Author's response to reviews

Title: Arthropod steroid hormone (20-Hydroxyecdysones) suppress IL-1beta-induced catabolic gene expression in cartilage

Authors:
- Shiow-Yunn Sheu (amel@tmu.edu.tw)
- Shin-Rong Ho (god940@gmail.com)
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Author's response to reviews: see over
To:

Editorial Board

BMC Complementary and Alternative Medicine

Date: 30th, Oct., 2014

Dear Editors:

Enclosed are files of the revised manuscript entitled “Arthropod steroid hormone (20-Hydroxyecdysone) suppress IL-1β- induced cartilage destruction” by Shiow-Yunn Sheu, Shin-Rong Ho, and Jui-Sheng Sun (MS: 9345583251422139). Thank you for your kindness to give us the chance of revision. We have learned a great deal from the comments and suggestions. We have revised and rewritten the manuscript in accordance with comments made by the reviewer. All the specific revisions have been highlighted by underlines and yellow text highlight color on the revised manuscript. We are now resubmitting this manuscript for possible publication in the esteemed Journal of BMC Complementary and Alternative Medicine.

Please direct the correspondence and phone calls about this to me at the following address and phone number:

Thank you for your attention to our paper.

Sincerely yours,

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To Reviewer #: 1

Ms. Ref. No.: MS: 9345583251422139

Thank you very much for your kind review and comments on our paper. We have learned much from your comments. After looking over your remarks, we feel confident that the revised manuscript addresses your concerns in detail and bring the paper into accordance with the standards expressed in your review. We are resubmitting the revised manuscript and would like to request a further review. We have responded to your original comments on a point-by-point basis below. Additional commentary on the revised manuscript would be highly appreciated.

1. ..... it requires language editing - some examples are provided below:
   p 2, line12 change "role" by "effect"
   p 2, line14 remove "was"
   p 2, line21 change "evidenced by down-regulated the enhanced" by "reduction of the enhancement of"
   p 4, line12 "promote", not "promotes"
   p 4, line20 "closely", not "clo" ?
   p 4, line24 "ingredient", not "ingredients"
   p 5, line3 "provides evidence that", not "provides a plausible value in that"
   Ans. Thank you for your comments; the language editing work have been done accordingly.

2. The experiments have been made with $10^{-8}$ M 20-hydroxyecdysone, but the choice of this value is only based on the results of MTT test. It would have been of interest to perform a dose-response experiment on one or two additional parameters.
   Ans. Thank you for your comments. Indeed, choice of concentration based on the results of MTT test is somewhat risky; however, the main purpose of the in vitro test is to validate its efficacy and the possible mechanism, we just choose one value for all test. We should and will perform a dose-response experiment on one or two additional parameters for the future in vivo study.

3. In the results section (p 9, line 25), the authors state "but it is not able to recover the cartilage phenotype", but it is necessary to develop such a sentence in details. Moreover, p 10 lines 2-7 contain twice the same ideas and this should be reorganized.
   Ans. The effect of Ecd was different in normoxic and hypoxic conditions. Although, “…with the pretreatment of $10^{-8}$M Ecd, it can scavenge IL-1β effect on Epas1, but there is also no effect on Col2a1 gene expression (Figs. 2).” [Page9, Line 19-20]
   However, “….. The Col2a1 gene expression was stationary under hypoxic condition; while in hypoxic condition, Ecd pre-treatment eliminated the IL-1β induced Col2a1 gene down-regulation (Fig. 4B). Ecd does have protective effect on Col2a1 gene expression under hypoxic condition.”[Page 10, Line 5-9]
   We revised the controversial statement in the revised manuscript as the following:
   …Ecd can reduce the catabolic effect of IL-1β on the cartilage explants; Ecd has protective effects on articular cartilage by inhibiting Epas1, MMP-3, MMP-13 and ADAMTS-5 genes expression.

4. Finally, some recent references are missing and should deserve proper citation, especially the last one:

Ans. Thank you for your comments; your mentioned above 3 references have been cited as Ref. 7, 14 & 15 in the revised manuscript, as the following:

Ref. 7

Ref. 14

Ref.15

5. In figure 5, the prefix "bio" is not necessary for "factors". Such a figure would gain more interest if more detailed mechanisms would be indicated. As such, it is not very useful.

Ans. Thank you for your comment, we revised the description and omit the prefix “bio” as your suggestion.

Thank you very much for your kind review and comments on our paper.

Sincerely yours,

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To Reviewer #: 2
Ms. Ref. No.: MS: 9345583251422139
Thank you very much for your kind review and comments on our paper. We have learned much from your comments. After looking over your remarks, we feel confident that the revised manuscript addresses your concerns in detail and bring the paper into accordance with the standards expressed in your review. We are resubmitting the revised manuscript and would like to request a further review. We have responded to your original comments on a point-by-point basis below. Additional commentary on the revised manuscript would be highly appreciated.

**Major concerns:**
1. No any experiments of IL-1beta-induced cartilage destruction were done in this manuscript. Otherwise the title needs to be changed to Arthropod steroid hormone (20-Hydroxyecdysone) suppress 1 IL-1β- induced catabolic gene expression in cartilage.
   Ans: Thank you, the title has been changed to the following:
   **Arthropod steroid hormone (20-Hydroxyecdysone) suppress IL-1β- induced catabolic gene expression in cartilage**
2. Authors conclude that Ecd may suppress IL-1beta-induced cartilage destruction via HIF-2alpha pathway. This is not true because only parallel experiments for epas1, MMP-3, MMP-13 and ADAMTS-5 genes expression were performed. If HIF-1alpha gene is knocked down, effects of Ecd on the MMP-3, MMP-13 and ADAMTS-5 genes expression were reduced. The conclusion can be accepted.
   Ans. Thank you of your comments, we added this concept on the end of “summary” as the following: … destruction via HIF-2α pathway. However, further researches by in-vivo HIF-1alpha gene knock-down and translational medicine are mandatory for the possible clinical application in the future.

