Author’s response to reviews

Title: Sesamin Stimulates Osteoblast Differentiation Through p38 and ERK1/2 MAPK Signaling Pathways

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Author’s response to reviews: see over
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*Journal of BMC Complementary and Alternative Medicine*

In-house Editor,
Miss Iratxe Puebla,

Manuscript number 7884870045593009, entitled “Sesamin stimulates osteoblast differentiation through p38 and ERK1/2 MAPK signaling pathways”

We thank the referees for review manuscript and would like to submit a revised manuscript in which we have addressed the comments and criticisms of the reviewers.

**Revised sections consisted of:**

1. **Abstract**
   - Introduction and result were shorten.
   - method section was added (page. 2 line 10-16)

2. **Material and methods**
   - Statistical analysis was analyzed by SPSS software. (page.8 line 8-10)

3. **Results**
   - Because of figure re-organization, the referred number of each picture in manuscript was changed.
   - Results of cell viability and proliferation were revised (page.8 line 15-23)
   - Band density analysis data and the rationale of unfound phosphorylated JNK MAPK protein signaling result was informed. (page.11 line 5-12)

4. **Discussion part**
   The discussion about cell cytotoxicity and proliferation rate effected by sesamin (page. 11 line 23-24, page.12 line 1-6) and competing interest of osteoclast differentiation effected by sesamin treatment and further investigation were added. (page. 14 line 5-9)

5. **Figure and legend**
   - All figures were rearranged as reviewer advice. The detailed of re-organized figures are
     - Figure 1 and 2 were combined as Figure1A-B
- Figure 3-6 were combined as Figure 2A-D
- Figure 7 was changed to be Figure 3
- Figure 8-9 were combined as Figure 4A-B
- The legends of all figures were revised.

Altogether, we upload response compiling to those reviewers’ comments. We sincerely hope that this will be found to be acceptable for publication in the Journal of BMC Complementary and Alternative Medicine and we look forward to an early response.

Sincerely yours,

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Response to reviewer 1
Major Compulsory Revisions

1) The authors described that “sesamin is able to up-regulate OPG and down-regulate RANKL gene expression”, “thus sesamin could control osteoclast differentiation and function” For this the authors should add the data that sesamin inhibit osteoclast differentiation and function in co-cultures of osteoblastic cells and osteoclast progenitor cells. The effect of sesamin on OPG and RANKL is only on gene expression level. The data are not sufficient to the conclusion.

Our response: The effect of sesamin on osteoclast differentiation was performed in preliminary by our colleague, using of osteoclast progenitor derived from peripheral blood mononuclear cells, inducing with osteoclast stimulating factor under the presence of vary concentrations of sesamin (1.25-10 µg/ml) after 7 days treatment. It was found that osteoclastogenesis was inhibited in dose-dependent manner as observed by TRAP staining. (Please keep this attached result as confidential communication) Sesamin effects on both osteoblast and osteoclast revealed the sesamin is bone formation stimulators not only enhance on osteoblast differentiation but also inhibit osteoclast differentiation. Nevertheless, this experiment did not clarify whether RANKL and OPG produced by osteoblast under
sesamin treatment lead to decreasing of osteoclast maturation and function or not. Hence, effects of sesamin on decrease RANKL and increase OPG expression in osteoblast should be elementary result that we will further investigate in co-culture system.

Figure 1. Osteoclast differentiation under sesamin treatment, osteoclast progenitors were cultured in the presence of osteoclast differentiation factors (M-CSF and RANKL) and vary concentrations of sesamin for 7 days. Staining with TRAP (purple) and PCNA (brown) showed that mature osteoclasts (the large multi-nucleated cells stained in purple) were continuously decreased along increasing of sesamin concentrations. The replicate data was shown in each row.

2) The authors concluded that sesamin do not affect cell viability. However, in figure 1 the treatment of sesamin (0.3-20 microg/ml) for 96 hr increased cell viability by 140%. This is significant and the conclusion is not correct. I recommend that they the explanation about this should be described in discussion.

Our response: From MTT assay, although, treatment of sesamin for 96 hr, increased cell viability up to 140%, percentage of cell survival did not increased in dose dependent manner responding to sesamin concentration and also contrasted with alamar blue result in the same treatment condition. Thus, it might not be postulating that sesamin enhanced cell viability. Accompany with the result from 24, 36, and 72 hour treatment, both MTT and alamar blue assay were showed that sesamin did not significally changed cell viability and proliferation compared with control. Therefore, sesamin at 0.3-20 µg/ml did not effect to cell viability or cell death.

