Author's response to reviews

Title: Effects of pamidronate disodium on the loss of osteoarthritic subchondral bone and the expressions of cartilaginous and subchondral OPG and RANKL in rabbits

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Version: 4 Date: 9 September 2014

Author's response to reviews: see over
Dear Dr. Roman Krawetz,

Thank you very much for your and the reviewer’s critical comments and thoughtful suggestions. We are pleased to know that our study is of general interest for BMC Musculoskeletal Disorders. We have revised the manuscript entitled “Effects of pamidronate disodium on the loss of osteoarthritic subchondral bone and the expressions of cartilaginous and subchondral OPG and RANKL in rabbits” (MS: 2824172881355222) in line with your and reviewer’s comments. All changes made to the text are in red so that they may be easily identified. We have asked for native English speakers to revise the paper before it was submitted this time. We don’t know whether it has reached your magazine’s standard. In addition, we have made the background and discussion more streamlined and straightforward and added the positive control. Furthermore, we have revised the title page at the front of the manuscript and added more details to all figure legends.

Once again, we acknowledge your comments and constructive suggestions very much and deeply appreciate your consideration of our manuscript for publication in BMC Musculoskeletal Disorders. If you have any queries, please don’t hesitate to contact me at the address below. Thank you and best regards.

Xiang-yang Chen
September 8, 2014

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Version: 4 Date: 5 September 2014

Author's response to reviews: see over
Thank you for consideration of our manuscript for publication in your journal.
We have reviewed the manuscript according to your reviewer’s comments. The following are our point-to-point responses to the reviewers’ comments.

Responses to Reviewer 1

Reviewer's report
Title: Effects of pamidronate disodium on the loss of osteoarthritic subchondral bone and the expressions of cartilaginous and subchondral OPG and RANKL in rabbits
Version: 3 Date: 5 August 2014
Reviewer: yin-gang zhang

Reviewer's report:
Osteoarthritis is a common disease in the elderly, at the early stage, main treatment are physical therapy and medication, at the lately, to be joint replacement surgery et al.. Authors adopted an animal model, to use Micro-CT, Safranin O and rapid green staining, immunohistochemistry, and Western blotting techniques, to investigate the effects of pamidronate disodium (PAM) on bone loss and the expression of OPG and RANKL in anterior cruciate ligament transection (ACL T)-induced osteoarthritis. Their conclusion is that PAM can significantly inhibit and even reverse osteoarthritic subchondral bone loss, thus alleviating the process of cartilaginous degeneration. And The mechanisms might be associated with its upregulation of OPG expression and inhibition of RANKL expression, thus increasing their ratio.

Major Compulsory Revisions

1. Background:
a. Background is sort of lengthy, to need to simply.
Thank you for your suggestions. We have simplified the background section of the paper to be more streamlined and straightforward.

We have already combined the first two paragraphs of this Section and deleted some repeated statements to highlight the importance of subchondral bone on the occurrence and development of OA.

We now change the description of OPG and RANKL to deepen relevant aspects of these molecules correlate with OA.

Page 3, Line 19: “OPG, which is also produced by the osteoblasts, acts as a soluble decoy receptor for RANKL; by interacting with RANKL, it prevents RANK activation and subsequent osteoclastogenesis, resulting in the inhibition of bone resorption [14]”

Page 3, Line 22: “OPG was transferred onto cartilage explants, resulting in a marked decrease in aggrecan cleavage and cartilage proteoglycan release, demonstrating the role of OPG in the regulation of cartilage catabolism [1].”

In the description of the PAM treatment of OA, we have deleted some lengthy statements and strengthen the description of the aim in our study.

Page 4, Line 13: “Therefore, drug intervention after the formation of early OA will be more clinically meaningful”

Page 4, Line 15: “whether PAM is able to prevent or reverse the progress of this early-stage OA has not been investigated”

b. Osteoarthritis of the mechanism would be briefly summarized, and propose the theory of PAM treatment of osteoarthritis.

We are grateful for your sincere suggestions. The mechanism of OA has been summarized to be more concise and now appears as follows:

Page 3, Line 1: “Osteoarthritis (OA) is a degenerative joint disease that is characterized by progressive cartilaginous degeneration, osteophyte formation, subchondral bone changes, and synovitis inflammation [1,2]. Although cartilage lesions are the main feature of OA, but increasing evidence indicates that it is also a bone disease [3,4]”

We now propose the theory of PAM treatment of osteoarthritis and appears as follows:

Page 4, Line 1: “As the goal of today’s medicine shifts more towards disease
early prevention rather than disease advanced treatment, subchondral bone might represent an attractive candidate for a therapeutic target for osteoarthritis. Therefore, Pamidronate disodium (PAM) and other bisphosphonates, bone-loss inhibitors, might be potential therapeutic drugs for OA [17].