**Minor changes:**
1. Page 2 line 2: In osteoarthritic (OA), should be changed to steoarthritis (OA).
   Ans. Thank you for tour comment; we have corrected this mis-spelling.
2. Add one sentence of effects of Ecd, and why IL-1beta was chosen in the background part.
   Ans. Thank you for your comments; we revised the controversial statement in the revised manuscript as the following:
   …. induce cartilage destruction. **IL-1β is a potent pro-inflammatory cytokine that is capable of inducing chondrocytes and synovial cells to synthesize MMPs.** The hypoxia-inducible factor-2alpha (HIF-2alpha, encoded by Epas1) is …. 

3. Page 2 line 11: "were" should be changed to "was".
   Ans. Thank you for tour comment; we have corrected this mis-spelling.
4. Page 2 line 17: The full names of term are described at the beginning, then the abbreviations can be used later.
   Ans. Thank you for tour comment; we have corrected this sentence.
5. Page 2 line 20: "was" should be changed to "were".
   Ans. Thank you for tour comment; we have corrected this mis-spelling.
6. Hope authors to correct some grammar mistakes in the revised manuscript.
   Ans. Thank you. The whole manuscript was proof-reading by a native English speaking colleague in our institute. We hope this version will be less grammar mistakes.
7. Where did the Ecd was got? how Ecd was prepared in vitro experiments?
Ans. 20-Hydroxyecdysone (Ecd) was obtained from Enzo Life Sciences; New York, USA. Stock solution of Ecd was prepared by adding 10mg Ecd to 2.08 ml 1XPBS, the final concentration of this Ecd stock solution was 10^{-2} M, then kept in -20°C condition. This statement had been added in the first paragraph (Materials and Methods) of revised manuscript as the following:

**Chemicals and reagents**
20-Hydroxyecdysone (Ecd) was obtained from Enzo Life Sciences; New York, USA. Ecd stock solutions (10^{-2} M) were prepared in phosphate buffer solution (PBS, Sigma Chemical, St. Louis, MO, USA) and stored at -20°C.

8. Only quantitative real-time PCR data were shown, but no RT-PCR data were found in the manuscript.
Ans.: Thank you for your comments, the subtitle of “RNA extraction, cDNA synthesis, reverse transcriptase polymerase chain reaction (RT-PCR) and quantitative real-time PCR” in “Materials and Methods” has been revised as “Gene expression analysis” (Page 7, last para).

9. How long pre-treatment of Ecd in vitro was not introduced?
Ans. The pretreatment of Ecd was 24 hours before experiment. This statement was described at the 2nd paragraph (Page 7) of material and Methods as the following:

**Articular cartilage explants culture**
Newborn ICR mice (3 days-old) were obtained from the laboratory center of the Medical College, National Taiwan University. Under sterile condition, fibrous tissues were removed, the articular cartilage explants were washed and then seeded to α-MEM (containing 1% FBS) one day before test. After 1 day’s pre-incubation with/ or without Ecd (20-Hydroxyecdysone: 10^{-8} M), IL-1β (5 ng/ml) was added for further test.

10. Add some description in Figure 5. Also the effect of IL-1 beta in this Figure should be added.
Ans. Thank you for your comments. The effect of IL-1 beta has been added into Figure 5, same as some other physical and chemical factors; some description in Figure 5 has also been added as the following:

Both physical and chemical factors may induce cartilage degradation via IL-1β/ HIF-2α pathway; 20-Hydroxyecdysone (Ecd) does have chondroprotective effect and can inhibit IL-1β- induced cartilage catabolism via HIF-2α pathway.

11. No statistical analysis was done in Figure 2, 3 and 4.
Ans. Thank you for your comment. Statistical analysis done in Figure 2, 3 and 4 was added in the revised manuscript.

12. The labeling in Figure 4 is difficult to understand.
Ans.: Thank you for your comments; the description about Figure 4 had been revised to make it more clear and easier to understand as the following:

**Fig. 4** Ecd protect cartilage explants both in hypoxic and normoxic conditions
The Epas 1 gene expression is enhanced under hypoxic condition. With the pre-treatment of 10^{-8} M Ecd, it can effectively eliminate the IL-1β enhanced Epas1 gene expression. The Col2a1 gene expression was stationary under normoxic condition. Under hypoxic condition, the pretreatment of 10^{-8} M Ecd can effectively eliminate the IL-1β down-regulated Col2a1 gene expression. Under hypoxic condition, IL-1β mediates HIF-2α–induced ADAMTS-5 gene expression of articular cartilage.
The pretreatment of $10^{-8}$M Ecd can effectively down-regulate MMP3 gene expression only under normoxic condition; while down-regulate ADAMTS5 genes expression both in normoxic and hypoxic conditions.

(Each value is the mean ± standard deviations; n = 8; *: p<0.05; **: p< 0.01; ***: p < 0.001).

13. In discussion section, please focus the discussion on the experimental data and what will be do next.

Ans. Thank you for your comments, major revision of “Discussion” was done and the discussion on the experimental data and what will be do next was added on the revised manuscript. Please refer to the revised manuscript for the detail.

Thank you very much for your kind review and comments on our paper.

Sincerely yours,

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