Author's response to reviews

Title: Qian Yang Yu Yin Granule-containing serum inhibits angiotensin II-induced proliferation, reactive oxygen species production, and inflammation in human mesangial cells via an NADPH oxidase 4-dependent pathway

Authors:

Kang Ding (zh_cb2013@163.com)
Yan Wang (hy_nf2013@163.com)
Weimin Jiang (ct_dg2013@sohu.com)
Yu Zhang (fr_gh2013@sohu.com)
Hongping Yin (ty_gf2013@sohu.com)
Zhuyuan Fang (zhuyuan_fang@163.com)

Version: 3
Date: 4 February 2015

Author's response to reviews: see over
February 4, 2015

Dr. Vivek K. Bajpai
Associate Editor, *BMC Complementary and Alternative Medicine*

Ms. Ref. No.: 1725045676145110
Revision of manuscript “Qian Yang Yu Yin Granule-containing serum inhibits angiotensin II induced proliferation, reactive oxygen species production, and inflammation in human mesangial cells via an NADPH oxidase 4-dependent pathway” by Ding, et al.

Dear Dr. Vivek K. Bajpai,

Thank you very much for your decision letter and advice. We have revised the manuscript, and would like to re-submit it for your consideration. We have addressed the comments raised by the Referees. Point-by-point responses to the Referees’ comments are listed below. All the changes are highlighted in the revised manuscript.

We hope that the revised version of the manuscript is now acceptable for publication in your journal.

I look forward to hearing from you soon.

With best regards,

Yours sincerely,

Zhuyuan Fang, Professor
Jiangsu Province Hospital of Traditional Chinese Medicine
No.155 Hanzhong Road, Nanjing 210029, China
Tel.: +86-139-159-05596
Fax: +86-25-8661-8942
E-mail: zhuyuan_fang@163.com
We would like to express our sincere thanks to the Referees for the constructive and positive comments. We have addressed these comments and point-by-point responses are listed below.

**Replies to Referee #1**

**Specific Comments**

1. Seropharmacological method is a well-accepted approach to investigate MOA of Chinese herb. In this study, the authors used valsartan as a positive control. For preparation of serum containing QYYYG and valsartan, animals were orally administered 10-fold clinic dosage of QYYYG or valsartan once daily for 7 days. So, how and why was 10-fold clinic dosage chosen here?

   **Answer:** We would like to thank the Reviewer for this thoughtful question. In this study, we administered QYYYG or valsartan to Wistar rats to prepare drug-containing serum. Therefore, it is essential to appropriately extrapolate the human dose to the equivalent animal dose. According to Reagan-Shaw et al. (*FASEB J.* 2008; 22:659-61.), the theoretical equivalent dose of QYYYG or valsartan for rats was 6.2-fold clinic dose for humans. However, this equivalent dose was calculated only based on the body surface area normalization method. Considering there might be differences in oral absorption and bioavailability between humans and rats, we used 10-fold clinic dose of QYYYG or valsartan for rats in order to obtain drug-containing serum with adequate pharmacological activity.

2. In Western blot analysis, the authors detected the effects of QYGS on Ang II-induced expression of inflammatory markers including TNF-α, NF-κB p65, and IL-6 in HMCs. It is well known that NF-κB is composed of homo- and heterodimeric complexes of five members including p50, p65 (RelA), c-Rel, p52, and RelB. Why did the authors only detect the expression of p65?

   **Answer:** It is well known that p65 subunit is the most transcriptionally active one among the five subunits of NF-κB (*Proc Natl Acad Sci USA.* 1993; 90: 9901–5.). It forms a dimer with p50, which is the most prominent dimeric form of NK-κB and hence is considered the prototype (*Adv Immunol.* 1995; 58:1-27; *Genes Dev.* 1995; 9: 2723-2735). The dimer p50/p65 plays a crucial role in inflammatory and immune responses (*J Immunol.* 2003;
However, p50 subunit is not an idea inflammatory marker when compared to p65, because it is produced from constitutive processing of p105 and has no intrinsic ability to activate transcription (Annu. Rev. Immunol. 2000; 18: 621-63; Science. 2001; 293: 1495-9). Therefore, we only detected the expression of p65 in this study.

3. In Western blot analysis, the data of relative expression of GTP-Rac1/total Rac1 should be shown.

Answer: Thanks for this valuable suggestion. We admit that the ratio of GTP-Rac1 to total Rac1 is more meaningful to evaluate the inhibitory effects of QYGS on the NOX4-dependent pathway. We have added the data of relative expression of GTP-Rac1/total Rac1 in Figures 4 and 6 of the revised manuscript. The main body text related to these figures has also been updated accordingly (Page 14, Line 285; Page 16, Line 312).

4. In "Statistical analysis" part, the authors stated "Differences between experimental groups are assessed for statistical significance using one-way analysis of variance (ANOVA), followed by least significant difference (LSD) or Games-Howell post-hoc multiple comparison tests." Is there any difference between the post-hoc comparison tests (LSD vs. Games-Howell)? The authors should clarify what variables were tested using the LSD posthoc method, what variables were tested using the Games-Howell method and why. In addition, two-tailed or one-tailed P-value less than 0.05 were considered significant?