2. In Materials and methods, grouping and sampling time, animals numbers were confused, please use the work flow chart.
   - We are so sorry to say that we did not realize this point. Thank you for your suggestions, we have added “a work flow chart” in the Animals and study design section (Page 5, Line 27).

3. Results:
   a. Figure 3, ACLT 4W, comparison with the other figures, is clearly not the same multiple.
      - We must apologize for being so careless. We have chosen the same multiple images for Figure 3E.

   b. Figure 7, RANKL immunohistochemistry, PAM-S and PAM-L group showed thinner cartilage, why?
      - Thank you for your sincere reminding. We apologize that the two images were confusing. We observe the sections which were incubated with antibodies against RANKL again, and find no significant decrease in the thickness of cartilage in PAM group compared with other groups. We have chosen the better quality image in PAM-L group this time for Figure 7E.
Responses to Reviewer2

Reviewer's report

Title: Effects of pamidronate disodium on the loss of osteoarthritic subchondral bone and the expressions of cartilaginous and subchondral OPG and RANKL in rabbits

Version: 3
Date: 11 August 2014
Reviewer: Giuseppe Musumeci

Reviewer's report:

1. General comment

a. First of all please revise the punctuation and space between the words throughout the text and add the appropriate abbreviations in all text (the PAM meaning must be specified in the introduction and not only in the abstract).

   • Thank you for your helpful comments and advice. We have revised the punctuation and space and added the appropriate abbreviations throughout the text.
   • the PAM meaning has been specified in the introduction and now appears as follows:

   Page 4, Line 3: “Therefore, Pamidronate disodium (PAM) and other bisphosphonates, bone-loss inhibitors, might be potential therapeutic drugs for OA [17]”

b. Please re-read the text carefully, there are some careless mistakes on the English language.

   • Thank you for your sincere reminding, and we have asked for native English speaker to revise our paper. Nevertheless, we don’t know whether it has reached to your standard.

c. Please strengthen better the aim of your study in the abstract and in the background section.

   • We are grateful for your sincere suggestion.
   • The following statements now appear in the first paragraph of the abstract of the paper:

   Page 2, Line 2: “Osteoarthritis (OA) is major health problems in the
increasing elderly population. It is crucial for the prevention and treatment of OA at the early stage. The present study investigated whether pamidronate disodium (PAM), a bone-loss inhibitor, can significantly prevent or reverse the progress of the early anterior cruciate ligament transection (ACLT)-induced osteoarthritis, and whether the therapeutic intervention will be associated with the regulating expression of osteoprotegerin (OPG), receptor activator of nuclear factor-k B ligand (RANKL), metalloproteinase-9 (MMP-9) and Toll-like receptor-4 (TLR-4) in cartilage and/or subchondral bone

- In the Background section, The statements now appears as follows:

Page 3, Line 24: “Thus, the roles of these cytokines secreted by the chondrocytes in the onset and progression of subchondral bone changes in OA are suggested but remain to be investigated”

Page 4, Line 9: “As the goal of today’s medicine shifts more towards disease early prevention rather than disease advanced treatment, subchondral bone might represent an attractive candidate for a therapeutic target for osteoarthritis. Therefore, Pamidronate disodium (PAM) and other bisphosphonates, bone-loss inhibitors, might be potential therapeutic drugs for OA”

Page 4, Line 13: “Therefore, drug intervention after the formation of early OA will be more clinically meaningful. Studies in this area are rare, and whether PAM is able to prevent or reverse the progress of this early-stage OA has not been investigated. In addition, If there were any effects, would they be associated with their mediations of the ratio of OPG to RANKL, MMP-9 and TLR-4 in cartilage and/or subchondral bone?”

- Please add also the MMP-9 and TLR-4 in the aim and in the conclusion of the study. It seems that these results were added later and weren’t present in all text.