3) There were not data of reference gene and protein in figure 8 and 9, respectively. The data have to be added in the figures.

Our response: In western blot experiment, the total form of MAPK proteins (p38, ERK, JNK), which constitutively expressed, were used as references for phosphorylated form.
4) For mineralization experiment the authors have used ADSCs, not hFOB1.19. Why have they used ADSCs? Are the osteoblastic makers in ADSC also induced by sesamin?

Our response: At first hFOB1.19 did not give pronounced alizarin red s staining quality that may be attributed to descending of cell efficiency after many culture passage or inadequate calcium supplement in recommend culture medium (DMEM/HAM F’12) The mineralization assay was performed in ADSCs instead. ADSCs are well study about differentiation capacity as same as stem cells from other sources. Although, we did not accessed osteoblastic marker’s in ADSCs, the positive staining with alizarin red s, dying specific dye for calcium deposition, may suggested that sesamin treated ADSCs is stimulated to differentiated into osteoblast.

In addition, our preliminary study on the effect of sesamin on primary mesenchymal stem cells development (figure.2) could imply the effect of sesamin on the progenitor cell stage. The results of osteoblastogenic markers expression suggested that sesamin enhanced osteoblast differentiation as partly indicated by alteration of osteoblastic gene expression in each stage of cell differentiation.

(Please keep this attached result as confidential communication)
Figure 2. Osteoblastogenic markers gene expression of bone marrow derived MSCs. The cells were cultured in the presence of osteogenic media supplemented with or without 5 µg/ml sesamin for 7, 14, 21 and 28 days. Then the expression of Type I collagen (COL1), Alkaline phosphatase (ALP), Osteocalcin (OCN) and bone morphogenetic protein-2 (BMP-2) were measured.

Minor Essential Revisions

1) I recommend figure rearrangement, for example, figure 1 and 2 could be put together in figure 1; figure 3-6 in figure 2 A-D; figure 8 and 9 in figure 4 A and B.

Our response: All figures were re-organized according to your suggestion.
2) In figure 5 RANKL and OPG indications were wrong. Please confirm them.

Our response: We apologize for our disordered results. The correct information is OPG expression was up-regulated and RANKL expression was down-regulated corresponding to sesamin treatment as mention in page 9.

Discretionary Revisions

The effect of sesamin on the BMP-4 and -7 gene and OC protein level had better to be adde, and Photograph with ALP staining of figure 6 experiment also shown in the figure.

Our response: For this study, we examined the effects of sesamin only on BMP-2, the good representative of BMPs that exhibit the strong osteoinductive and osteoblast differentiation stimulator in all developmental stage since differentiation of MSC to osteocyte, whereas other BMPs such as BMP-6,4,7 play combination role in some stages. [1, 2] For ALP we examined only ALP activity by using enzymatic activity detection assay but not staining assay for this enzyme.

Response to reviewer 2

Reply the comments

Major Compulsory Revisions:

1) Statistical analysis. The authors should state the p values for which a difference is considered significant (*) or highly significant (**). Please re-organize the method section accordingly.

Our response: We re-analyzed our results with using SPSS software. The method section and the asterisks represented for signification levels were modified, that one (*) and two (**) asterisk indicated for p values less than 0.05 and 0.01, respectively. The legend in all of figures, which are statistical- analyzed, were informed.

2) Figure 3 and Figure 4. The sesamin-mediated increase of COL1, ALP and BMP-2, normalized to GAPDH is convincing. The increase of Runx2 and OC is not. I suggest to include more data on SD to differentiate COL1, ALP and BMP-2 expression from Runx2 and OC. The indication p<0.05 is in my opinion not acceptable (please note that * and p<0.05 are values for both BMP-2 and Runx, which behave very differently).

Our response: Although, we interpreted again on the signification level of ALP, COL1, BMP-2, Runx2 and OC, the analyzed data indicated that all of those genes were significantly increased according to sesamin treatment in same p values. We thought that the fold of expression of ALP, COL1 and BMP-2, that seem higher difference than Runx2 and OC, is due to cells are need high amount of mRNA products during bone formation process. In addition, Runx2 can be regulated both at transcriptional and post-transcriptional levels. Despite the minor changed of Runx2 expression by sesamin treatment, this transcription factors should be modulated by phosphorylation stimulation of Runx2 was mediated by MAPK signaling pathway.[1] As the reason for OC expression level, this gene is recognized as late
stage of osteoblast differentiation, [2] thus during early stage of osteoblast development might have low response as well.

