Author's response to reviews

Title: The effect of psychological stress on iron absorption in rats

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Author's response to reviews: see over
Dear professor Rikki Graham,

First of all, thank you very much for positive comments on our manuscript (MS: 1216934215268244). We also appreciate very much for the reviewer’s favorable comments and very pleased to revise our manuscript based on the reviewer’s suggestions.

Response to the reviewer’s comments:

Comment 1

1) I assume ethical approval has been given for this protocol as I cannot see a statement to this effect.

Response: All animal treatments were strictly in accordance with international ethical guidelines and the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals, and the experiments were carried out with the approval of the Committee of Experimental Animal Administration of the University.

2) Hepatic hepcidin expression needs to be included on these rats to confirm the temporal pattern of iron absorption.

Response: We investigated the expression of hepatic hepcidin on these rats, and found that psychological stress up-regulated expressions of hepcidin after 3 day, which was shown in detail in our previous study(Zhao M, Chen JB, Wang W, Wang L, Ma L, Shen H, Li M: Psychological stress induces hypoferremia through the IL-6-hepcidin axis in rats. Biochem Biophys Res Commun 2008,373: 90-93.)

3) Spleen iron and ferroportin expression should be presented to demonstrate the hypoferremia is not due to retention of iron by macrophages. It is likely macrophages contribute significantly to the hypoferremia measured and be the more potent factor than intestinal iron absorption.

Response: in our previous study we measured the iron of liver and spleen by atom absorption method on these rats, and found that the iron in liver and spleen increased after 3 day’s psychological stress(Wei CL, Huang XQ, Zhou J, Chen JB, Li M: Effect
of stresses on iron in serum, liver, spleen and bone marrow of rats. J Prev Med Chin PLA 2008 26: 14-17). In this study we demonstrated that iron apparent absorption decreased and iron significantly accumulated in the apical poles of villous enterocytes in 3 d and 7 d psychological stress groups. From analysis above, It is possible that PS lead to the decreased iron absorption and iron redistribution in body induced the decreased serum iron and bone marrow iron and inhibited the synthesis of hemoglobin (Hb) and erythropoiesis. They all contribute to the hypoferremia after psychological stress.

4) Importantly, the nature of the intestinal iron measurements are indirect rather than direct such as using isotopic measurements, the preferred method. Moreover the experimental design also may be problematic, that is PS is likely to increase intestinal transit. The fact that defecation was a response to PS indicates loss of control of bowel movements. Since non haem iron absorption is confined to the duodenum any rapid/uncontrolled gastric emptying will make iron absorption inefficient and this may account for the decreased iron absorption measured. This can be estimated by the period of the red coloration appearing in the feaces (not included) but still this is not as good as measuring isotopic iron absorption in duodenal situ gut sacs which is direct.

Response: It is true that the nature of the intestinal iron measurements are method we used in this study is also a good and precise method which is widely used by many scientists. (Vaishali Agte, Madhavi Jahagirdar, Shashi Chiplonkar. Apparent absorption of eight micronutrients and phytic acid from vegetarian meals in ileostomized human volunteers. Nutrition 21 (2005) 678–685.)

5) The way in which the protein was harvested from the duodenum appears suboptimal. The lack of protease inhibitors in the buffers used to recover protein from the duodenum is fraught with problems as the duodenum contains high levels of pancreatic derived proteases which can rapidly degrade the protein.
Response: It is true that The lack of protease inhibitors in the buffers used to recover protein from the duodenum is fraught with problems as the duodenum contains high levels of pancreatic derived proteases which can rapidly degrade the protein. So we put a protease inhibitor mixture containing 4-(2-aminoethyl)benzenesulfonyl fluoride, trans-epoxysuccinyl-L-leucylsmido(4-guanidino)butane, bestatin, leupeptin, aprotinin, and sodium EDTA (Sigma) into the butter, which we didn’t mention in the former paper.

Comment 2

6) The title was too confirmed about the effect of PS on iron absorption in rats. Should it be “effect of PS on iron absorption in rats”.

Response: we agreed with reviewer’s opinion. So we changed the title into “The effect of psychological stress on iron absorption in rats”.

7) page 2 the 2nd line: “in rats” should be deleted. Page 2 the 9th line: “that” should be deleted. Or the sentence should be altered as “And we found that the iron apparent absorption and accumulation were decreased in the apical poles of villous ------”. There are many “the”s in this manuscript, please confirm their necessary usage. For example, page4 the 9th line: the control group and the PS group. page8 “Results: PS induced changes in CORT, ACTH and NE”, and page 9 “PS induced changes in ferritin and FPN1 protein expressions”, “in” should be replaced by “of”. page9 “Perl’s staining”: the whole sentence has grammar mistakes. page10 the last 5th line: “It was conclude that------” should be altered as “It was concluded that”. There are numerous misspellings and grammatical errors that will have to be corrected.

Response: According to reviewer’s opinion, we asked a native English speaking colleague to help up copyedit the paper carefully. The detail of language corrections is showed in the paper.

