Author's response to reviews

Title: A possible involvement of Nrf2-mediated heme oxygenase-1 up-regulation in protective effect of the proton pump inhibitor pantoprazole against indomethacin-induced gastric damage in rats

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Author's response to reviews: see over
Response letter to reviewer’s comment

Here there were the comments to reviewers including handling editor in a point-by-point manner, which were included in a revised manuscript and we provide a cover letter in revised manuscript.

Response to the comments of Handling Editors

The reviewers all felt that your manuscript was interesting and had potential. However, they all also raised a number of issues that I feel need to be adequately addressed before this submission could be considered for publication.

➔ Here you can find the response letter to reviewer’s comment in a point to point manner as well as revised manuscript comprising of comments.

In addition to responding to the comments provided by the reviewers, you should also indicate in the legend to each figure how many independent experiments are represented by the data shown (also “M ± SD” should be “mean ± SD” and should be in the legend rather than in the figure itself).

➔ Yes, we did revise the description in this revised manuscript according to your comments.

Also, please make the following formatting changes during revision of your manuscript. Ensuring that the manuscript meets the journal’s manuscript structure will help to speed the production process if your manuscript is accepted for publication.

After reading through your manuscript, we feel that the quality of written English needs to be improved before the manuscript can be considered further.

We advise you to seek the assistance of a fluent English speaking colleague, or to have a professional editing service correct your language. Please ensure that particular attention is paid to the abstract.

Research involving human subjects (including human material or human data) that is reported in the manuscript must have been performed with the approval of an appropriate ethics committee. Research carried out on humans must be in compliance with the Helsinki Declaration (http://www.wma.net/en/30publications/10policies/b3/index.html ). A statement to this effect must appear in the Methods section of the manuscript, including the name of the body which gave approval,
with a reference number where appropriate.

→ No human data were used in this study.

*Please include a title page at the front of your manuscript file. It should contain, at minimum, the names, institutions, countries and email addresses of all authors, and the full postal address of the submitting author.*

→ All the informations of all authors were put into title page and we did do improvement by native English expert, who is working with me as journal ME of Clinical Endoscopy because I am positioned as Senior Deputy Editor.

**Response to the comments of Reviewer I**

In this manuscript by Lee HJ and his/her co-workers, it has been demonstrated that pantoprazole prevented NSAID-induced gastric injuries through the expression of HO-1, independent of its acid suppression. This basic research has abundant persuasive data, and was very interesting for most of gastroenterologists. I hope that the authors revise this manuscript as following comments, and plan the clinical investigation in near future.

**Major**

1. *In your paper, the mechanism that ZnPPIX inhibited the expression of HO-1 induced by pantoprazole (Figure 3.) remains unclear. The authors should discuss this issue.*

→ As can be seen in Figure 3B, 100 μM pantoprazole increased the expressions of both VEGF and HO-1, whereas co-treatment of pantoprazole and ZnPPIX did not increase the expression of VEGF as well as HO-1. These changes were further prominently seen in 300 μM pantoprazole treatment. Similar changes were seen in angiogenic factors after pantoprazole as exemplified with PDGF, bFGF, HIF-1α, and VEGF as well as HO-1 (Figure 3C). In addition to the changes of angiogenic factors seen in Figure 3C, in vitro angiogenesis assay showed same result that ZnPPIX abolished the angiogenic actions of pantoprazole. All of these experiments consistently showed that pantoprazole induced HO-1 and resulting angiogenic actions. All these descriptions were done revised in this revised manuscript.
2. In Figure 4B, IL-1 expression was faint in the group treated with indomethacin (500μM) plus pantoprazole (100μM) plus ZnPPIX (50μM). The author should discuss the reason why.

→ The changes of indomethacin-induced TNF-α clearly showed the blocking action of pantoprazole through HO-1 regulation, but clearly indomethacin increased IL-1β than before. However, PPI showed blocking actions of IL-1β except with co-treatment of 300μM pantoprazole and 50μM ZnPPIX. However, lower results from qRT-PCR showed the result that some blocking action of pantoprazole on IL-1β expression and reversal with ZnPPIX.

