Author's response to reviews

Title: Does Low-Intensity Pulsed Ultrasound Treatment Repair Articular Cartilage Injury? - A Rabbit Model Study

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Author's response to reviews: see over
Dear Editor of BMC musculoskeletal disorders

We would like to submit this revised manuscript entitled “Does Low-Intensity Pulsed Ultrasound Treatment Repair Articular Cartilage Injury? – A Rabbit Model Study” by Prof. Dr. Shan-Wei Yang, Dr. Chien-Lin Kuo, Professor Shwu Jen Chang, Professor Po-Chou Chen, Yen Ting Lin, Professor Ioannis Manousakas and Professor Shyh Ming Kuo for publication in BMC musculoskeletal disorders. We designed a study to investigate the effect of LIPUS on articular cartilage repairing in a rabbit cartilage injury model. The manuscript is written and formatted in accordance with the latest Instructions for authors.

We have studied these valuable comments very carefully and have made revisions and corrections accordingly. And, we hope that they would meet your approval. The reviewers’ comments were answered following:

# Response to comments of reviewer : Caroline Hoemann

**Question 1:** Methods: Please give the age in months post-natal (12 weeks), of the rabbits used in the study. It is stated that “matured” rabbits (near matured, control group can compared) were used, but the kg weight suggests they were skeletally immature, less than 6 months old. It is important to report the actual age of the rabbits, and whether the rabbits are skeletally immature.

**Answer:** Experimental rabbits in this study were post-natal 12 weeks. Even near-matured, not matured rabbits were selected in the study, control group was designed in the study to compare. It still can explain the difference between the experimental and control group.

**Question 2:** Statistical tests: the one-way ANOVA test is not appropriate for the non-parametric histological scoring data, a non-parametric statistical test should be used (i.e., Mann Whitney U).

**Answer:** It was a mistake of statistical test. We have corrected the statistical test as a Wilcoxin signed rank test to analyze the Mankin score in the revised manuscript.

**Question 3:** How far away was the RF source (LIPUS) from the condyle target tissue?

**Answer:** Because the LIPUS need a jelly as a medium to transduce, the source of LIPUS was applied on the skin of knee joints covered by jelly.

**Question 4:** Burst microwave radiofrequencies at 1.5 MHz, at 30 mW/cm2, may have different effects if the condyle were placed immediately adjacent to the source, or around 1 meter away, where most hand-held RF meters are able to register a peak signal. How did the authors decide on the particular exposure frequency and duration?
Answer: Yes, it is difficult to determine the actual exposure dose of articular surface. However, in our study the machine of LIPUS was a commercial product. The energy of LIPUS was fixed as 30 mW/cm². We did not test the effect in different doses. It is a limitation in our study. We have described this limitation in the revised manuscript.

Question 5: Is it possible that the tissue “FT” in panel C of Figure 5 is simply incompletely debrided cartilage?
Answer: Fibrous tissue (FT) was a new formed tissue above the residual cartilage and it was not a cartilage tissue under the histological exam.

Question 6: Did the authors examine other rabbit initial defects to ensure that all cartilage was fully removed with no remnants?
Answer: Yes, we have tested MRI for all experimental rabbits to ensure the defect after surgical removal of cartilage.

Question 7: Can the authors perhaps explain better why was only the cartilage removed?
Answer: In our study, we want to evaluate the cartilage repair in “severe” chondral injury. So we designed a rabbit model to present only deep cartilage loss of knee joints without subchondral bone damage to control study, which mimicked severe chondral injury clinically.

Question 8: If pulse frequency was shown to have an inflammatory effect on bone fracture repair, then perhaps a better model would have included microfracture or marrow stimulation in the cartilage defect
Answer: Yes, LIPUS may be stimulate the subchondral bone to induce unknown effect, such as repair of cartilage or osteo-formation. However, no significant chondral repair was found in the result of our model. It may be need another design and study to test above hypothesis.

Question 9: Pulse frequency emission propagates more than 2 feet, they explained that the rabbits were kept 2 per cage, and most probably grouped in one larger cage support;
Answer: It might be more suitable that each rabbit was housed in each cage. However, the cages were limited in our laboratory at that time. We just described the fact of study. In fact, the cages used in the study were big sized, 120x80x80 cm with enough space for movement of rabbits.

