Author's response to reviews

Title: Polygonum viviparum L. induces vasorelaxation in the rat thoracic aorta via activation of nitric oxide synthase in endothelial cells

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Author's response to reviews: see over
Dear Editor,

Please accept our sincerest gratitude for BMC Complementary and Alternative Medicine’s conditional consideration for publication of our paper (Manuscript ID: 1483743711810511. Article title: *Polygonum viviparum* L. induces vasorelaxation in the rat thoracic aorta via activation of nitric oxide synthase in endothelial cells) upon minor revisions. In the revised manuscript, we completely addressed the majority of the valid experimental recommendations raised by the reviewers. In addition to following the writing and typesetting guidelines set forth by BMC Complementary and Alternative Medicine, we also strived to improve the readability and clarity of our paper through major English grammar revisions. Additional or revised parts of the manuscript for the editors and referees are presented in red. Furthermore, we add a co-author, Dr. Wen-Yu Yu, in author group. Basing on so much professional knowledge she provided when paper revising; therefore, our group decides to place her name as the third author.

Thank you for the notice. About the method of rate sacrificed, it had been published at JAVMA (JAVMA, Vol 218, No. 5, March 1, 2001). At p.684, the article, Report of the AVMA Panel on Euthanasia, mentions that animals might be stunned to induce loss consciousness. Moreover, stunning by a blow to the head is the primary option in
small animals with thin craniums. The file of Taipei Medical University animal Euthanasia was been attached in the new revised manuscript.

I had emailed each author on this manuscript to send separate email to editorial biomedcentral for confirming their approval. You should receive their approval email in couple days. If you have any questions or need more information, please feel free to contact with me.

Thank you for your kind assistance in processing our manuscript.

Sincerely,

Chien-Ming Hu, PhD.
Reply to the editors and reviewers’ comments

Answers to Reviewer 1:

1. On page 5, …the rats were killed by exsanguinations from the carotid artery….We usually use the term of “sacrifice” instead of “kill”. Before sacrifice, do rats anesthetize by injecting or inhaling with anesthetize drugs, such as ketamine or isoflurane or others for humane treatment? Can anesthetize drugs affect the vasorelaxation or contracture in the isolated aorta rings? For what reasons?

Answer: The term of “kill” was revised to “sacrifice”. I appreciate the reviewer's expert comment. Indeed, the anesthetize drugs may affect the vasorelaxation or contraction in the isolated aorta. Therefore, the animals were sacrificed by exsanguinations from the carotid artery under lose consciousness by knocking medulla instead of using anesthetize drugs.

2. In your previous studies, 5 µM ACh could successful relax 3 µM PE-induced aortic contracture, but why did you use 10 µM ACh in this study?

Answer: Thanks for this question. Certainly, the dose response curve of ACh-induced relaxation is approximate at 5 µM, however, we used higher concentration of ACh (10 µM) to secure ACh-induced well relaxation by ACh in aorta.

3. Why do you choose these two NO inhibitors (L-NAME and L-NMMA) for PV-induced vasorelaxation? Can you cite references in the text?

Answer: L-NAME is non-specific NO inhibitor; L-NMMA is specific NO inhibitor.
We already added the references of L-NAME and L-NMMA citation in the text.

4. As you mentioned that HUVECs in 6-well plates were incubated with or without various concentrations of PV (10, 30, 50, and 100 µg/ml) for determination of nitric oxide (NO) production; however, no data was shown in the concentration of 50 µg/ml in Fig. 3. Does it lost? Whether cells were treated for 24 h in text or 1 h in figure 3 legend?

Answer: Thanks for reviewer’s cautious correction; we just measured PV 3, 10, 30 and 100µg/ml to affect NO production in HUVECs. In figure 3 legend, cells do be treated for 1 h; however, it is typo in the text that “…. PV (10, 30, and 100 µg/ml) and ACh (30 µM), as a positive control, for 24 h”. The error has been revised.

5. The whole name of BAPTA-AM?

Answer: The whole name of BAPTA-AM is “1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid”.