3. In Figure 4A, NOX-1 expression was inhibited by treatment with pantoprazole. Why?

→ NADPH oxidase-1 (NOX-1) was significantly increased after indomethacin as seen in Figure 4A, whereas NOX-1 was not induced with co-treatment of indomethacin and pantoprazole, suggesting indomethacin induced NOX-1, leading to oxidative stress. These result was further validated with ESR (electron spin resonance) measurement that indomethacin induced significant levels of oxidative stress in gastric epithelial cells.

4. Figure 4D is absent (p. 10).

→ Thank you for your correction. The figure 4 was initially composed of four sub-figures, but was organized into three. We corrected these in revised manuscript.

Minor

1. This paper has many wrong spells. The authors should revise more carefully.

p1, 300 M >>>> 300 μM, p2, 500 M >>>> 500 μM, TNF- >>>> TNF-# .......

→ Thank you so much for your kindness and consideration. In this revised manuscript, these were all corrected.
2, p8 Figure 2B >>>>> Figure 2E?, Figure 2A >>>>>>> Figure 3A?

→ Thank you so much for your kindness and consideration. In this revised manuscript, all of these miswriting were all corrected.

Response to the comments of Reviewer II

The author reported that pantoprazole can protect stomach against NSAIDs-induced damages by not only acid suppression but also Nrf2-driven HO-1 induction which occurs the improvement of NSAIDs-induced ischemia, attenuation of adhesion molecule, and decreasing inflammatory mediators. This study is interest. *In vitro* angiogenesis assay, the author reported that pantoprazole increase expression of angiogenic factors and activate NSAIDs-induced angiogenesis. However, there are some problems.

**Major**

1. The author reported that pantoprazole improve NSAIDs-induced ischemia. However, in this study, although pantoprazole increase expression of angiogenic factors and activate NSAIDs-induced angiogenesis, it is not enough persuasive to conclude that pantoprazole improve the ischemia. The authors should discuss about this point.

→ Yes, like your comments, in this revised manuscript, we clearly described the explanation that Figure 3A and 3B showed increased angiogenic growth factors with increasing dose of pantoprazole, resulting in increased *in vitro* angiogenesis shown in Figure 3D. Also we added more evidences that decreased HIF-1α-DNA binding activities were shown in gastric homogenates treated with pantoprazole under indomethacin challenge, suggesting the ischemic condition was improved than indomethacin alone group.

2. In Figure 2A, the author demonstrated that Western blots for either cytosolic keap1 or nuclear Nfr2 in a different time in the presence of 300 M pantoprazole. However, the author did not describe whether the lower concentration of pantoprazole could increase cytosolic keap1 and nuclear Nfr2 or not.
As you know, pantoprazole is not a professional agent to induce these kind of phase II enzyme response as well as HO-1 induction, in which condition what we have identified was that pantoprazole additionally could induce HO-1 through Nrf2 activation by Keap1 inactivation. As can be seen in Figure 1A, 300 μM pantoprazole showed high efficiency of HO-1 induction. This is why we have checked Keap1 inactivation when stimulated 300 μM pantoprazole, maximally inactivation of Keap1 1hr after pantoprazole. Though 100 μM pantoprazole also inactivated Keap1 and Nrf2 activation, but the experiment was shown on 300 μM pantoprazole.

3. Figure4 and Figure5 showed that indomethacin alone did not increase the expression of HO-1. However, indomethacin induced inflammation. Some reports found that indomethacin increased the expression of HO-1. The authors should discuss about this point.

The results from Figure 5 were done with gastric homogenates, in which indomethacin attenuated HO-1 expression (Figure 5B), whereas co-treatment of indomethacin and pantoprazole can preserve and increase HO-1, reflecting protection from indomethacin-induced gastric damages. The results from Figure 4 were drawn from in vitro cell model showed insignificant changes in HO-1, whereas co-treatment of indomethacin and pantoprazole could preserve or increase HO-1. Therefore, please understand the theme of our investigations focusing onto the changes of HO-1 relevant to pantoprazole administration.

