Author's response to reviews

Title: Angelica sinensis Extract Inhibits RANKL-mediated Osteoclastogenesis by Down-regulated the Expression of NFATc1 in Mouse Bone Marrow Cells

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Author's response to reviews: see over
Dear Dr. Armelle T. Mbaveng and Reviewers

On behalf of my co-authors, we thank you very much for giving us an opportunity to revise our manuscript. We deeply appreciate you and reviewers for the positive and constructive comments and suggestions on our manuscript entitled “Angelica sinensis Extract Inhibits RANKL-mediated Osteoclastogenesis by Down-regulated the Expression of NFATc1 in Mouse Bone Marrow Cells” (#MS: 696274612966216).

We have studied these comments carefully and made revisions which are marked in red in the paper (Manuscript with Tracked Changes submission). We have tried our best to revise our manuscript according to the comments, and these changes will not influence the content and framework of the paper. We are confident that the new version of the manuscript is easier to understand and has a more fluent scientific discourse. The point-by-point responses for all these comments are as following:

Referee 1:

Comment:

“Please provide an explanation in the section of Materials and methods for the following:
- On the cytotoxicity assays, BMMs were cultured on the presence of M-CSF. RANKL was not added.
- Different culture times were used, i.e. 4 days for the evaluation of TRAP-positive cells, 3 days for the cytotoxicity assays”

Response: We appreciate this constructive comment and we authors are so sorry for some of the part “Materials and methods” were poorly presented in our manuscript, and thank you for the kind hearted and patient review. Current molecular findings have demonstrated that the differentiation of osteoclasts is mainly depending on two essential cytokines, macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa B (RANK) ligand (RANKL) [1, 2]. M-CSF is crucial...
for providing proliferation and survival signals to osteoclast precursor cells and upregulating RANK expression that is prerequisite for osteoclast differentiation [1]. Since M-CSF, not RANKL, is first stage key stimulate factor for the osteoclast precursor cells proliferation, many previous studies showed the protocol of the “Cell Viability Assay” by only added the M-CSF during the BMMs culture, for the mainly reason is to test the cytotoxicity of the different drugs during the very beginning of the osteoclast precursor cells differentiation [3]. Moreover, RANKL is second stage key factor to stimulate the osteoclast precursor cells for further differentiation, therefore, we evaluated the cytotoxicity assays on day 3 (only M-CSF) and TRAP-positive cells on day 4 (M-CSF+RANKL).

**Comment:**

“- *Use of RAW 264.7 cells in the clonogenic assay*”

**Response:** We used two cell lines and two methods (BMMs for XTT and RAW 264.7 for Clonogenic assay) to evaluated the cytotoxicity of the AS.

**Comment:**

“- *Reason for the use of different concentrations of RANKL in the different experiments, and also for the different time treatments with RANKL*”

**Response:** Because NF-κB is known to be redox sensitive, as well as one of the key transcription factors activated by RANKL during osteoclast differentiation, RANKL also induces the activation of MAPKs, including JNK, ERK, and p38, which are known to play crucial roles in osteoclast differentiation [4] and to be redox sensitive. In response to NF-κB activators, such as TNF-α, the inhibitory protein IκB, which binds to NF-κB and sequesters it in the cytoplasm under resting conditions, is phosphorylated and subsequently degraded by the ubiquitin-proteasome system, thereby allowing NF-κB to translocate to the nucleus and activate gene expression [5]. Therefore, we used different methods and different concentraions of RANKL as previous studies demonstrated [6].
Comment:
“- Use of DMSO in the real time RTP-PCR and Western blot analysis”
Response: DMSO was set as blank-control group.

Comment:
“Specify the details of the experiments in the materials and methods section instead of in the Figure legends
Check the reference list – e.g. references 2 and 8 are the same; references 5 and 10 are the same: references 1 and 17 are the same”
Response: We appreciate your comments and suggestion. To address this concern, we have double checked and proofread our manuscript very closely for the mistakes and revised some part of “Materials and methods” and “References” for more clearly to understand, and we hoped that we have identified and corrected all the spelling and grammatical errors.

