Author's response to reviews

Title: Effect of a Herbal-Leucine mix on the IL-1beta-induced cartilage degradation and inflammatory gene expression in human chondrocytes

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Author's response to reviews:

Editor-in-Chief
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Please find the revised research article (MS: 9816785705512274) entitled “Effect of a Herbal-Leucine mix on the IL-1beta-induced cartilage degradation and inflammatory gene expression in human chondrocytes” for favor of publication in BMC Complementary and Alternative Medicine. We have carefully addressed the comments of the reviewers. This has resulted in substantial improvement of the manuscript.

I hereby declare that no substantial portion of the manuscript has been published or is under consideration for publication elsewhere and that its submission for publication has been approved by all of the listed authors.

Please feel free to contact me if you have any concern.

Sincerely yours,
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Editorial requests
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Author list: We note that the author list on the manuscript file and on the submission page do not match, could you please clarify which author list is correct and ensure that this is written in exactly the same way on the revised manuscript and the submission page.
Author’s response: Author’s list has been corrected on the submission page.

Ethical approval? Please revise the statement under the Methods section to indicate that the study was approved by the Institutional Review Board at Case Western Reserve University.

Author’s response: The statement for ethical approval has been revised in the Methods under subheading “cell isolation and human chondrocytes culture”.

Competing interests- You indicate that the herbal product employed in the study is a commercial product, we therefore feel that this should be declared under the Competing interest section, please also revise the declaration to indicate any relationship between the authors and the manufacturer of the product and whether the manufacturer provided any funding for this study.

Author’s response: Product is “intended” to be commercialized by Vital g-Netics, LLC but is indeed not yet released. The company only provided the mixture for this exploratory study but was not involved in either the planning or execution of the studies. None of the authors have a conflict of interest – we have no commercial contracts with Vital g-Netics. This statement has been mentioned in the competing interest section.

Reviewer # 1 report

Specific minor comments:

Abstract – line 11 – aggrecan
Author’s response: corrected

Methods – It is written that chondrocytes at 80% confluence were used and in the next paragraph it is mentioned 1x10⁶ cell/ml – does it mean seeding density? Please, clarify!

Author’s response: Yes, 1x10⁶ cell/35 mm dishes indicate seeding density and when chondrocytes reached at 80% confluence were used for treatment. To avoid the confusion this has been clarified in the Methods under subheading “cell isolation and human chondrocytes culture”.

In Cell viability and Nitrites assay experimental setup should be written, not only description of the assay itself

Author’s response: As suggested, the description of experimental setup has been added on the “Cell viability” and “Nitrites assay” subheadings of the Methods section.

RT-PCR – line 5 – Trizol
Author’s response: Corrected

Primers and probe that you have used – were they Taqman Gene expression Assays or you have designed them? Assay number or sequence should be written.
Author’s response: The primer probes used for the Real Time PCR were TaqMan Gene expression assays. Applied Biosystem does not provide primer sequences of these assays. But, the Assay ID number has now been provided in the methods section under subheading “Quantitative real-time-PCR (RT-PCR)

Was the expression of GAPDH validated – the same expression in all experimental conditions?

Author’s response: The GAPDH was used as a reference control to study the expression level of specific gene. The expression of GAPDH was consistent in between the different treatment groups of each experiment. The expression of each gene was normalized with the GAPDH expression in the corresponding treatment group, relative expression levels was determined by ##Ct method and compared with untreated control.

All text – better to write THM concentrations (1 and 5µg/ml) than (1 - 5µg/ml) –you have two concentrations, not from 1 to 5.

Author’s response: Corrected

Results – Figure 4 is missing!!! (Figure 3 is presented 2-times!)

Author’s response: This was an inadvertent error. Figure 4 has now been submitted for ACAN and COL2A1 with revised manuscript.

Discussion – NF-#B, not NK-#B (line 25)

Author’s response: Corrected

Last paragraph – concerning GAG release – I wouldn’t say that 16% is a dramatic inhibition

Author’s response: The word “dramatically” has been removed from the discussion section last para.

Reviewer’s 2 report

Comments to the Authors:

1. The introduction should contain literature data informing the reader what is unknown and what remains to be studied etc, and not only a summary of what the state of knowledge is on each topic.

Author’s response: Introduction has been edited and improved as per suggestion.

2. The major concern is the characterization of such extracts. It is usual that herbal extracts of the same plant give different results due to differences in chemical composition (plant origin, extraction conditions, artifacts formed during plant conservation or extraction, etc.). Therefore, to give reproducible results extracts must be perfectly characterized with respect to chemical composition. How do we know the effect of every active compound in a composition of plant extracts?
Again, the results indicate that these extracts counteract IL-1beta effects. With such complex extracts, the possibility exists of non specific effects.

Author’s response: Commercially available plant extracts were used. They are standardized extracts and so that confers processes of uniformity and consistency. The amino acid, L-Leucine, is of pharmaceutical grade and is also commercially available.

