Author's response to reviews

Title: Bee venom effects on ubiquitin proteasome system in hSOD1G85R-expressing NSC34 motor neuron cells

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Author's response to reviews: see over
Dear Editor-in-Chief of BMC Complementary & Alternative Medicine

Ref: MS 7848617389180323
Title: Bee venom effects on ubiquitin proteasome system in hSOD1G85R-expressing NSC34 motor neuron cells

On the behalf of my co-authors, I would like to thank you for arranging the re-review of our manuscript and for your invitation to submit a revised version. We appreciate the effort of the reviewers, and believe that their constructive suggestions have resulted in a stronger manuscript for readers of the BMC Complementary & Alternative Medicine.

Yours faithfully,

Eun Jin Yang, PhD
Dear Reviewer,

RE: Bee venom effects on ubiquitin proteasomesystem in hSOD1G85R-expressing NSC34 motor neuron cells (MS: 7848617389180323)

We appreciate the minor comments from the reviewer regarding our manuscripts. According to his (her) suggestions, my colleagues and I have made the appropriate changes in a point by point manner. The responses are detailed as follows:

Reviewer 1 Comments to Author:
This study by Kim and co-workers describes the potential therapeutic effect of BV in ALS. In particular they look at BV effect on aggregation of mutant G85R SOD1 and potential degradation pathway. The experiments suggest proteasome dysfunction due to mutant SOD1 protein, and BV restores the function. However, some experiments suggest that BV can have the opposite effect on the control cells. There are several points made that should be pursued rather than commented on.

Major compulsory revisions
1. The method of counting aggregates should be clarified. Figure 2 graph shows % aggregation. What is % aggregation? In the legends, a distinction is made in the scattered filamentous aggregates and condensed aggregates. What are the differences and how are those represented in the quantification? From the representative figures, it is unclear what the scattered filamentous aggregates represent because they are also present in the GFP cells. Does BV affect a specific type of aggregate in the G85R cells?

Answers and revised points>
We inserted the method of cell counting aggregates in the Materials and Methods as reviewer’s comments. The % aggregation is the percentage of aggregated cells among the GFP-positive cell which transfected cells as described in the Materials and Methods. In addition, we combined filamentous and condense forms for quantification of aggregates. Immunocytochemical staining showed that GFP-control presented widespread and diffuse pattern relative to wild-type or mutant G85R hSOD1 cells, whereas wild-type or mutant G85R was observed as cytoplasmic aggregates. Since we counted filamentous and condense forms, we didn’t examine whether BV affects a specific type of aggregate.
We marked the changes in yellow.
2. It is mentioned in the discussion that the effect of BV on reducing aggregation resulting in cell survival should be examined. I think that this is a simple experiment that should be added to this manuscript.

**Answers and revised points**

As a reviewer’s comment, we added the result on cell survival in Figure 2 and changed the discussion. We marked the change in yellow.

3. In figure 3, there is an evident difference in the BV treated cells transfected with GFP. While BV seems to decrease ubiquitination in G85R GFP cells, it increases ubiquitination in GFP transfected cells. This result seems to contradict the claim on BV effect on mutant SOD1.

**Answers and revised points**

As a reviewer’s comment, we observed that BV treatment in only hSOD1G95R-overexpressed cells increased proteasome activity and decreased ubiquitination but not GFP-transfected cells. From this result, we think that BV effects are various responses to expressing proteins because BV consists of a variety of bio-active components including enzymes, peptides, and proteins as described in the Introduction. Therefore, we think that the research needs to determine which component of BV is involved in UPS function in eliminating ubiquitinated hSOD1G95R-GFP protein.

4. The claim of BV not activating autophagy in Figure 4 is confusing. I think the point is made to say that rather than autophagy, proteasome mediated degradation is activated by BV (with the use of ISG15 WB). LC3 II is one marker of autophagy process, but it is a process that is in flux as it fuses with the lysosomes. I think other markers of the pathway should also be checked. It is also unclear compared to controls (WT and GFP) if there is any significant accumulation of LC3 II in the mutant cells. Furthermore, Figure 4 BV reduces LC3 II, but in all cells. It is not explained why that would be or whether that is significant.