6. In Fig. 4, no explanations for the time courses of HUVECs (5×10^5 cells/well) incubated with or without concentrations of PV (3, 10, and 30 µg/ml) or ACh (30 µM) for the short-term incubation of 0.5 h or long-term exposure for 24 h?

Answer: Thanks for reviewer’s comment. We have explained why the short- or long-time courses were done, and the explanations were added in the section of “PV induced eNOS phosphorylation, cytosolic Nrf2 degradation, and HO-1 protein expression”

7. Could the authors draw a flow chart to explain the possible pathways of
PV-induced vasorelaxation?

**Answer:** Yes, the flow chart of possible pathways in PV-induced vasorelaxation is ready which shown in Figure 5.

**Answer to reviewer 2:**

# 1 My main criticism for this study is the method used to measure the effects of the extract of P. viviparum (PV) in rat aortic rings. Starting in the methods section, the description of incubation of PV in vessels is partially missing. The authors’ description includes a single concentration of PV (100 mcg/ml) as the only one tested in aortic rings when cGMP was measured (sorry, no page numbers in the submission to allow an easy location of sentences). However, nothing is described for the experiments used to evaluate the ability of the extract to induce vasorelaxation or how the results were analysed. With this lack of information and the short description of the ability of PV to cause relaxation, interpretation of figure 1 was a challenge. A closed inspection of the typical records provided in the submission revealed that the relaxation induced by Ach in aortic rings pre-contracted by phenylephrine was not greater than 90%, as expected in endothelium-intact rings (Fig. 1, A). Moreover, PV-induced relaxation (at 100 mcg/ml) showed in Fig. 1C was very small (perhaps 25%). How exactly the magnitude of PV-induced relaxation in endothelium-intact rat aortic rings was measured and analyzed? Figure 1C describes the relaxation obtained after PV incubation as a ratio of PV/Ach. In order to better describe these results, which are the main point for the rest of investigation, the authors must present the effects of PV as percentage of relaxation considering the maximum contraction elicited by phenylephrine in preparations with at least
80% of relaxation to Ach. These procedures must be described in details in the Method Section.

Answer: Thank you very much for providing us so well comments.

a.) The description of PV incubation was added in the section of “Preparation of rat aorta and tension recording ex vivo” of Methods.

b.) The reviewer referred that there is nothing to describe the evaluation of extraction ability inducing vasorelexation or how the results were analysed. Indeed, we have mentioned the relationship of cGMP and vasorelaxation and PV treatment in section of “ Formation of cGMP was elevated by treatment with PV in rat aorta” of Results.

c.) The reviewer referred that “PV-induced relaxation (at 100 µg/ml) showed in Fig. 1C was very small (perhaps 25%)”. Indeed, PV-induced relaxation (at 100 µg/ml) showed in Fig. 1C is 72.16 ± 3.49 %, but was not 25%.

d.) The reviewer suggested that we must present “the effects of PV as percentage of relaxation considering the maximum contraction elicited by phenylephrine in preparations with at least 80% of relaxation to ACh”. Above sentence was already described in details in the Methods Section.

#2 The authors justified their study using rat aortic rings and exploring the relaxation and effects of PV on the nitric oxide/guanylate cyclase pathway taken into account the popular usage of this plant to “boost blood circulation to dissipate blood stasis”. However, the model adopted is conventionally used to study anti-hypertensive effects. Is this plant used against other cardiovascular diseases such as hypertension? This relationship should be explored in the discussion and the disadvantages of the experimental model adopted clearly considered.
**Answer:** According to literature report, PV can ameliorate blood circulation to dissipate blood stasis in traditional medicine usage. However, it was no literature reported that PV used against hypertension until now.

#3 The process of preparation of the extract must be included in the article.
Thinking about science the reader cannot be precluded of this information. Is it an alcoholic, aqueous, hydroalcoholic solution? Which parts of the plant are popularly used? Which parts were used in the extract? The source of plant also must be more detailed. For instance, was it collected in the winter or summer? Is there a voucher specimen?
**Answer:** Thanks for reviewer’s suggestion. The process of preparation of the PV extract was added in the Methods. The other detail information of PV were described in our previous publication [4].