Comment 3

8) The description of the stress protocol should be improved.

Response: We improved the description of the stress protocol as follows: Briefly, a
communication box was divided into Room A and Room B with a transparent acrylic board. Room A included 10 little rooms with a plastic board-covered floor and Room B included 10 little rooms with a metal grid-exposed floor for electric insulation. Rats in Room B were randomly given electrical shock (0.6 mA for 1 s) for 30 min (60 times) through the floor and exhibited nociceptive stimulation-evoked responses, such as jumping up, defecation and crying; rats in Room A were only exposed to the responses of rats in Room B to establish PS model. PS was given to rats for 30 min every morning (10:00–10:30) for 7 days. At the end of the exposure, the rats were kept in the cages for another 4 min before they were taken out. Animals in the control group were only kept in the cages for 4 min without receiving any stress.

9) The way the hypothalamus and serum was collected and then stored should be described.
   **Response:** We described the way the hypothalamus and serum was collected and then stored as follows: Blood samples were collected from the heart followed by centrifuging at 3 000 g for 15 min, and the supernatants were obtained and stored at −80°C for further determination. Then the rats were perfused through the left cardiac ventricle with ice-cold phosphate buffered saline (PBS; pH 7.4) to flush out the plasma. Hypothalamus and duodenum were quickly removed and snap frozen in liquid nitrogen, and kept in a -80 °C freezer till use.

10) The name of the companies for the ELISA kits for corticosterone, ACTH and norepinephrine as well as the CV for each assay should be given.
   **Response:** Contents of noradrenalin (NE) in hypothalamus, corticosterone (CORT) and adrenocorticotropic hormone (ACTH) in serum were analyzed using commercially available ELISA kits (R&D Systems, Inc., USA). Coefficient of variation (CV) values for NE, CORT and ACTH were 13%, 15% and 11% separately.

11) How was the duodenum fixed: formalin then embedded in paraffin or frozen?
   **Response:** Perfused duodenum was fixed by formalin then embedded in paraffin,
which was sectioned at 30 μm on a sliding microtome into free-floating tissue sections.

12) In the Western blot section, no details on the HEPES-EDTA buffer are given (use of protease inhibitor, sucrose?). There are also no details on the electrophoresis and transfer procedures.

**Response:** the detail of the Western blot section was described as follows: Aliquots of the lysates containing 50 μg of protein were diluted in 2× sample buffer [50 mM Tris, pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 0.1% bromophenol blue, and 5% β-mercaptoethanol] and heated for 5 min at 95° before SDS-polyacrylamide gel electrophoresis (PAGE) on a 10% gel and subsequently transferred to a pure nitrocellulose membrane under condition 200 mA and 120 min.

13) The values for ACTH are enormous (300-400 ng/mL when the usual values range between 10 and 100 pg/mL): is it a problem with the assay or a mistake in the units?

**Response:** we are so sorry for making a mistake in the unit of the values for ACTH. And we also found that there are reports about the values of ACTH of rats, which is out of that range between 10 and 100 pg/mL. (Celso R. Franci, Janete A. Anselmo-Franci, Samuel M. Mccann: The role of endogenous atrial natriuretic peptide in resting and stress-induced release of corticotropin, prolactin, growth hormone, and thyroid-stimulating hormone. Proc. Nail. Acad. Sci.1992,89: 11391-11395. and N. Ono, W. K. Samson, J. K. Mcdoalald, M. D. Lumokin, J. C. Bedran De Castro, S. M. Mccann. Effects of intravenous and intraventricular injection of antisera directed against corticotropin-releasing factor on the secretion of anterior pituitary hormones. Proc. Nail. Acad. Sci.1985,82: 7787-7790.)

14) In the discussion and conclusion, the authors use the results of one of their recent paper to conclude on the role of hepcidin and IL-6 in the reduced iron intestinal absorption they observed. However, they did not actually measure the iron apparent absorption in rats treated with anti-IL6 antibody to draw firm conclusion. I would
therefore suggest being less affirmative in their conclusions and discuss other possible mechanisms.

**Response:** we can elucidate the possible mechanisms from this study. In order to confirm it, further study would be carried out in the coming future, which regard effect on iron apparent iron absorption in rats treated with anti-IL-6 antibody after ps.

15) 1- Figure 1 B: are the iron intake in mg as written or mg/d? Figure 1C: what is the actual unit of fecal iron content: mg/g of faeces, mg excreted / d? Figure 1D: is it the percentage or the ratio?

**Response:** The iron intake is mg/d. The actual unit of fecal iron content is mg/d. It is the percentage.

According to the editor’s comments, we have made an extensive and careful correction of format and syntax throughout the manuscript adhere to the guidelines and standards required for publication in “BMC Gastroenterol”.

We believe that the revised manuscript is in good shape after revision according to the reviewer’s and editor’s suggestions. Please consider for acceptance to be published in “BMC Gastroenterol” and let us know any further information of the reviewing process at your earliest convenience.

Thank you very much for your consideration.

With best regards,

Sincerely yours,

Hui Shen