- The reviewer’s comment is true, MMP-9 and TLR-4 were studied as a supplemental description to the therapeutic effects of PAM. We have added the MMP-9 and TLR-4 in the aim and in the conclusion of the study and the statements now appears as follows:

Page 2, Line 6: “whether the therapeutic intervention will be associated with the regulating expression of osteoprotegerin (OPG), receptor activator of nuclear factor-k B ligand (RANKL), metalloproteinase-9 (MMP-9) and Toll-like receptor-4
(TLR-4) in cartilage and/or subchondral bone”

Page 2, Line 25: “The mechanisms might be associated with its upregulation of OPG expression, and downregulation of RANKL, MMP-9 and TLR-4 expressions”

Page 14, Line 7: “This protection occurred through the mediation of the upregulation of OPG expression and inhibition of RANKL, MMP-9 expressions in the cartilage and subchondral bone, and TLR-4 expression in the cartilage”

2. Abstract
a. Please reformulate better the abstract adding the suggestion above.

• Thank you for your suggestions. We have simplified the background section of the paper to be more streamlined and straightforward and made some improvement based in line with the suggestions in “General comment” section.

b. Please reformulate the following sentence of the results section with the correct verbs: “Micro-CT, Safranin O and rapid green staining analyses indicated that PAM treatment for two or ten weeks could completely prevented or reversed osteoarthritic subchondral bone loss and cartilage surface erosion..”

• Sorry for our careless. Change made as indicated by the reviewer on Page 2, Line 18.

3. Background
a. Please reformulate this section, the description of OPG and RANKL must be improved and updated. The authors should deepen different recent and relevant aspects of these molecules correlate with OA. I recommend seeing the following recent and interesting suggested paper and commenting them: (e.g. “In rat with glucocorticoid-induced osteoporosis, RANKL is downregulated in bone cells by physical activity (treadmill and vibration stimulation training). Histol Histopathol. 2013;28:1185-1196”. – “The effects of physical activity on apoptosis and lubricin expression in articular cartilage in rats with glucocorticoid-induced osteoporosis. J Bone Miner Metab. 2013 May;31(3):274-84”.

• We are grateful for your sincere suggestion. We have studied the two articles and reformulated the description of OPG and RANKL completely.
• Improved and updated description of OPG and RANKL section appears as follows:

Page 3, Line 16: “A molecular composed of OPG/RANK/RANKL has been described as a critical system for controlling osteoclast biology. RANKL, synthesized by osteoblasts, binds to the RANK on the osteoclast precursor membrane, and is an essential factor for osteoclast differentiation and bone resorption [12,13]. OPG, which is also produced by the osteoblasts, acts as a soluble decoy receptor for RANKL, by interacting with RANKL, it prevents RANK activation and subsequent osteoclastogenesis, resulting in the inhibition of bone resorption [14]”

• Deepen different recent and relevant aspects of these molecules correlate with OA section appears as follows:

Page 3, Line 14: “Subchondral bone mass is maintained through a balance between formation and resorption during the development of OA. In the bone resorption process, regardless of the pathology, the osteoclast is the exclusive resorptive cell but osteoblasts[11]”

Page 3, Line 21: “It was recently reported that OPG was transferred onto cartilage explants, resulting in a marked decrease in aggrecan cleavage and cartilage proteoglycan release, demonstrating the role of OPG in the regulation of cartilage catabolism [1]”

b. Also please add some information about pamidronate disodium (PAM), other bisphosphonates, MMP-9 and TLR-4 protein.

• Thank you for your helpful advice, we have added some information about pamidronate disodium as follows:

Page 4, Line 4: “Kadri A et al have ever proposed that the level of bone resorption influences cartilage metabolism and that inhibition of pamidronate might prevent the progression of OA [18]”

• some information about other bisphosphonates appears as follows:

Page 4, Line 9: “Additionally, in the latest study indicated that Zoledronic acid, in a high-dose regimen, proved to be chondroprotective in a well-established animal model of OA [20]”

• and some information about MMP-9 and TLR-4 protein appears as follows:

Page 3, Line 26: “Recent study has shown that cartilage damage of OA is
caused by the disruption of a shift in the balance between catabolic and anabolic capacities of chondrocytes [15]. Catabolic activities of OA chondrocytes are related to the elevated release of cartilage degrading enzymes, such as matrix metalloproteinases (MMPs) [16], while in anabolic activities, toll-like receptor 4 (TLR4) has been shown a crucial role in inflammatory signalings in human chondrocytes [15].

4. Materials and Methods

a. Please specify the manufactures used for antibodies, kits, equipment and other chemicals with their correct addresses in all materials and method section.

- The manufactures used for antibodies, kits, equipment and other chemicals have been specified with their correct addresses in all materials and method section.

b. Animals and study design section

Line 10, please replace fixed.

