Author's response to reviews

**Title:** Can the disorder of immune response in bone cause the steroid-induced femoral head osteonecrosis? The investigation on Toll-like receptor 4 signaling pathway

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**Author's response to reviews:** see over
Dear Mr. Reynaldo Aldea Jr. & Dr. Gethin Thomas:

On behalf of my co-authors, we thank you very much for giving us an opportunity to revise our manuscript, we appreciate editor and reviewers very much for their positive, valuable and constructive comments and suggestions on our manuscript entitled “Can the disorder of immune response in bone cause the steroid-induced femoral head osteonecrosis? The investigation on Toll-like receptor 4 signaling pathway”, (ID: 9241816491001632). Those comments are all valuable and very helpful for revising and improving our paper, as well as the important guiding significance to our researches.

We have studied reviewer’s comments carefully and have made revision. The revised portions have been marked in red in the paper. We have carefully considered and studied them and tried our best to make correction and revise our manuscript according to all comments, and we hope meet with approval. According to the comments and suggestions of reviewers, the main corrections have been performed in the paper and the responses to the reviewer’s comments, point to point, listed below. All authors have read and approved the final revised version.

We would like to submit the revised manuscript for your kind consideration and express our great appreciation to you and reviewers for the comments on our paper. We hope the manuscript can be accepted for publication in your journal. Looking forward to hearing from you.

Thank you and best regards.

Yours sincerely,

Kunzheng Wang

List of responds to the reviewer’s comments:

1. Responses to SHUNICHIRO OKAZAKI:
   Comment 1: Could the authors perform RNA isolation and protein isolation with same femoral head? Size of rat femoral head was under 5mm.
   A: We thank reviewer very much for his kind and rigorous comments. In fact, we didn’t use the same femoral head from the same rat to perform RNA isolation and protein isolation. We are so sorry that we didn’t describe clearly in the origin manuscript, and the revision is: “The left femoral heads of all rats were preserved in -70 °C cryogenic freezer immediately after sacrifice, half of which were performed RNA isolation and the others were performed protein isolation, respectively.”

   Comment 2: To my knowledge, TLR4, MyD88, NF-kB and MCP-1 are not cytokines.
   A: We thank reviewer very much for his kind and rigorous comments. We are very sorry for our mistaken writing, and “cytokines” had been modified to “signaling molecules” in the revised manuscript.

   Comment 3: TAK 242 can’t purchase from Takeda Pharm Co.
A: We are very sorry for our negligence of explaining it. In fact, we didn’t directly buy the TAK242 from Takeda Pharm Co. because of some actual difficulties, and we indeed bought it from Haoyuan Chemexpress Co., Ltd., Shanghai, China (The CAS No. is 243984-11-4). This company said that their commercial TAK242 (Resatorvid) was standard and same as the Takeda Pharm Co., and have been registered, extensively used in the experiments of some projects in university. Therefore, in order to highlight this drug, we had marked the Takeda Pharm Co. We express our sincere apologies to you for our mistake. We have modified the real company in our manuscript (Haoyuan Chemexpress Co., Ltd., Shanghai, China).

Comment 4: The authors defined osteonecrosis as the diffuse presence of empty lacunae or pyknotic nuclei of osteocytes in the bone trabeculae, accompanied by surrounding bone marrow cell necrosis or myelofibrosis. However, I could not recognize these findings in Figure 3. Higher power magnification is needed. And histopathological appearance in Figure 3 is different from that of previous reports (Ichiseki et al. 2011, Okazaki et al. 2012, Janke et al. 2013).

