Author's response to reviews

Title: Standardized butanol fraction of WIN-34B suppresses cartilage destruction via inhibiting the production of matrix metalloproteinase and inflammatory mediator in osteoarthritis human cartilage explants culture and chondrocytes

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Author's response to reviews: see over
Standardized butanol fraction of WIN-34B suppresses cartilage destruction via inhibiting the production of matrix metalloproteinase and inflammatory mediator in osteoarthritis human cartilage explants culture and chondrocytes

Dear Tom Rowles PhD

Executive Editor, BMC Complementary and Alternative Medicine

Editorial comments:

- We notice that, although you have included references in your text, there is no reference list in your manuscript.

Answer) I have included references in manuscript.

- We recommend that you ask a native English speaking colleague to help you copyedit the paper.

Answer) Thank you very much for your comment. I corrected by a native speaker of English for submission of revision manuscript.

Thank you very much for the kind comments for the reviewers, and giving us the opportunity for revision. I have revised the manuscript according to the reviewer's comments as follows;

We hope that these revisions fulfill all the reviewers’ concerns. On behalf of the research team, I would like to thank you for your effort in improving our manuscript.

Sincerely yours,

Dong-Suk Park, O.M.D., PhD.
Reviewer's report

Title: Standardized butanol fraction of WIN-34B suppresses cartilage destruction via inhibiting the production of matrixmetalloproteinase and inflammatory mediator in osteoarthritis human cartilage explants culture and chondrocytes

Version: 3 Date: 2 August 2012
Reviewer: EunAh Lee

Reviewer's report:
The question that this study is trying to answer is well defined and the data soundly support the conclusion. Especially, authors examined the appropriate target components to explain the protective mechanism of this new drug. But, there are several points as follows to be amended before acceptance.

1. The identity of the PBMCs obtained from buffy coat was not commented in this manuscript.

Answer) I have attached the results of CBC counting of healthy donors by CBC counting for blood transfusion (lymphocytes; 30.2%).
However, data concerning the viability of chondrocytes is included instead of data on the viability of PBMCs (Figure 2B) in consideration of another reviewer comments. The results of the revised figure are described on page 13, lines 14-19 of the revised manuscript.

2. As the WIN-34B showed differential effect on MAPKs compared with MF and CA, please comment about the implication in down-regulation of pERK and pJNK by WIN-34B for anti-inflammatory effect or tissue protective effect.

Answer) Thank you for your comments. We have now described the differential effect of WIN-34B on MAPKs compared with MF and CA for cartilage protective effect in the Discussion section of the revised manuscript (page 17, lines 26-27; page 18, lines 1-7; lines 10-17).

3. The data presented in this study is so soundly presented that it is in fallible to understand.
However, the written expression at some parts cause misunderstanding. I strongly suggest that the English need to be edited by native speaker.

**Answer**) Following your comment, the manuscript was revised in concert with a native English speaking science editor.

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Needs some language corrections before being published

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: Standardized butanol fraction of WIN-34B suppresses cartilage destruction via inhibiting the production of matrix metalloproteinase and inflammatory mediator in osteoarthritis human cartilage explants culture and chondrocytes

Version: 3 Date: 2 August 2012
Reviewer: YUNJONGLEE
Reviewer's report:

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.

Previously, Huh et al. showed the analgesic and anti-inflammatory effects of WIN-34B, a butanol extract from two herbs, in several animal models including monosodium iodoacetate (MIA)-induced osteoarthritis animal model. And, in this study, they revealed that WIN-34B inhibited the production catabolic factors and stimulated the production of matrix proteins in human osteoarthritis (OA) cartilages through suppression of ERK, JNK, and p38 MAP kinase pathways. Because there has no established effective treatment in OA, their results may be some worthwhile findings. However, but several short comings should be addressed.

- **Major Compulsory Revisions**

1. They used WIN-34B that contains 3.11% of chlorogenic acid (CA) and 0.64% of
mangiferin (MF), as previously described. Therefore, 40-200 ug/mL of WIN-34B has 1.24-6.22 ug/mL of CA and 0.26-1.28 ug/mL of MF. But, as standard molecule, they used 40-120 ug/mL of CA and 40-200 ug/mL of MF. Why were CA and MF used at much higher concentrations in this experiment? Chen et al. used 0-20 uM (about 7 ug/mL) of CA to study its effect on rat chondrocytes (Int Immunopharmacol. 2011 Jan;11(1):23-8.). Furthermore, CA and/or MF at these high levels revealed cytotoxic effects (Fig. 2). In this context, the chondroprotective effect of CA or MF may be underestimated. Moreover, the effects of CA and/or MF at these levels could not offer additional information on the biologic effects of WIN-34B.