- Thank you for your reminding. We have chosen the “positioned” instead of “fixed” on Page 5, Line 5.

Line 14, please explain better the ACLT technique.

- Change has been made as indicated by the reviewer and the statements now appears as follows:

  Page 5, Line 9: “A medial parapatellar incision was made. After the patella was dislocated laterally, the knee was flexed maximally so that the anterior cruciate ligament could be readily visualized and identified. It was then transected with a No. 12 blade. An anterior drawing test was performed gently to confirm that the ACL was transected completely. The joint was irrigated with sterile saline and closed. The joint capsule was closed with a running suture of 4-0 nylon, and the skin incision was closed with running mattress sutures of 3-0 nylon.

Line 18, please correct the font size.

- Sorry for our careless. We have corrected the font size on Page 5, Line 15.

c. Histology and OARSI score section
Lines 18, 19 please fix the symbols.

- Sorry for our careless. We have corrected the symbols on Page 6, Line 14 and 15.

Line 20, please add cartilage samples.

- Thank you for your reminding. Change has been made as indicated by the reviewer on Page 6, Line 16.

d. Immunohistochemical localization of OPG and RANKL section
Line 27 please add cartilage and subchondral bone sections

- Thank you for your reminding. Change has been made as indicated by the reviewer on Page 6, Line 24.

Please add the positive control

- Thank you for your helpful comments and advice. We have added the positive control and the negative control in additional file 1 and the statements now appears as follows:

Page 6, Line 29: “On negative control slides, non-immune goat serum was substituted for the primary antibody. On positive control slides, the brown-yellow precipitate was developed as the final product (see Additional file 1)”

e. Why did the authors not analyze also MMP-9 and TLR 4 protein in both cartilage and subchondral bone? Please explain it or add these results to strengthen the findings to better readers understanding. Please add the relative results and figures about these two proteins.

- Special thanks to you for your good comments. We are grateful for your sincere suggestion. After careful discussion, we consider that the purpose of this study on MMP9 and TLR4 is to measure the amount of the proteins expressions in cartilage and subchondral bone and then investigate the change regularity of the proteins expressions in cartilage and subchondral bone after PAM treatment. Of course, Immunohistochemistry can more sensitively reflect the distribution of the proteins expressions and be more conducive to investigate the treatment mechanism of PAM. Thus, we would make an intensive study of MMP-9 and TLR 4 protein in the future research. Once again, We thank the reviewer for drawing our attention to it.
5. Discussion

a. Page 12, line 27 the word “osteoporosis” is in different font, please fix it.

- Sorry for our careless. We have corrected the font on Page 13, Line 7.

b. The discussion is too long and boring, please shorten this section.

- Thank you for your suggestions. We must apologize that we did not do well. We have simplified the discussion section of the paper to be more streamlined and straightforward.
- We have reformulated the occurrence mechanism of OA and deleted some lengthy statements.

Page 11, Line 8: “Some studies indicate that increased subchondral bone remodelling could lead to bone resorption initially [1,28]. Mechanically, the subchondral bone supports the overlying cartilage and absorbs the forces transmitted by the joint. Mineralization of the subchondral bone and increased rigidity reduce its buffering and load balancing capacities and accelerate the cartilage covering process [3,29]. However, some scholars have posited that changes in subchondral bone could exist during or after cartilaginous degeneration [10]. Therefore, the development of OA originates from cartilage or subchondral bone alterations remains controversial.”
- We have deleted a lot of discussion related to the ACLT-induced OA model and just introduced the clinical relevance of the model.

Page 12, Line 9: “We considered the ACL transection rabbit model for the study of cartilage lesions, as it reproduced all of the OA-associated lesions in arthrosis. Moreover, the ACLT model has a significant risk factor for development of post-traumatic osteoarthritis (PTOA), which lose the proteoglycan and collagen molecules from cartilage in the first few weeks after anterior cruciate ligament injury [31]”
- We have reformulated the molecular mechanism of PAM, and added some information about bisphosphonates, MMP-9 and TLR-4 protein and have deleted some lengthy statements.
- Some information about bisphosphonates appears as follows:

Page 11, Line 23: “Although some animal experiments have shown beneficial effect of bisphosphonates on subchondral bone and prevent the progression of OA, there is no study evaluating a selected sample of patients with early OA due to
ethical reasons. In a 2-year trial, risedronate, also a member of bisphosphonate family, decreased biochemical markers of cartilage degeneration and partial symptoms, but did not prevent the radiographic progression of the advanced OA.”