Answer: Thanks for this valuable suggestion. In this study, LSD post-hoc test was used to perform multiple comparisons when equal variance was assumed; while Games-Howell post-hoc test was used when variances were unequal (Basic Statistics and Pharmaceutical Statistical Applications, Third Edition, Edited by James E. De Muth, ISBN-13: 978-1466596733, p261). We have addressed this issue in the Statistical Analysis part of the revised manuscript (Page 12, Line 249). Additionally, two-tailed P-value less than 0.05 were considered significant.

5. Page 10, Line 12, the authors stated "Calculations of the expression levels were carried out using the absolute curve method." Please provide details or a reference to this method.
Answer: We have added a reference (Ref. 18) to the absolute standard curve method in the revised manuscript.

Replies to Referee #2

Specific Comments

1. QYYYG is a Chinese herbal formula instead of a Chinese patent drug. So, please briefly describe the preparation process in the Methods section.

Answer: We have added a paragraph to describe the preparation of QYYYG in the Methods section of the revised manuscript (Page 5, Line 111).

2. The authors prepared QYYYG-containing serum for the whole experiments after oral administration of QYYYG (including the positive control valsartan) to rats. I totally understand that this method is common for seropharmacological study of Chinese herbal formula. However, the metabolism of QYYYG in rat might be different with that in human body. So, if this difference exists, can it affect the reliability of the results? I strongly suggest that the authors discuss this issue in the Discussion section.

Answer: Thanks for this valuable comment. According to Cao et al. (Pharm Res. 2006; 23:1675-86), rat and human show distinct expression levels and patterns for metabolizing enzymes in the intestine. Therefore, it is reasonable to assume that the metabolism of QYYYG in rat may be different with that in human, and that the use of QYYYG-containing rat serum might introduce a bias into the results of this study. Nevertheless, in a pilot study, we used HPLC method to identify the main metabolites of QYYYG in human and rat serum and found that the metabolite profiles were similar in both species. This finding indicates that QYYYG-containing rat serum can be a good tool for investigation of the molecular mechanism of action (MOA) of QYYYG in the treatment of hypertensive nephropathy. However, we admit that further studies using QYYYG-containing human serum are required to validate the present findings and firm up the conclusions.

We have added a paragraph to discuss this issue in the Discussion section of the revised manuscript (Page 21, Line 425).
3. Please explain why the authors chose 10-fold clinic dosage of QYYYG and valsartan for preparation of drug-containing serum.

Answer: We would like to thank the Reviewer for this thoughtful question. In this study, we administered QYYYG or valsartan to Wistar rats to prepare drug-containing serum. Therefore, it is essential to appropriately extrapolate the human dose to the equivalent animal dose. According to Reagan-Shaw et al. (FASEB J. 2008; 22(3):659-61.), the theoretical equivalent dose of QYYYG or valsartan for rats was 6.2-fold clinic dose for humans. However, this equivalent dose was calculated only based on the body surface area normalization method. Considering there might be differences in oral absorption and bioavailability between humans and rats, we used 10-fold clinic dose of QYYYG or valsartan for rats in order to obtain drug-containing serum with adequate pharmacological activity.

4. In the Discussion section, the authors claimed that 2,3,5,4’-tetrahydroxystilbene-2-O-#-D-glucopyranoside (THSG) in Polygoni Multiflori Radix (a component of QYYYG) may be mainly responsible for the suppression of AngII-induced ROS generation and inflammation in HMCs. In this study, however, the authors used QYYYG-containing serum for all experiments instead of QYYYG. Considering QYYYG should be metabolized during the serum preparation, I am not sure if there was still THSG in the drug-containing serum. Did the authors examine the content of THSG in drug containing serum?

Answer: Actually, we used high performance liquid chromatogram (HPLC) method to examine the content of THSG in the prepared QYYYG-containing serum and confirmed there was still THSG in the serum. Please see the figure below for your reference.
(a) THSG detected by HPLC in a representative QYYYG containing serum sample; (b) THSG standard for HPLC analysis

However, considering these data are not directly relevant to the present study, we didn’t show them in our manuscript.

5. In addition to THSG, are there any other active substances that may be responsible for the suppression of Ang II-induced ROS generation and inflammation in HMCs? The authors should discuss this issue in the Discussion section.

Answer: Previous studies have reported that some active substances extracted from Corni Fructus, such as 7-O-galloyl-D-sedoheptulose and loganin, have potent protective effects against diabetic renal damage or glycerol-induced renal damage through elimination of ROS and inhibition of inflammation (J Pharm Pharmacol. 2012; 64: 1730-40; Eur J Pharmacol. 2010; 648:179-87). Therefore, we presume that these substances may also be responsible for the suppression of Ang II-induced ROS generation and inflammation in HMCs; but further studies are needed to verify this presumption.

We have added several sentences to discuss this issue in the Discussion section of the revised manuscript (Page 19, Line 377).

6. Page 7, line 16, please change "optical density (OD)" to "absorbance"

Answer: According to this comment, we have changed "optical density" to "absorbance" in the revised manuscript (Page 8, Line 165).