Answer) Thank you for your comments.

i) Prior to our study, we showed that the effective doses of WIN-34B, CA, or MF require high-concentration in cartilage explants culture more than chondrocytes. The pieces of cartilage explants culture were thick, so it was difficult to infuse WIN-34B, CA or MF compared with chondrocytes.

ii) Also, we performed an additional experiment using cartilage and chondrocytes from OA patients. OA cartilage is hard and thick compared with rabbit chondrocytes (Int. Immunopharmacol. 2011 Jan;11(10:23-8). Therefore, the effective dose of CA on OA cartilage is different to the chondrocytes of rabbit (Int. Int Immunopharmacol. 2011;11(1):23-8.).

iii) The effect of MF or CA on the viability of chondrocytes (with or without IL-1β–induced chondrocytes) did not exceed IC₅₀ at concentrations up to 200 μg/ml (Fig. 2B).

2. In the previous animal models, you administered 100-400 mg/kg of WIN-34B to investigate its effect in MIA-induced OA models. In the present in-vitro study, 40-200 ug/mL
of WIN-34B was used. As far as I am concerned, there has been no background information about the in vivo concentrations of WIN-34B. What is your supporting evidence?

Answer) The component of WIN-34B, the dried flowers of *Lonicera japonica* and dried roots of *Anemarrhena asphodeloides*, are recommended for the optical range for human treatment (g/kg) in Donguibogam and are used at the Kyung Hee University Oriental Medicine Hospital.

3. To study cytotoxic effects of WIN-34B, PBMCs were used. Because you focused the effect on cartilage and chondrocytes are the only cell types present in cartilage tissue, the cell viability test should be performed using isolated chondrocytes, instead of PBMCs.

Answer) Acknowledging your comment, we included data on the viability of chondrocytes instead of the PBMC viability data in the revised Figure 2B, and have described the revised results of Figure 2B on page 13, lines 15-20 of the revised manuscript.

4. A fixed concentration of IL-1beta (10 ng/mL or 5 ng/mL?) was used in the present study. This is supra physiologic levels even IL-1beta increases in OA patients.

Answer) The IL-1β concentration of 10 ng/mL has been correctly included on page 7, line 19.

5. For amplification of beta-actin mRNA, PCR was performed for 30 cycles. Figure 3 & 5 showed thick bands of beta-actin and the PCR bands might be saturated. Compared to the PCR cycle numbers of the other genes, the cycle number of beta-actin PCR should be reduced.

Answer) Following your comments, we performed 27 cycles of PCR and corrected
6. In histological analysis, a quantification method to compare stain intensities was not clearly described. In figure 4, the imaging of IL-1beta control seems out of focus.

Answer) In the revision, we described the quantification method in the Histological analysis portion of the Methods section (page 9, lines 24-27). In Figure 4, the IL-1beta control displayed clearly reduced proteoglycan content or collagen deposition, because it was pale stained and not stained in a focused pattern.

7. Why were the levels of GAG and type II collagen measured at different times? They have to consider cytotoxic effects of CA and MF. Please, show time-concentration curves of GAG and type II collagen.

Answer) A previous study described the degradation of GAG and type II collagen, and their release at different times by IL-1α in rabbit articular cartilage explants (Biol Pharm Bull 29(7):1408-1413). Also, we added the results of time response and dose effect of IL-1β (Figure 3A, page 13, lines 7-14) and the time-dependent effect of GAG release and type II collagen of WIN-34B, CA and MF in IL-1b-stimulated human cartilage explants culture (Figures 3B and 3D; page 13, lines 25-26 and page 14, lines 1-7 and 9-11).

8. Aggrecanases, MMPs, PGE2, and NO production are regulated by NF-kB as well as MAP kinases pathways. The effects of WIN-34B on NF-kB pathway deserve to be further investigated. It is known that induction of MMP-13 require p38, JNK, and NF-kB but MMP-1 does not require JNK or NF-kB.

Answer) Thank you for your comments. Our study reported that WIN-34B inhibits the
inflammatory response by inactivating IκB-α phosphorylation and mitogen activated protein kinase pathways in IL-1β stimulated human fibroblast-like synoviocytes (J. Ethnopharmacol 2012). We have also added that WIN-34B showed a differential effect on MAPKs compared with MF and CA for cartilage protective effect in the Discussion section of the revised manuscript (page 17, lines 26-27; page 18, lines 1-7 and 10-17).

9. In figure 5, IL-1beta seemed to induce ADAMTS-5. However, ADAMTS-5 mRNA is not upregulated by IL-1beta although ADAMTS-4 mRNA is induced. Please, recheck beta-actin bands (please, refer to comment #3).

Answer) We double-checked for ADAMS-5 mRNA expression after the correction of β-actin. Figure 5A has been corrected.