- some information about MMP-9 and TLR-4 protein appears as follows:

  Page 13, Line 16: “MMP-9 was considered an important class of proteinase that can degrade all components of the complex extracellular matrix in terms of cartilage degradation [16]. Furthermore, many studies have shown that upregulation of MMP-9 results in destruction of articular cartilage of OA patients [35,36].”

  Page 13, Line 28: “Thus, we considered the results at least provided an additional explanation regarding the control of joint inflammation and mild degeneration in the knee joint of the rabbit after PAM treatment”.

c. Moreover, in the conclusion add the results finding about MMP-9 and TLR-4 protein in both cartilage and subchondral bone.

- Thank you for your reminding. Change has been made as indicated by the reviewer on Page 14, Line 8.

d. Clinical relevance of your work and some important suggestions for the scientific community must be added.

- Thank you for your suggestion. We have added the clinical relevance of our work and appears as follows:

  Page 12, Line 7: “These results indicate that PAM treatment stopped bone loss in the subchondral bone and even reversed the pathological lesions on the subchondral bone after long-term therapy.”

  Page 12, Line 10: “the ACLT model has a significant risk factor for development of post-traumatic osteoarthritis (PTOA), which lose the proteoglycan and collagen molecules from cartilage in the first few weeks after anterior cruciate ligament injury [31].”

  Page 12, Line 27: “Thus, these data provide further support that the subchondral bone loss and cartilage lesions were closely relevant and the improvement of microstructure and remodelling at ACLT-induced subchondral bone following the treatment with PAM, initiated at 4 weeks after joint surgery, would markedly contribute to avoid the progression of cartilage damage in our experimental
model.”

Page 13, Line 28: “Thus, we considered the results at least provided an additional explanation regarding the control of joint inflammation and mild degeneration in the knee joint of the rabbit after PAM treatment.”

- We have added some important suggestions for the scientific community and appears as follows:

Page 11, Line 23 and 30: “Although some animal experiments have shown beneficial effect of bisphosphonates on subchondral bone and prevent the progression of OA, there is no study evaluating a selected sample of patients with early OA due to ethical reasons.

Thus, further studies are necessary to evaluate the importance of timing of treatment with anti-resorptive agents for improved treatment at the early stage of OA.”

6. Figure legends
a. Please reformulate and add more details to all figure legends.

- We are grateful for your sincere suggestions. We have reformulated and added all the information that was available to all figure legends.

b. Please use capital letters to explain each image in figures 1 and 3

- Thank you for your reminding. We had use capital letters to explain each image in figures 1 and 3 and appears as follows:

Page 18, Line 6: “Figure 1 Experimental design to study the effect of PAM on subchondral bone loss upon the progression of the early ACLT-induced osteoarthritis. (a) Experimental scheme. (b) Typical Micro-CT images selected of a highly representative subchondral bone sample for each group. (A,B) Sham-operated group, (C) short term treatment with PAM, (D) long term treatment with PAM, (E-H) ACLT-induced osteoarthritis at 2, 4, 6 and 14 weeks. Marked differences in the microarchitecture of subchondral bone are observed.”

Page 18, Line 20: “Figure 3 Effects of PAM treatment on cartilage surface erosion. The sections were stained with safranin-O (red stain) for glycosaminoglycans,
rapid green for bone. (A) The Sham group, no erosion-like changes in the cartilage surface. (B,C) short term and long term treatment with PAM group, compared with the ACLT group at 6 and 14 weeks, chondrocytes and matrices in the PAM group were significantly increased, fibroses of the cartilage surface were significantly reduced. (D) ACLT group at 2 weeks, no significant changes were observed in the cartilage surface. (E) ACLT group at 4 weeks, staining of safranin O had been partially lost and various degrees of surface fibrosis, even cracks, cell loss, cell cloning were observed in the surface layer of the hyaline cartilage. (F,G) ACLT group at 6 and 14 weeks, chondrocytes of the hyaline cartilage surface were massively reduced, fibroses were extensive, osteophytes had formed, vertical fractures had increased and safranin O staining in the cartilaginous area was significantly reduced. Bar represents 200 µm.”

c. Please revise the symbols in figures 5a and 5b.
- Thank you for your reminding. We have revised them accordingly.

We tried our best to improve the manuscript and made some changes in the manuscript. These changes will not influence the content and framework of the paper. And here we marked in red in revised paper. We appreciate for Editors/Reviewers’ warm work earnestly, and hope that the correction will meet with approval. Once again, thank you very much for your comments and suggestions.