Author's response to reviews

Title: Oryeongsan suppressed high glucose-induced mesangial fibrosis

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Version: 3 Date: 11 October 2014

Author's response to reviews: see over
Response to Review Comments

Oryeongsan suppressed high glucose-induced mesangial fibrosis

We thank to reviewers for the invaluable comments and suggestions. We hope this revision has improved the manuscript to a level of their satisfaction. We revised the text in the Introduction, Materials and Methods, Results, and Discussion (red colored part).

Editorial comments:

- Please include the email addresses of all manuscript authors on your title page.
  
  We included the email address of all authors as you indicated.

- Please include details in your manuscript on who formally identified the plant material used in your study to ensure its authenticity.
  
  Herbarium voucher specimen of Oryeongsan (No. HBH112) was kindly provided from Korea Institute of Oriental Medicine, Daejeon, South Korea. Thus, we ensured its authenticity as an experimental material. We added in the Methods parts.

- Please include a Conclusions section after your Discussion.
  
  We included a Conclusion part as you indicated.

- Please include Authors’ Contributions and Acknowledgements sections in your manuscript. Details on the information that we would ask you to include in these sections, as well as formatting guidelines, can be found through the following link: http://www.biomedcentral.com/bmccomplementalternmed/authors/instructions/researcharticle#formatting-contributions
  
  We include authors’ contribution and acknowledgments properly.
Reviewer's report

Title: Oryeongsan suppressed high glucose-induced mesangial fibrosis

Version: 1 Date: 4 August 2014

Reviewer: Shou-Hsien Huang

Reviewer's report:

**Major Compulsory Revisions**

1. Fig. 2A, it seems that Oryeongsan have bi-effect for collagen IV protein expression at low or high concentrations. Oryeongsan up-regulated collagen IV at low concentrations; however, Oryeongsan down-regulated collagen IV at high concentration. I wonder whether 50 µg/ml Oryeongsan might induce cell death and cause the decrement of collagen IV levels.

Supplement Fig. 1 and Fig. 2: Firstly, to assess the effect of Oryeongsan on cytotoxicity in mesangial cells, the cells were pre-incubated with various concentrations (1 – 100 µg/ml) of Oryeongsan for 24 h. As shown in Fig. 1, Oryeongsan alone did not alter cell viability at the range of 1 – 100 µg/ml. In addition, LDH assay was performed. As shown in Fig. 2, Oryeongsan alone did not alter LDL release level at the range of 1 – 200 µg/ml. In addition, Oryeongsan treated with HG (25 mM) did not change LDL release level. Thus, these results demonstrated Oryeongsan itself did not affect mesangial cell cytotoxicity.

Supplement Figure 1. Cell viability and cell number

Supplement Figure 2. LDH release by Oryeongsan with/without HG
2. In Fig.3A, it seems that Oryeongsan have bi-effect for TIMP-2 protein expression at low or high concentrations. Oryeongsan down-regulated TIMP-2 at low concentrations; However, Oryeongsan up-regulated TIMP-2 at high concentration. The authors should explain the controversial results.

In Figure 3A: We improved western blotting of TIMP-2 in Fig. 3A. These data suggested that Oryeongsan significantly attenuated HG-induced TIMP-2 expression. Thus, Oryeongsan suppress high-glucose induced renal fibrosis via the regulation of ECM accumulation in renal mesangial cells.

**Minor Essential Revisions**

1. There were mismatch of the picture of collagen IV mRNA gel bands and the histogram of its mRNA expression. The collagen IV mRNA levels with Oryeongsan treatment were lower than vehicle in gel bands; However, the collagen IV mRNA levels with Oryeongsan treatment were higher than vehicle in histogram.

In Figure 2B: We remove collagen IV mRNA data by RT-qPCR assay (Figure 2B) to improve and emphasize the credibility from the result of realtime RT-PCR.

2. Authors mentioned that Oryeongsan can regulate TGF-beta, NF-κB, but what is the Oryeongsan downstream target actually?

Actually Oryeongsan suppress high-glucose induced renal fibrosis and TGF-beta expression through regulating the NF-κB pathway. Emodin attenuates HG-induced TGF-β1 and fibronectin expression in mesangial cells through inhibition of NF-κB pathway (Ref 1). In addition, NF-κB/AP-1 pathways was involved in suppression of HG-induced proliferation and extracellular matrix accumulation in mesangial cells (Ref 2).


3. Authors mentioned that Oryeongsan can regulate NF-kB and IkB. NF-kB and IkB both are regulated by IKK-αβγ complex. Will Oryeongsan regulate IKK-αβγ complex?

We did not test the regulation of IKK-αβγ complex on Oryeongsan. Future study is required to clarify the regulation of IKK-αβγ complex on Oryeongsan.