#4 Clearly describe how the animals were killed. Were it anesthetized before exsanguination?
**Answer:** The part of animal sacrifice was added in revised manuscript. There is no anesthetize drugs using before animal sacrificed. Actually, the animals were sacrificed by exsanguinations from the carotid artery under lose consciousness by knocking medulla.

#5 The number of experiments performed for each experimental group was described as equal or greater than 3. A group of 3 samples is usually considered too small for functional studies for statistical purposes, although it is commonly accepted for biochemical or molecular approaches. However, to allow a better
interpretation of the significance of the statistical tests adopted information such as “each experiment was repeated more than 3 times” is useless. The authors must revise this point and complete the groups for at least 5 experiments per group in the functional study.

Answer: Thanks for reviewer’s comment. Indeed, each experiment was tested for at least 5 individual determinations. We already revised description of figure legends.

# 6 The discussion must be improved. The significance of the results obtained can better explored. Since part of the results suggest the involvement of nuclear factor E2-related factor and HO in the effects of PV on vessels, the description of the function and relevance of this pathway is much more important than the description of the classical findings related to the discovery of nitric oxide.

Answer: Thanks for reviewer’s comment. The relevance of Nrf2 and PV-induced vasorelaxation had been provided in “Discussion section”. Heiss et al. reported that activating Nrf2 can elevate the bioavailability of NO by triggering eNOS phosphorylation and reducing eNOS protein expression by HUVECs [38].

Answer to Reviewer 3:

Major Compulsory Revisions

1. Conclusion: It should be rewritten based on the results obtained here, so it can provide manifest interests to the readers. Also there are some grammatical errors. You should check it.

Answer: Thanks for reviewer’s comment. The conclusion was rewritten from just the results. We also strived to improve the readability and clarity of our paper through major English grammar revisions.
Minor Essential Revisions

1. I found a few strange phrases and typo like: Methods: "maintained under a1-g tension"? Results: "PV-induced rat aortic relaxation"? "No scavenger suppressed brazilin-induced vasorelaxation"? Figure legends: Figure 4: "Phosphor-eNOS" and "ach" ? Discussion: "In the context of the cardiovascular system, knowledge that Nrf2 possesses antioxidant and anti-inflammatory characters can be of benefit in the onset of endothelial dysfunction [38]." This sentence should be corrected. It may conveys the wrong message.

Answer: Thanks for reviewer’s comment and suggestion. The part of strange phrases was revised. "A detailed description of PV was given in our previous publication” was deleted, and we added “PV is a perennial herb that arises from a short, thickened rhizome that appears massive, distorted or uncinated. The stem, which ranges from 10 to 30 cm in height and terminates in a narrow, dense flowering spike, is simple, erect and smooth and bears few leaves. The best survey period lasts from approximately late June to early September” as the background.

2. Background: "A detailed description of PV was given in our previous publication [4]." It may be deleted.

2. Figure legends( Figure 1, 3 ) and Table 1: I would recommend that authors delete the "as described in "Methods"

Answer: Thanks for reviewer’s recommend. "as described in "Methods" was deleted in Table 1, Table 2, Figure 1 and Figure 3.

3. Explain what Nfr2 is in Discussion.
**Answer:** Thanks for reviewer’s suggestion. We already explained what Nrf2 is in Discussion. “The nuclear factor E2-related factor 2 (Nrf2) play a critical role by interacting with cognate DNA-binding domains in the HO-1 promoter to up-regulate \( ho-1 \) gene transcription [39]. Cytoplasmic Nrf2 is bound to the Kelch-like ECH-associated protein 1 under general conditions; however, the Nrf2/Keap1 complex can be disrupted by some compounds, which allows the translocation of Nrf2 into the nuclei [40].”

4. **The paper is requiring editing for proper English usage by a native English speaker.**

**Answer:** Thanks for reviewer’s recommendation; the part of this manuscript was revised, and we also strived to improve the readability and clarity of our paper through major English grammar revisions.