4. Figure4 and Figure5 showed that ZnPPIX decreased the expression of HO-1. I think that ZnPPIX inhibit only activity of HO-1. Can ZnPPIX decrease the expression of HO-1?

ZnPPIX is inhibitor of HO-1 enzyme. This is why we have co-administered pantoprazole and ZnPPIX to observe whether HO-1 inhibitor abolished the protective action of pantoprazole relevant to indomethacin-induced gastric damages in Figure 5. In in vitro experiment, the purpose of co-administration of HO-1 inhibitor was to observe whether the protective properties of pantoprazole were through HO-1 induction. ZnPPIX was also known to either inhibit HO-1 activity or its transcription. 

5. Figure3C showed that pantoprazole increased the expression of HIF-1α. However, Figure5 showed that pantoprazole decreased HIF-1α-DNA binding. It seems contradictory.
The discrepancy might come from the interpretation of result as well as experiment condition, the former HIF-1α have reflected angiogenic actions, while the latter result from EMSA have reflected that pantoprazole improved

6. The authors think all PPIs may induce HO-1 from the discussion. However, Yoda et al. (J Physiol Pharmacol. 2010 Jun;61(3):287-94) reported that lansoprazole, but not omeprazole, induced HO-1. The authors should refer this paper and discuss about this point.

Thank you very much for your comment. Here in this revised manuscript, we added the reference you have suggested as follows; Yoda Y et al, Prevention by lansoprazole, a proton pump inhibitor, of indomethacin-induced small intestinal ulceration in rats through induction of heme oxygenase-1, J Physiol Pharmacol 2010, 61: 287-294 in conclusion description as reference 31. We have also checked the HO-1 induction according to kinds of PPI before the current study and found that pantoprazole>lansoprazole>omeprazole have induced HO-1, whereas rebeprazole and omeprazole was somewhat weak in this induction in gastric epithelial cells. We speculated the in vitro aqueous condition might affect HO-1 inducing capacity since pantoprazole in aqueous status was proven to be most potent and stable and differed in target with Yoda’s experiment, stomach and small intestine.

Minor

1. There is Figure2E which show the change of DCF-DA fluorescence after different dosing of pantoprazole. However, in Figure legend, there is no description of Figure2E. Is Figure2E the part of Figure2D?

Yes, it is Figure 2D. Please understand mis-numbering for figure was done during figure organization. Thanks a lot for correction.

2. In p.8 l.23, the author described “PPI also increased expression of VEGF, mRNA and protein levels(Figure2A) in RGM-1 gastric mucosal cells.” However, not Figuer2A but Figure3A showed the expression of VEGF.

Yes, Figure 3A is correct. Please understand the mis-numbering was done during organization of Figures.
3. In p.9 l.16-18, the author described “Additionally indomethacin challenge significantly increased the expressions of NADPH oxidase-1(NOX-1) as seen in Fig.4B”. However, Weston blotting of NOX-1 is shown in Figure4A.

→ Yes, Figure 4A is correct. Thanks for correction.

4. In p.10 l.16, the author described “ICAL-1”. Is it mistake?

→ Yes, it was ICAM-1. Thank you for correction.

Response to the comments of Reviewer III

This manuscript describes the induction of the stress-responsive enzyme heme oxygenase-1 (HO-1) by the proton pump inhibitor pantoprazole through activation of Nrf2. In an in vitro cell culture study, pantoprazole induced activation of Nrf2 as evidenced by increased nuclear translocation and subsequent ARE binding of this transcription factor. The typical NSAID, indomethacin enhanced the transcription of some proinflammatory and adhesion genes, which was suppressed by pantaprazole. In an in vivo animal gastritis model, patoprazole protected against indomethacin-induced gastric erosion, which was attenuated by the pharmacologic HO-1 inhibitor. Overall, the finding are interesting and provide the novel protective effects of proton pump inhibitors against NSAIDs-induced gastric damage. There Are several issues that should be incorporated into the manuscript for the better revision.