A: We are very sorry for our deficiency in the figure 3, and we had change the figures of new and higher power magnification which more clearly demonstrated osteonecrosis according to the reviewer’s comments. Considering the suggestions, we had enlarged the figure 3 to the 200 times magnification. In our study, we had also successfully established the model of osteonecrosis by a simple high-dose steroid injection. Because of the animal and experimental conditions were various, so the appearance observed were more or less different. We think it was suitable as long as if only it showed the femoral head osteonecrosis. According to many scholars’ modeling experiences, the observation of osteonecrosis in Wister rat was better and more obvious than SD rat, but both of rats were all successfully established the model of osteonecrosis. The professors (Ichiseki et al. 2011, Okazaki et al. 2012, Janke et al. 2013) all used Wister rats to copy the osteonecrosis but we used the SD rats due to the limited conditions. Moreover, the objective of this study was to explore the effects of TLR4 signaling pathway in the osteonecrosis, as LPS was the ligand of TLR4, it could have a direct impact on the results of this experiment. Therefore, we couldn’t use LPS and methylprednisolone to establish the model in order to reduce the experimental errors and bias. Thence, we had used a simple high-dose steroid injection to perform the model of osteonecrosis which was different from the previous reports (Ichiseki et al. 2011, Okazaki et al. 2012, Janke et al. 2013). We thought the osteonecrosis were more obvious (including the numbers of empty lacunae and marrow fat cells were more) in the previous reports (Ichiseki et al. 2011, Okazaki et al. 2012, Janke et al. 2013) than ours, probably because of LPS could play some roles in promoting the osteonecrosis. A simple high-dose steroid injection in SD rat could also copy the steroid-induced femoral head osteonecrosis in our study. We have cited the related reports (Ichiseki et al. 2011, Okazaki et al. 2012, Janke et al. 2013) to the reference in our manuscript for their dedication to the osteonecrosis study and the help to us.
Comment 5: Why are TLR4, MyD88, NF-kB p65 and MCP-1 (positive staining, mRNA and protein) of Group A higher than Group N? Group A rats received TLR4 inhibitor, TAK242. Please discuss. It is well known that steroids suppress NF-kB activation.

A: We thank the reviewer’s comments and suggestions about the expressions of the related factors in TLR4 signaling pathway. Both group A and B were all given the methylprednisolone to modeling, and the observation in fact were the expressions of TLR4, MyD88, NF-kB p65 and MCP-1 (positive staining, mRNA expression and protein level) of group A and B higher than group N. Compared with the control group, there were significant differences in group B, and no significant in group A. The reasonable interpretation probably was: TLR4, MyD88, NF-kB p65 and MCP-1 could be activated and overexpressed by a series of signaling pathway, and under the influence of the glucocorticoids, TLR4 signaling pathway was one of an important pathways. TAK242 maybe couldn’t completely resist the factors to overexpress, and we had also observed the actual and significant results in our study. Moreover, the purpose of our study was just only to clarify the TLR4 signaling pathway exists conversely. As is known, the effects of glucocorticoids were multiple and not fully understood, especially used in the case of high doses. Furthermore, NF-kB was also much involved in multiple pathway, and the role was also relatively complex, it couldn’t be said for sure that the use of steroid could completely suppress it, and there were also many references to support that steroid could induce NF-kB and its ligand to overexpress, then the osteoclasts were activated. We can guarantee that the observation in our experiment were true and reliable.

Comment 6: In conclusions, the authors described that TLR4 signaling pathway plays a role in the pathogenesis of steroid-induced femoral head osteonecrosis. It was already reported previously (Okazaki et.al 2009, 2012). There is no priority.

A: We thank reviewer for pointing out the priority of our paper, and we have some explanations for our study. In fact, Okazaki’s research have developed a new rat model of steroid-induced femoral head osteonecrosis that closely mimics the features of human osteonecrosis, and found the femoral head osteonecrosis could be caused by disruption of the systemic immune response via the toll-like receptor 4 signalling pathway. They had used the LPS and methylprednisolone to establish the model, and the observed factors and research perspective weren’t exactly same as ours. They had assessed the consequences of osteonecrosis on femoral head histology, the systemic immune response and lipid synthesis. The histopathological and biochemical analyses were performed, the plasma triglyceride, the total plasma cholesterol concentrations and the plasma concentrations of IL-1β, IL-2, IL-4, IL-6, IL-10, GM-CSF, IFN-γ and TNF-α. The observed times were just from week 1 to week 4 after the last methylprednisolone injection. Thence, the conclusions of Okazaki’s study was LPS and methylprednisolone induced osteonecrosis of the femoral head in rats and this was associated with a disruption of the innate immune system and lipid synthesis. These findings suggest that the TLR4 signalling pathway plays an important role in the pathogenesis of femoral head osteonecrosis.
However, the focus of our work is on further explaining the the pathogenesis of femoral head osteonecrosis via the TLR4 signaling pathway. We used a simple methylprednisolone injection to established the femoral head osteonecrosis, and the detection factors and the experimental methods were also different, so the description perspective was discriminated. We initially confirmed that the TLR4 signaling pathway played an important role in the steroid-induced femoral head osteonecrosis from both positive and negative aspects, and further found and clarified that the disorder of immune response caused by excessive steroid could induce the osteoclasts to excessive activation and proliferation by the effects of abnormal activation of TLR4 signaling pathways, which is very different from Okazaki’s works, and this was also the major contribution of our work. We have cited the related reports (Okazaki et.al 2009, 2012) to the reference in our manuscript for their dedication to the osteonecrosis study and the help to us.