3) In all the Figures, please state how many independent experiments (treatment of cells with the indicated concentrations of sesamin) were performed.
   
   Our response: In cytotoxicity and proliferation assay, experiments were performed in triplicate wells, while all other experiments were performed in three independent experiments. The numbers of experiments were informed in figure legends.

4) For legends to Figure 8 and 9 the quantification of the western blottings is required.
   
   Our response: The quantification of band density was analyzed and normalized to total form. The band density values were showed in the figures.

5) In the methods the authors state that used antibodies against p38, ERK, JNK, phosphorylated p38, phosphorylated ERK, and phosphorylated JNK: what antibodies they used for JNK in Figure 8 and 9?
   
   Our response: We also performed experiments for determine the presence of JNK after sesamin treatment by using mAb against phosphorylated as well as other phosphorylated MAPK signaling proteins, We found that there was no JNK expression on sesamin treatment. For that reason, the results in figure8 and 9 illustrated only JNK total form. The absence of JNK activation in osteoblast cells were concordance with other previous researchers reported that osteoblast differentiation was activated through MAPK signaling pathway p38 and ERK but not through JNK activation. [3, 4]

6) Figure 5 indicates, at least apparently, that sesamin induces and increase of RANKL, but a decrease of OPG. Surprisingly, the authors state (results section,
that after 24 hours of treatment, sesamin (at 2.5 and 5.0 µg/ml) up-regulated OPG gene expression, while the expression of RANKL was significantly decreased. (Fig.5). Which is right, the Figure or the text?

Our response: We apologize for our disordered results. The correct information is OPG expression was up-regulated and RANKL expression was down-regulated corresponding to sesamin treatment.

Minor Essential Revisions.

1) Figures 3-5. Why the authors decided to take 24 hour treatment to study the effects of sesamin. What is the effects of sesamin after 24 or 72 hours treatment? This issue is of interest, especially considering that sesamin has no effect on cell growth.

Our response: Because sesamin treatment for 24 hr can enhance the osteoblastogenic genes expression as shown in Figure 3-5, thus we think that results should be adequate for reflected the effect of sesamin on osteoblast differentiation. We hypothesize that sesamin can ameliorate osteoblast differentiation, by increasing ALP activity and mineralization processes. The effect of sesamin on cell growth was reflected by alamar blue assay that sesamin treatment up to 96 hour did not significantly alter cell proliferation rate.

2) Legends to Figures 8 and 9 are unclear and should be changed. Please describe in the legend the experiments and the results, not the interpretation. This should be discussed in the text, or by including the conclusion in the Title of the legend.

Our response: We revised the legend of the figures as your advice.

3) The legend to Figure 5 does not describe what it is shown in the panel. No ratio RANKL/OPG is shown.
Our response: We revised the legend of the figures as your advice.

4) At page 9 the authors state that results on JNK are not shown. Please include these data.
   Our response: The JNK expression was shown only in total form. Because of in all of western blotting experiment, the expression of phosphorylated JNK was not found.

Discretionary Revisions.

1) Comment the possible effects of sesamin on osteoclast differentiation and/or apoptosis and possible future experiments for reaching relevant milestones in this research area. As the author know, the balance osteoclasts/osteoblasts is very important in the therapy of bone-related disease.
Our response: Discussion on this interested issue was added. Our ongoing study is investigating the sesamin effect on osteoclast differentiation that preliminary result from our colleague presented that sesamin had well inhibition effect on this cells differentiation. (Please keep this attached result as confidential communication) However, this result needs more confirmation and the co-culture system will be investigated for the overall scheme of sesamin effect on both bone cells.

Figure 1. Osteoclast differentiation under sesamin treatment, osteoclast progenitors were cultured in the presence of osteoclast differentiation factors (M-CSF and RANKL) and vary concentrations of sesamin for 7 days. Staining with TRAP (purple) and PCNA (brown) showed that mature osteoclasts (the large multi-nucleated cells stained in purple) were continuously decreased along increasing of sesamin concentrations. The replicate data was shown in each row.

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References