Referee 2:
Comment:
“Major comments”
“1. The inhibitory effect of AS on osteoclastogenesis should be confirmed by using a single compound or mixed compounds composed by the combination of single compound.”
“2. Since AS was known as a suppressive activity in inflammatory diseases, this study should be proven by using animal models with inflammatory (collagen, RANKL, LPS or interleukin)-induced osteoporosis to suggest a possible positive modulator of AS against osteoporotic bone diseases, especially in inflammatory-mediated bone destruction.”
Response: We appreciate these constructive comments. It is a very valuable comment.
Angelica sinensis (Oliv.) Diels (AS), a member of the Umbelliferae family, is one of the most popular traditional medicines in China. The AS rhizome contains more than
80 compounds, most of which have been isolated and identified [7]. Among these, ferulic acid, ligustilide, and butylideneephthalide are considered the active ingredients of the AS root [8]. Asian people frequently use AS as a daily dietary supplement because of its health-promoting anti-inflammatory-, anti-cancer-, anti-oxidative-, and anti-hepatotoxict effects, coupled with a unique pleasant flavor. Additionally, our previous study demonstrated that AS extract could inhibit wear debris particles-induced bone resorption by attenuating proinflammatory cytokines. However, as the reviewer’s pointed out, there still has absence of knowledge of the pharmacokinetics of AS on osteoclastogenesis, its metabolism, and whether the effects of AS and/or its products are effective as a single compound or mixed compounds. Further, we will work on investigate the therapeutic mechanisms of AS in the animal model, for more definitive studies of the therapeutic mechanisms of AS and the pharmacokinetics of AS.

Comment:

“Minor points”

1. Reference number 1 and 17 are overlapped and references are not decorated to reference format with some error misprints such as comma and space.
2. Because this study has no data related to osteoclast activation and function, the last sentence of Introduction part is removed or required to modify the sentence.
3. The MS described different concentration of MCSF and RANKL in osteoclast differentiation as mentioned in the section of Abstract and Methods.”

Response: We appreciate these constructive comments and we authors are so sorry for the errors. And we have double checked and proofread our manuscript very closely for the mistakes and revised some part of “Introduction” for more clearly to understand, and we hoped that we have identified and corrected all the spelling and grammatical errors.
Comment:

Major compulsory revisions

TRAP expression alone was shown in the culture. Bone resorption should be shown because it has been reported that TRAP is not an exclusive marker for mouse osteoclasts in cell culture (Modderman WE et al. Bone. 1991;12:81-7).

Response: We appreciate these constructive comments. It is a very valuable comment. In our experiment, osteoclasts were generated from mouse BMMs in the presence of M-CSF plus RANKL to verify the effects of AS extract in osteoclastogenesis, during the experiments we identified those TRAP+ cells were osteoclasts, the methods were as previous studies described [9,10]. Furthermore, we used RT-PCR and Western blot to demonstrated the inhibition effects of AS on different osteoclasts gene expression such as: OSCAR and NFATc-1.

Comment:

Minor Essential revisions

1) ‘Angelica sinesis’ in the 1st paragraph in Discussion should be ‘AS’.
2) ‘Yang et al.’ in the 1st paragraph in Discussion should be ‘we’.

Discretionary revisions

1) The 1st paragraph in Discussion may be described in Introduction.

Response: We appreciate these constructive comments and we authors are so sorry for the errors. And we have double checked and proofread our manuscript very closely for the mistakes and revised some part of “Introduction” for more clearly to understand, and we hoped that we have identified and corrected all the spelling and grammatical errors.

Furthermore, we also declare: No conflict of interest exits in the submission of this manuscript, and manuscript is approved by all authors for publication. I would like to declare on behalf of my co-authors that the work described was original research that has not been published previously, and not under consideration for publication elsewhere, in whole or in part.
Again we would like to express our great appreciation to you and reviewers for comments on our paper, and we would like to resubmit this revised manuscript to “BMC”, and we hope it is acceptable for publication in the journal.

Looking forward to hearing from you soon.

With best regards,

Sincerely yours

Jinyu ZHU

REFERENCES

3. Han Bok Kwak, Byeong Ki Lee, Inhibition of osteoclast differentiation and bone resorption by rotenone, through down-regulation of RANKL-induced c-Fos and NFATc1 expression Bone 46(2010) 724–731