1. Abstract, line 4: its acid suppressive action is not suffice to explain # its acid suppressive action does not suffice to explain.

→ Thank you so much for your very comprehensive correction. We have used your correction in this revised manuscript.

2. The role of Nrf2 and HO-1 in adaptive stress response need to be briefly mentioned in a
Background or Method session of the abstract.

→ Yes, we did add description about Nrf2 and HO-1 in this revised manuscript. Thanks.

3. page 1, line 3 from the bottom: HO-1 … inductions after pantoprazole were significantly associated with the increased expressions of … The underlined words are used in a singular noun. The corresponding verb (were) also needs to be corrected accordingly. Same correction need for the first line, p. 3 (expressions # expression) and many other locations throughout the text.

→ We improved whole descriptions in this revised manuscript including your pointing.

4. p. 2, line 4: .. because HO-1 inhibitor abolished these changes. In vivo mice model of … # .. because a HO-1 inhibitor abolished these changes An in vivo mice model of.. Numerous grammatical corrections are needed elsewhere (Introduction, Material & Methods, Results and Discussion parts)

→ We improved whole descriptions in this revised manuscript including your pointing.

5. keap1 should be abbreviated as Keap1 (capital for the first letter).

→ Thank you for your correction.

6. There is large standard deviation in the mRNA expression of HO-1 shown in Fig. 1A histogram. The average induction is less than 2 fold. In this respect, it would be better not to include the mRNA data as Western blot data alone clearly support the HO-1 induction by pantoprazole.

→ Yes, we removed histogram according to your comment.

7. The number and the concentration unit should be apart (e.g., 300uM # 300 uM)

→ Thank you for your corrections.
8. p. 8, line 15 & 16: As seen in Figure 2D, 3 uM pantoprazole exerted clear scavenging action of DMPO-adduct-generating hydroxyl radicals. This concentration of the compound is not the one used in the experiment

→ Please understand the experiment of ESR measurement is performed in chemical reaction, not in cell based condition. This is why the used concentrations of pantoprazole were different according to experimental condition, cell free condition and cell based condition.

9. p. 8, line 19-21: These chemical results drawn from ESR were further validated with biological test using DCF-DA fluorescence measurement, showing that pantoprazole showed significant DCF-DA reduction in a dose dependent manner (p<0.05, Figure 2B). Fig 2B does not reflect the statement in this sentence. The ROS scavenging activity of pantoprazole may not be associated with its anti-inflammatory and gastroprotective effects against indomethacin and the EPR data does not necessarily be included in the manuscript. If the authors would like to relate the antioxidant effects of this PPI inhibitor with its protective effects on indomethacin-induced inflammation, a series of experiments using a conventional antioxidant will be necessary.

→ It was Figure 2D, not figure 2B, stating the direct antioxidative action of pantoprazole. Since fundamental action of HO-1 is associated with antioxidative property, the HO-1 induced after pantoprazole might play antioxidative action. Interestingly since indomethacin induced oxidative stress directly and indirectly, the antioxidative action of pantoprazole also contributed cytoprotections of HO-1. We would like to keep Figure 4D, but we have stressed your comments in this revised manuscript. Thanks.

10. The manuscript title: Nrf2-mediated heme oxygenase-1 induction of PPI confers adaptive survival response to NSAID-induced gastric damages. The title is rather general and does not properly reflect the work the authors conducted. As the authors used indomethacin as a typical NSAID and pantoprazole as a representative PPI inhibitor, the title should specify the names of compounds used. In addition, the majority of data are derived from in vitro cell culture studies. The suggested title would be: title: A possible involvement of Nrf2-mediated heme oxygenase-1 upregulation in protective effect of the proton pump inhibitor pantoprazole against indomethacin-induced gastric damage in rats.

→ Thank you so much for your very kindness. We put the title as the reviewer have suggested in this
revised manuscript. Thanks a lot for your very generosity.

11. While scientifically sound, the manuscript needs editorial and grammatical improvement.

→ Revision and English corrections by fluent native English speaker were done in this revised manuscript.