zinc (abundant in meat and beans), rather than using medicines.

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English usage. Please note that use of an editing service is neither a requirement nor a guarantee of publication. Free assistance is available from our English language tutorial (https://www.springer.com/gb/authors-editors/authorandreviewertutorials/writinginenglish) and our Writing resources (http://www.biomedcentral.com/getpublished/writing-resources). These cover common mistakes that occur when writing in English.

Response: Thank you very much. To improve the quality of our manuscript, the writing has been edited by professional editors at Editage, a division of Cactus Communications,. The “Certificate of English editing” has been provided.

We appreciate for your warm work earnestly, and hope that the revised manuscript will meet with approval. Thank you very much again.

With kindest regards to you.

Yours sincerely,

Lixin Shang