**Answers and revised points**

As shown in Fig. 4A, the expression of active LC3 II increased significantly by G85R overexpression compared to WT. It means G85R overexpression induces the activation of autophagy pathway. However, BV treatment reduced the expression of active LC3 II in GFP and WT expressed cells even though it was not significant compared to saline-treated GFP and WT overexpressed cells. Furthermore, BV treatment reduced LC3 II expression increased by G85R overexpression. From those results, we suggest that BV affects UPS function but not autophagy pathway.

As a reviewer’s comment, we will confirm the relevance of autophagy on BV effects using other autophagy markers in the further study.
5. Sumoylation of SOD1 is mentioned as a reason for mutant SOD1 aggregates not being suppressed by BV while proteasome function is activated, not autophagy. Demonstration of sumoylation of mutant G85R in their system should be provided. The claim “BV treatment reduces aggregation of proteins specifically through ubiquitination and not SUMO modification” appears inappropriate. First, the aggregation of mutant SOD1 was not decreased according to figure 2. Sumoylation was not checked, and the competition between ubiquitination and sumoylation is only speculative.

**Answers and revised points**
As reviewer’s comment, we corrected the sentence and marked it yellow.

6. Is this effect specific to G85R mutant? Does it affect other mutants such as G93A, G37R, A4V?

**Answers and revised points**
We have not examined the effect of BV in other mutants (G93A, G37R, and A4V) yet. However, we will investigate BV effects on other mutants in the further study.

7. It is not indicated in the manuscript (methods or elsewhere) how the BV is obtained and how it is applied to cells (vehicle?), except for the concentration used (2.5 ug/mL). It appears that BV contains multiple active compounds that could potentially be relevant for ALS treatment. There is no mention of within this amount of BV used, what is potentially the useful compound, particularly to what they point out in regards to proteasome function and aggregate/protein degradation. From what I understand, in order for BV to be effective a significant amount of bee sting would be needed. In the least, it should be discussed what are the potential compounds in BV that might affect protein degradation.

**Answers and revised points**
We added the description on BV treatment in the materials and method section. In addition, we discussed on a bioactive compound from BV in the discussion.

**Minor essential revisions**
1. A publication [7] is cited as reporting presence of misfolded or ubiquitinated SOD1 in primary MN with different SOD1 mutations. I believe this citation is incorrect, as in [7], there is no such claim.

**Answers and revised points**
We corrected it as a reviewer’s comment.

2. First sentence on pg 4 is unclear. Is it suggesting that SOD1 is degraded by autophagy under circumstances of macroautophagy and lysosomal proteolysis inhibition?
Reviewer 2 Comments to Author:

In this article Kim and co-workers set out to investigate the effect of bee venom (BV) on the protein clearance system in cells expressing either wild-type or mutant SOD1 associated with ALD. They show that SOD1 reduces proteasome activity but this can be restored by BV administration. In addition, they provide evidence that BV causes a reduction in total ubiquitin conjugates and ubiquitinated mutant SOD1. These data support that BV has an effect to rescue UPS-impairment in ALS models. The abstract and introduction explain well the purpose of the work. All the results are also clearly described and concise; they are well-represented in figures, and justified and discussed appropriately. So, I have few minor concerns to be addressed:

1. What is RIPRA buffer (page 12)?

   **Answers and revised points**
   
   We corrected RIPRA to RIPA and marked it in yellow color.

2. If the reduction of misfolded SOD1 protein was consistent, the effect on protein misfolding should be considered.

   **Answers and revised points**
   
   We added on the effect of misfolding SOD1 in the discussion and marked it in yellow.

3. There is lack of details what the crucial focus of the paper, BV. The author should provide its more detailed information, for example, where this is produced and what this contains.

   **Answers and revised points**
   
   We added more detailed information on BV and marked it in yellow color.