**Level of interest:** An article whose findings are important to those with closely related research interests  
**Quality of written English:** Acceptable  
**Statistical review:** No, the manuscript does not need to be seen by a statistician.  
**Declaration of competing interests:**  
I declare that I have no competing interests
Reviewer's report

Title: Oryeongsan suppressed high glucose-induced mesangial fibrosis

Version: 1  Date: 18 July 2014

Reviewer: LEE-TIAN L. T. CHANG

Reviewer's report:
I review the revised manuscript carefully. Authors revised most of my original question. But, some questions still got no respond. Red ones are no response ones. Blue ones are my new comment. If authors can reply these comment directly and correctly, this manuscript can be accepted. Attached file is my comment.

1. This is an in vitro study, but title of this manuscript is too strong to demonstrate renoprotective effect of oryeongsan without in vivo observation.

   We corrected the title of this manuscript. “Oryeongsan suppressed high glucose-induced mesangial fibrosis: Renoprotective role in diabetic nephropathy” → “Oryeongsan suppressed high glucose-induced mesangial fibrosis”

2. Concentrations of 10 and 50 ug oryeongsan/ml apparently inhibit cell proliferation induced by HG (Fig 1A). But these ratios were lower than control one (w/o HG treatment). Did it mean high concentrations of oryeongsan own cytotoxicity on HG-treated cells?

   Ø Author did not explain this question in his resubmitted manuscript.

   Supplement Fig. 1 and Fig. 2: Firstly, to assess the effect of Oryeongsan on cytotoxicity in mesangial cells, the cells were pre-incubated with various concentrations (1 – 100 µg/ml) of Oryeongsan for 24 h. As shown in Fig. 1, Oryeongsan alone did not alter cell viability at the range of 1 – 100 µg/ml.

   In addition, LDH assay was performed. As shown in Fig. 2, Oryeongsan alone did not alter LDL release level at the range of 1 – 200 µg/ml. In addition, Oryeongsan treated with HG (25 mM) did not changed LDH release level. Thus, these results demonstrated Oryeongsan itself did not affect mesangial cell cytotoxicity.
3. “HG” labelled in X axis of lower panel of Figure 3A was disappeared. Meanwhile, why higher concentrations of oryeongsan did not inhibit HG-induced TIMP-2 expression?

Ø Author did not explain this question in his resubmitted manuscript.

In Figure 3A: We improved western blotting of TIMP-2 in Fig. 3A. These data suggested that Oryeongsan significantly attenuated HG-induced TIMP-2 expression. Thus, Oryeongsan suppress high-glucose induced renal fibrosis via the regulation of ECM accumulation in renal mesangial cells.

4. SB431542, a TGF-beta type I receptor inhibitor, was used through Figure 2-6. But author only discuss it on Figure 6. Did author have any tests of oryeongsan related with this drug?

In result: We corrected it. We described that explanation of Oryeongsan related with SB431542 at result part.

5. Again, “HG” labelled in X axis of lower panel of Figure 4A was disappeared. Moreover, lower concentrations of oryeongsan seem significantly inhibited p-smad-2, smad-4 and smad-7 expression induced by HG. But, * and # were not labelled in lower panel of Figure 4A, especially smad-7. It means dose-dependent response of oryeongsan was not show in Figure 4A, but Figure 4B was.
In Figure 4A: We corrected it (Figure 4). We look over statistical significance test of figure 4A again. As a result, lower panel of Figure 4A had statistical significance and thus we did label in lower panel of Figure 4A. However, although pretreatment of 10 μg/ml Oryeongsan significantly inhibited Smad-7 expression, that was not up-regulation. So we did not label that. We confirmed that Oryeongsan regulated Smads protein expression in dose-dependent.

6. Figure 5 showed significant variation on intensity of DAPI and p-smad-2. These variations will induce false results on translocation. We need arrows to point these translocations.

Ø Please adding the explanation on arrows in legend of Figure 5.

In Figure 5: We marked arrows to point p-smad-2 translocations in Fig.5

7. ICAM-1 expression of 50 ug oryeongsan/mL of Figure 6B was not seen. But same experiment of Figure 6C show 1.5 fold ICAM-1 expression on 50 ug oryeongsan/mL. Did it mean Figure 6 B was not representative one?

Ø Author change the bar in new Figure 6C. It is an unusual modification. Author did not explain it. If possible, please provide all repeated ICAM-1 plots.

In Figure 6B: We remove ICAM-1 mRNA data by RT-qPCR (Figure 6B) to improve and emphasize the credibility from the result of realtime RT-PCR. Thus, it is confirmed that Oryeongsan significantly decreased HG-induced ICAM-1 mRNA and protein expression in a dose dependent manner.

8. There are two Figure 6 in this manuscript.

Manuscript Page 14, line 23: We corrected it.

9. Legend of Figure 7 did not explain NE and CE. What is the mean “Respective blot data” in legend?

In legend of Figure 7: We explain NE and CE in figure 7 legend.