10. In figure 6c, they showed IL-1beta levels in the media. For stimulation of cartilage explants, culture medium containing 10 ng/mL of IL-1 beta (= 10,000 pg/mL) was replaced every 3 days for 3 weeks. But, using ELISA, all levels of IL-1 beta in the conditioned media were less than 1000 pg/mL. They have to explain the low concentrations of IL-1 beta in the media containing 10 ng/mL of IL-1 beta.

Answer) IL-1β is coupled with IL-1β-receptor and then neutralized or incorporated into chondrocytes. Therefore, the level of IL-1β in conditioned culture medium is lower than the amount of IL-1β treatment. Also, we need further experiments to determine the pharmacokinetics or metabolomics of WIN-34B in condrocytes or in animal models of osteoarthritis.

- Minor Essential Revisions
1. For each experiment, the sample size is not clear.

**Answer** We added the sample size information in the legends to Figures 2-6.

2. In Abstract, they described that "WIN-34B is used for arthritis treatment in East Asian countries." However, WIN-34B has not been used as anti-arthritic agents in human being until now.

**Answer** We corrected the component herb of WIN-34B instead of WIN-34B in the Abstract (line 5).

3. In Methods, they described that "Experimental groups consisted of IL-1 -unstimulated control group, IL-1 -treated group (10 ng/ml), .... Cartilage pieces were placed in 48-well plates and treated with 5 ng/ml human recombinant IL-1.." Which levels of IL-1beta did they use? 10 ng/mL versus 5 ng/mL of IL-1beta?

**Answer** We used the same concentration of 10 ng/ml IL-1β for cartilage explants culture and chondrocytes. This has been corrected in the Methods section (page 7, line 19).
Reviewer's report

Title: Standardized butanol fraction of WIN-34B suppresses cartilage destruction via inhibiting the production of matrixmetalloproteinase and inflammatory mediator in osteoarthritis human cartilage explants culture and chondrocytes

Version: 3 Date: 4 August 2012
Reviewer: Li-Dong Wu
Reviewer's report:
This study was designed to test the cartilage protective effects of WIN-34B and its standard compounds on IL-1β-induced human cartilage explants culture and chondrocytes. The results demonstrated that WIN-34B more effectively improved the cartilage protective effect by modulating MMPs, ADAMTSs, TIMPs and inflammatory mediators, and possibly by inhibiting MAPK pathways as evidenced by RT-PCR, ELISA, histological analysis and Western blot. The authors concluded that WIN-34B has potential to be used in the treatment of osteoarthritis. It seems no related reports on it.
However, some questions remain:

- Major Compulsory Revisions

1. In vitro, as the authors pointed out the MMPs play an important role in the induction and progression of OA. Therefore, the evidence provided in the manuscript, in the “Culture of chondrocytes and treatment” part, the western blot data, seems too weak to support the hypothesis, both the protein synthesis and the activity of these enzymes (MMPs, ADAMTS) should be measured in vitro.

Answer) We added the results of the protein expression of ADAMS-4, MMP-1, and
MMP-13 to Figure 5C and in the revised manuscript (page 11, lines 23-25; page 12, lines 1-16; page 15, lines 9-12).

2. As in the legend of Figure2A, a high concentration of CA (more than 100 µg/ml) was cytotoxic in the presence of IL-1#. In PBMCs(Figure2B), MF or CA at 10µg/ml inhibited PBMC viability by more than 20% in the absence or presence of IL-1#, suggesting a possible cytotoxic effect at this concentration. However, MF or CA was used in the experiments at concentrations from 40 to 200µg/ml, please explain these.

Answer) We assessed the viability of chondrocytes, and revised Figure 2B. The viability of MF or CA did not exceed IC$_{50}$ at concentrations up to 200 µg/ml in chondrocytes (with or without IL-1β–induced chondrocytes) (page 13, lines 18-19).

3. Most important, we can not find any REFERENCES.

Answer) We apologize for the mistake, The references are now included.

- Minor Essential Revisions

4. The qualitative result of Safranin O staining is not clear. This paper has some problems including English grammar and spelling. So, proof reading this manuscript by native speaker.

Answer) The revision was done by a native English speaking science editor.

5. Some passages of the manuscript are imprecise. The discussion part is brief. Please describe more about the reasons of the difference therapeutic effect between WIN-34B and other compounds.
Answer) We added information concerning the differential effect of WIN-34B on MAPK pathway compared with MF and CA for cartilage protective effect in the Discussion section of the revised manuscript (page 17, lines 26-27; page 18, lines 1-7 and 10-17).

6. If necessary, the authors can analysis the pharmacokinetics of WIN-34B in vivo, include the WIN-34B serum concentration data and discuss the consequences.

Answer) We plan to analysis the pharmacokinetics or metabolomics of WIN-34B in animal model of osteoarthritis.

Level of interest: An article whose findings are important to those with closely related research interests.

Quality of written English: Needs some language corrections before being published

Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.

Declaration of competing interests: I declare that I have no competing interests.