Comment 7: More references are needed in discussion.
A: We thank the reviewer’s comments and suggestions about the description in discussion. It is really true as reviewer suggested that more references are needed in order to better support our study. Considering the reviewer’s suggestions, we have added more related references in the discussion, and all had been marked in red.

Comment 8: Quality of written English: Needs some language corrections before being published.
A: Thank you very much for your kind advice, we are very sorry for our quality of written, and we have polished and corrected the language with the help of a professor of English.

2. Response to Xinghuo Wu:
Comment: Quality of written English: Needs some language corrections before being published.
A: Thank you very much for your kind advice, we are very sorry for our quality of written, and we have polished and corrected the language with the help of a professor of English.

3. Responses to additional reviewer:
Comment 1: Groups A and B, and in “Abstract”groups A and B, and Group N in the section of” Experimental protocols”!How to label the groups? The author should make the group consistency and standardized. And the reader can’t understand the meaning of Group A and B in the abstract.
A: We are very sorry for our negligence of the description of the groups’ labels in the abstract. It is really true as reviewer’s suggestion and we have made the groups consistency and standardized in the manuscript according to the reviewer’s comments. The groups are as follows: group A is the treatment group, group B is the model group and group N is the control group.
Comment 2: Dose the drug TAK242 play the same effect on human and rats/?
A: We are very sorry for our negligence of not enough explaining the drug of TAK242. Rats possess 90 percent genes similar to humans’, and many studies of drug or diseases had been selected the rat model to make further research. The objective of our study was just to prove an existence of the TLR4 signaling pathway, and the research didn’t focus on the drug in current study. Maybe the TAK242 didn’t play the same effect on human and rats absolutely, but according to the previous studies, as the pharmacology and pharmacokinetic studies in animal models, it had a very high degree of similarity between human and rats, and we ensured that we will make further research and exploration about the optimal dose and pharmacokinetics of TLR4 antagonists in the rat model.

Comment 3: Fig. 2 Compared with the group N, the concentrations increased significantly in the group B, but no significant in the group A. I can’t understand the meaning of the sentence. According to the Fig 2, the concentrations of TRAP were significantly increased in group A, compared to Group N, such the time points week 10, week 12. But the authors described “….. but no significant in the group A.” Very confused.
A: We are very sorry for our negligence of explaining the results, the Fig.2 was really correct: Though the TRAP concentration in the treatment group increased than the control group, but the difference had no significant (P>0.05). The revision had attached in the blow of Fig.2 in red. We are very sorry for our incorrect writing in our manuscript.

Comment 4: Fig.3 Please label pathological alteration of trabecular osteonecrosis. The figures and legends should be consistency. I can see new bone formation in both Group A and Group B, how to explain?
A: We are very sorry for our negligence of explaining the results. Considering the Reviewer’s suggestion, we have labelled the pathological alteration of trabecular osteonecrosis in figure 3 and made the figures and legends consistent. According to the previous related studies, it is reasonable that the process of bone repair and bone formation also started when the bone destruction appeared, so the new bone formation were observed, furthermore, when the destruction of bone resorption exceeded bone formation, the osteonecrosis would happened.

Comment 5: Fig.4 The figures should be in high quality. How to get the data of the statistical charts. The author should provide the methods
A: It is really true as reviewer suggested that the quality of fig.4 weren’t high, and it probably because of the low magnification (100×). The observation was also unclear. Considering the reviewer’s comments, we have revised and changed the new magnification of figure 4 at 200 times. Furthermore, all data were analyzed according to the average area of positive staining in each graph completely, and the analyses of integrated optical density were performed using IPP 6.0 image analysis software. They just only reflected the qualitative expression of each factor.
Comment 6: The author should describe the results more specific, and should explain the results. In many parts, the authors just described the results. The readers want to get more information from the presentation.
A: We are very sorry for our negligence of explaining the results, and it is really true as reviewer suggestions. We have re-written some results according to the reviewer’s suggestion and added the specific descriptions and explanations. All revised portion have been marked in red in the revised manuscript.

4. Responses to editorial comments:
Comment 1: Please include a 'Competing interests' section between the Conclusions and Authors' contributions. If there are none to declare, please write 'The authors declare that they have no competing interests'.
A: We thank the editorial comments and have added the ‘competing interests’ section between the ‘Conclusions’ and ‘Authors contributions’, and all had been marked in red.

Comment 2: Please include an 'Authors' contributions' section before the Acknowledgements and Reference list.
A: We thank the editorial comments and have added the ‘Authors contributions’ section before the ‘Acknowledgements’ and ‘Reference’, and all had been marked in red.