Question 10: therefore, if the rabbits were exposed to the 20 minutes of pulse frequencies daily for 3 months while in their cage, it is possible that they had no truly untreated rabbits in their study.
Answer: Treatment of LIPUS was performed outside the cage. All rabbits can move freely in the cages and move outside the cage for 30 minutes everyday.
**Question 11:** It would also be interested to ask as a non-mandatory question, whether they saw any other biological effects of the pulse frequencies on repair of the arthrotomy suture site, or the joint. Their title may not have the right term, "ultrasound" should probably be changed to "microwave".

**Answer:** LIPUS is different from microwave. LIPUS is a kind of ultrasound with low intensity, not microwave.

#Response to comments of Reviewer: Kiyohito Naito

**Question 1:** This study investigates the effect of low-intensity pulsed ultrasound (LIPUS) for repair articular cartilage and bone defect in animal experimental model. This animal model is not suitable for evaluation of the cartilage repair capacity, because there are no chondrocytes in articular cartilage layer.

**Answer:** Severe chondral injury with loss massive chondral and deep to subchondral bone was a common problem clinically. Our animal model was presented this kind of injury. We want to clarify the repairing effect of injured side after LIPUS treatment, even no remnant chondrocytes in injured zone. It is the soul of our study.

**Question 2:** This study is not appropriate for a discussion of cartilage repair. As you know, LIPUS enhances the endochondral ossification by promoting chondrocyte proliferation and chondrocyte activities. However, in your animal models, there are no chondrocytes in articular cartilage layer. Because your model has defect not only articular cartilage but also subchondral bone. Of course LIPUS can not play role to repair cartilage via chondrocyte. Although, we can see fibrotic tissue in the defect in with-LIPUS-treatment group, this result is due to the promotion of bone formation by LIPUS.

**Answer:** We designed the study to test the repairing effect of severe chondral injury under LIPUS treatment, which was different from mild chondral injury in previous studies. In the result, no significant therapeutic effect and only fibrotic tissue in the defect were observed. Although the reviewer considered it was a simple concept, the study gave a strong evidence base to clarify the effect of LIPUS in severe chondral injury under this animal study.

# Response to Associate Editor's Comments:

**Question 1:** The author evaluated the cartilage repair only at 3 months after the operation. In the cartilage repair model in rabbit, investigations at several time point should be necessary, such as 4, (8,) 12, or 24 weeks after the surgery. Shorter and longer follow-ups should be included.

**Answer:** In most studies about rabbit cartilage repair, 4 or 8 weeks was too short to evaluation the healing. So we chose 12 weeks to evaluate the repair status. More repair maybe observed in longer
follow up, however in our study, we arrange MRI for rabbit first at 12 week. Filling defect was observed in MRI. So we supposed cartilage defect was repaired at that time (12 weeks) during the study.

**Question 2:** The authors performed MRI images to evaluate the cartilage healing. However, the purpose to use the MRI for this study is not clear. What do they want to see using MRI, cartilage repair or sub-chondral status, or others? What the MRI examination add the new information besides gross and histological findings? Please clarify why they performed MRI.

**Answer:** We arrange MRI to evaluate the status of repair. Since filling defect was observed from MRI, we supposed the defect was repaired. Then rabbit was sacrificed for morphology and histology evaluation.

**Question 3:** Why did they use the Mankin score for histology rather than Pineda’s score or Wakitani’s score?

**Answer:** Mankin scoring system was combined score of structure (0 – 6 points), matrix staining (0 – 4 points), and cellular abnormalities (0 – 3 points). Tissue repair can be evaluated layer by layer histologically in this scoring system, with lower score indicating more healthy cartilage.

**Question 4:** In Figure 5, histology should be identified including normal adjacent cartilage and defects or repaired area.

**Answer:** We have marked normal adjacent cartilage radial zone (RZ) in figure 5C. Fibrous tissue (FT) in figure 5c is indicated repaired zone.

**Question 5:** Please revise your ethics statement to clarify that their study protocol had been approved by your institutional animal ethics committee.

**Answer:** We have stated that “the present investigation conforms to the Guide for the Animal Use Protocol of Institutional Animal Care and Use Committee of I-Shou University (IACUC-ISU99010)” in the section of material and methods in the text.

Thank you for your consideration, and we look forward to receiving your reply comments.

Sincerely,

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