The purpose of the study was not to determine which of these phytochemical elements conferred the actions on gene expression and chondroprotection. It is true that there could be dominant actions of one plant extract and within that, certain chemical entities but we studied the effects of a combination of these extracts. Please note that somewhat similar results on chondroprotection, reduced expression of iNOS and NO production were obtained some 6 years ago with the combination of maca and cat’s claw (Ref 19). That suggests that the results are indeed reliable over time. It is also important to note that the results are consistent with the ethnomedical uses of these plants – for joint health and inflammation.

Are the results merely the result of non-specific effects of these plants? While that is always a possibility it seems highly remote. There was a clear dose dependency in the studies. In addition, these are actions that are quite unique, in that we know of no other reports that display similar actions. Clearly with additional research, including that performed by other investigators, the results and suggestions raised by this research will be placed in perspective.

3. The authors have to perform experiments with control cells (with normal, healthy chondrocytes), not only with OA-chondrocytes. They have to demonstrate that the induced mechanism is specific to “healthy and unhealthy” chondrocytes. It is important to show data from control experiments.

Author’s response: It is important to note that these are chondrocytes obtained from surgical specimens. They are superficially regarded as “normal" but we do not have IRB approval to take cartilage from individuals that are normal or without a need for surgery. In our opinion no IRB will approve such studies anywhere in the world. The closest we can come to this is to get cartilage samples from trauma or accident victims but in such cases it is not possible to get an accurate history. Moreover, such cases are not routine or regular and to wait to include these may delay the work indefinitely. We respectfully submit that the results as presented here and in the numerous previous publications with this technique, has value.

4. It has been previously shown that pro-inflammatory effects of IL-1β and TNF-β in OA are regulated by activated transcription factor “Nuclear transcription Factor kβ”. Herbal-Leucine mixture extracts most probably inhibit NF-kβ signaling pathway though their ability to scavenge free radicals. This was not investigated in this work. Why did the authors not look for activation of NF-kβ?

Author’s response: We have studied the effect of HLM on the IL-1β-induced activation of NF-kβ in OA chondrocytes. We found that HLM (5 and 10µg/ml) significantly inhibited the activation of NF-kβ in OA chondrocytes stimulated with
IL-1β (Figure 6). These results have now been included in the manuscript and respective changes have been made in the manuscript Method section, figure legend and conclusion.

5. It would make sense to show a picture of the structure in the manuscript. The behavior of the cells in the presence or absence of the extract should be shown. The spectrum of the methods performed is very limited for specific demonstration and evaluation of the complex process of ECM production by chondrocytes. Some histological data needs to be supplemented with Alcian blue staining for proteoglycans and GAGs.

Author’s response: The herbal-Leucine mix (HLM) used in this study is not a single purified compound, it is a mixture of three extracts and Leucine thus structure of the mixture cannot be obtained or shown. We have already shown that HLM has no effect on the cell viability at the concentration (1 and 5µg/ml) used in this study (Figure 1). Additionally there was no change on the chondrocytes morphology in HLM treated chondrocytes compared to control group (figure attached is only for reviewers, not for publication). The cartilage from different patients was used to prepare chondrocytes and we have shown the data of GAGs release in cartilage explants (Figure 5), which shows that HLM inhibits IL-1β-induced proteoglycan loss from the cartilage.

6. All extracellular matrix components are measured exclusively at the gene expression level. This, by itself, has very little value. Proteins are the key players in chondrocyte biology. Messenger RNA is simply the transcript and may not reflect important change at the protein level. Therefore, it would make more sense to evaluate these also with western blot analysis at the protein level.

Author’s response: As suggested by the reviewer we performed these additional studies and the culture supernatants were analyzed for the effect of HLM on MMP-9 and MMP-13 production in IL-1β-stimulated OA chondrocytes using ELISA. We found that the inhibitory effect of HLM on MMP-9 and MMP-13 expression is at both mRNA and protein level of in IL-1β-stimulated OA chondrocytes. These results are added in the manuscript (Figure 3 C and D) results section and respective changes have been made in the manuscript Method section, figure legend and discussion.

7. Fig. 3A/B: Why did the authors treat the chondrocytes with IL-1β for 6 h for MMP-9 and 24 h for MMP-13? Why did they not use the same incubation time for IL-1β for both MMPs? This should be clarified.

Author’s response: We have used both time points 6 h and 24 h to study the effect of HLM on the IL-1β-induced MMP-9 and MMP-13 production. HLM extract has also down-regulated the MMP-13 expression at 6 h. But, we found maximum change for MMP-9 at 6 h and for MMP13 at 24 h.

8. Fig. 4A and B are the same pictures like Fig. 3A and B, but in the results and Fig. Legends of the MS state they should be for ACAN (A) and COL2A1 (B). This should be clarified.

Author’s response: This was an inadvertent error on our part. Figure 4 has now
been submitted for ACAN and COL2A1 with revised manuscript.

Quality of written English: Needs some language corrections before being published.
Author’s response: Effort has been made for the language correction to improve manuscript.