Author's response to reviews

Title: Apoptosis associated with Wnt / beta-catenin pathway leads to steroid-induced avascular necrosis of femoral head

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Author's response to reviews: see over
Dear Prof Daniele Noel,

We thank you and the reviewers for the insightful and constructive feedback on our manuscript (Manuscript ID:9229815791587902) titled “Apoptosis associated with Wnt / β-catenin pathway leads to steroid-induced avascular necrosis of femoral head”. We have taken the comments and suggestion into consideration and revised our manuscript accordingly. We also attached our point-by-point response to this letter. We hope that our response and revision have fully addressed the reviewers’ concerns and our manuscript is ready for publication.

Thank you again for the positive feedback.

Best regards,

Chen Zhang, MD
Reviewer 1

1. Fig 2 The necrosis was evaluated by “the basis of the diffuse presence of empty lacunae or pyknotic nuclei of osteocytes in the bone trabeculae, accompanied by surrounding bone marrow cell necrosis or myelofibrosis”, furthermore in rat treated with MPS the authors find that “There were large fat cells in the marrow...” and “The bone trabeculae were in disorder and part of them was fractured” and these effect were more important inSFRP-1 treated rat but the histological section showed by Zhang et All in the paper do not illustrate all this change, only empty osteocyte lacuna were showed. I’ll appreciate to see the histopathological staining section of all the femoral head or part sufficient to appreciate the difference between the tree groups. The magnification sowed by the authors does not allow appreciating this effect.

Response: We thank the reviewer for the comment. We have revised our fugues in fig.1 to appreciate the difference between the tree groups.

2. Fig3 A tunnel assay performed on femoral head section was showed. Apoptosis was evaluated by counting the ratio between tunel positive cell and total cell. But never the author says if all the cells were counted (osteocyte, lining cell, and bone marrow cell) or only osteocyte.

Response: We thank the reviewer for the comment. We have revised the sentence in line 147 and now it reads: “Apoptosis was evaluated by counting the ratio between TUNEL positive cells and total cells.”

3. Fig3 All the pictures are focused on trabeculae and it is hard to evaluate what’s happened in bone marrow. Furthermore it is really hard to distinguish the positive cell from negative: picture with better contrast or arrowed positive cell will be a great improve for the quality of this data.

Response: We thank the reviewer for the comment. We have revised our fugues in fig.2 to appreciate the difference between the tree groups. Meanwhile, we have added the arrow to distinguish the positive cell.
4. **Fig3** Secondly, the authors showed some section of 8 weeks of SFRP-1 and MPS treated rat as group C (in fig 2) they evaluate de number of empty lacunae at 30% but in the same group on figure 3 no empty lacuna was see, it will be more convincing if the section used for the illustration was chosen more carefully and show really what it is described in the text. The foto of group B tunel at week 4 seem to over expose for DAB revelation.

Response: We thank the reviewer for the comment. We have revised our fugues in fig.3 to appreciate the difference between the tree groups.

5. **Fig4** Westerns blot showing total b-cat and c-myc are exposed, this assay raise some crucial questions. If in group C we can see a real decease on b-cat expression and c-myc but the lane with sample from group B has no difference with the blot of control rat (groupA), except for c-myc revelation at 4 week.But authoprs quantification show a significant difference between lane A and lane B so please change the western blot picture to fit with the graph result

Response: We thank the reviewer for the comment. We have revised our fugues in fig.4 to appreciate the difference between the tree groups.

6. **Fig4** Furthermore the blot for B-cat and c- myc for week 2 are exactly the same, I ‘am sure that is a mistake than please correct this error.

Response: Sorry for the mistake. We have revised our fugues in fig.4 carefully.

7. Because b-cat is also involved in other bone processes (such cadherin activation) total b-cat may not totally reflect a variation of wnt activity, for that it will be great to improve your data to monitor phosphor b-cat or active b-cat instead of total b-cat

Response: We thank the reviewer for the excellent suggestion. We have added the supplementary experiments to detected activated-β-catenin expression and revised our manuscript in related sections.
8. **Fig 5** An Immunohistochemistry was performed against b-cat and c-myc and the automatic analyze show difference of staining between the tree groups at each point. But once again this result is not visible on the fotos figure; please enhance the quality of the staining to bring more evidence of a diminution of b-cat expression in group B.

**Response:** We thank the reviewer for the comment. We have revised our fugues in fig.5 to appreciate the difference between the tree groups.

9. About the discussion it will be more interesting if the authors have more discussion about the expression of SFRP-1 and other wnt inhibitor such dkk-1, sclerostin which are known to be greatly involved in bone regulation.

**Response:** We thank the reviewer for the comment and great suggestion. We have added the discussion about the expression of SFRP-1 and its backgrounds details in discussion part.

10. **Minor Essential Revisions:** Line 113 “To prevent infection, each rabbit was intraperitoneally injected with 100,000 U of penicillin” I think that authors have committed one mistake making confusion between rat and rabbit.

**Response:** Sorry for the typo. We have deleted “rabbit” in the sentence and now it reads: “To prevent infection, each rat was intraperitoneally injected with 100,000 U of penicillin”

**Reviewer 2**

1. The major limit of the study is the absence of pure quantitative evaluation of apoptosis. Evaluating the number of apoptotic cells, empty lacunae on histological slides are not really quantitative. The quality of the labeling, and mainly the location of the section in the femur sample could influence the number of labeled cells and not be representative of the all tissue. Quantification of caspase activity is probably more relevant to determine the degree of early apoptosis.
Response: We thank the reviewer for the excellent suggestion. We have added the supplementary experiments “caspase-3 activity assay” to quantify caspase activity and revised our manuscript in related sections.

2. Reduction of B-catenin and c-myc targeted gene as quantified by western blotting indicates a modulation of the canonical wnt pathway in the model. However, the expression of other genes targeted by the canonical pathway and involved in apoptosis should be evaluated such as the anti-apoptotic Bcl2 or Bcl-X factors.

Response: We thank the reviewer for the excellent suggestion. We have added the supplementary experiments “Bcl-2 and Bax protein expression detected by immunohistochemistry and Western blot” to detect the apoptosis pathway signaling molecules expression and revised our manuscript in related sections.

3. Minor essential revisions: A proofreading of the manuscript by a native speaker is required.

Response: Thanks for the comments. We have appointed “American Journal Experts (AJE)” editing service company (http://www2.aje.com/) to polish the manuscript.