Author's response to reviews

Title: Pulsed electromagnetic fields stimulation prevents steroid-induced osteonecrosis in rats

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Author's response to reviews: see over
Dear Dr. Norton:

Thank you very much for your letter and advice regarding our manuscript entitled “Pulsed Electromagnetic Fields stimulation prevents steroid-induced osteonecrosis in rats” (MS: 1493820835571298). We have revised the manuscript, and would like to re-submit it for your consideration. We have addressed the comments raised by the reviewers, and the amendments are highlighted in red in the revised manuscript. Point by point responses to the reviewers’ comments are listed following this letter.

This manuscript has been edited and proofread by an English editor through Elsevier WebShop.

We hope that the revised version of the manuscript is now acceptable for publication in your journal.

I look forward to hearing from you soon.

With best wishes,

Yours sincerely,

Hao Peng (corresponding author)

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Replies to Reviewers and Editor

First of all, we would like to thank both reviewers and editor for their positive and constructive comments and suggestions.

Replies to Reviewer Dr. Roel Kuijer:

1. The manuscript needs editing. Most of the paper has been written in proper English, some sentences clearly need editing.

   Answer: This manuscript has been edited and proofread by an English editor through Elsevier WebShop. We have highlighted all changes in red in the revised manuscript.

2. Figure 1. The authors should consider providing a photograph of a negative control section for comparison. In general it is very difficult, but not impossible, to discriminate between control and diseased tissue using histological techniques.

   Answer: Thank you very much for the suggestion. We have added histological pictures of all the three groups in the revised manuscript (Fig. 2).

3. Old fashion PCR has been used followed by agarose gel-electrophoresis and densitometry of photographs. This procedure is prone to making errors. Using a simple gel documentation system could avoid making such errors. The main problem
is the saturation of bands. Gel documentation system software can correct for saturation, but once photographed, this is much more difficult, if not impossible. The best way would be to make multiple photo’s of the same gel with different exposure times and analyze them all.

**Answer:** Thank you for the suggestion. Fortunately, we had photographed all the agarose gel at different exposure times, such as 40 ms, 60 ms, 80 ms, 100 ms and 120 ms. In the revised manuscript (Fig. 4), we chose the best photograph from them (with exposure time 80 ms), adjusted the brightness and contrast using Photoshop program in a systematic fashion, analyzed the optical density of the bands, and performed statistical analysis by SPSS. The results were updated.

4. The same problem is present in the X-ray analysis of the Western Blots. Illumination of X-ray film does not result in a linear increase in density, if films are not pre-exposed. Secondly, the limit of X-ray illumination is approximately 1 OD. Also is this procedure saturation occurs rapidly. Looking at figure 4 it is likely that almost all bands are saturated, which makes the represented densities in 4B an underestimation of the true values. This also can be resolved by making multiple autoradiographs with different exposure times. By far the best way of doing this is using a Phosphorimager which has a linear range up to 4 OD.

**Answer:** Thank you very much for the suggestion. We do agree that there are some limitations of using X-ray film for Western Blot analysis. We took your advice and photographed all the X-ray films with different exposure time, and
chose the best one from them for analysis. In the revised manuscript (Fig. 5), we adjusted the brightness and contrast using Photoshop program, analyzed the optical density of the bands, and performed statistical analysis by SPSS. The results were updated.

5. The authors should briefly discuss how they would use this method in the clinic and which indications they consider relevant.

Answer: Thank you for the suggestion. A new paragraph was added (paragraph 1, page 14) to the revised manuscript. As a preventive therapy, PEMF could be used in combination with corticosteroid for treatment of many clinical conditions, such as AIDS and SLE, which require high-dose corticosteroid treatment. Daily treatment of PEMF is both time consuming and demanding; patients may find it preferable to perform the treatment at night. During the treatment, the coils were installed separately on the sides of the bed to generate an electromagnetic field on the gluteofemoral area.

6. The study could be greatly improve if the limitations mentioned in the discussion, were addressed. The authors should try to find evidence for PEMF-stimulated inhibition of adipogenesis.

Answer: Thank you for the suggestion. One limitation of this study is that we measured only serum markers of adipogenesis, including TG, TC, LDL and HDL, but did not evaluate the direct effect of PEMF stimulation on adipogenesis in
steroid-treated rats. We measured only serum markers of adipogenesis because we hypothesized that PEMF can prevent osteonecrosis by decreasing serum lipid levels. Steroid treatment could increase adipogenesis. Lipid lowering agents were used to prevent osteonecrosis and revealed satisfactory results. The data indicated that hyperlipidemia may be one of the pathological mechanisms of steroid-induced osteonecrosis. Therefore, the prevention of hyperlipidemia might also prevent steroid-induced osteonecrosis. We consider that PEMF could prevent osteonecrosis because several studies have shown that it can decrease serum lipid levels. Ishida et al. also found that PEMF did not affect bone marrow fat cell size and hypothesized that its preventative effect on steroid-induced osteonecrosis occurs via a mechanism independent of lipid metabolism. Therefore, in the present study, we measured only serum markers of adipogenesis in order to explain the underlying mechanism of PEMF stimulation in preventing steroid-induced osteonecrosis. The revised text can be found in paragraph 3 on Page 12.

Replies to Reviewer Dr. Leo Massari:

1. Was the environmental temperature controlled during exposure?

Answer: Yes. Rats were housed under standard laboratory conditions. The environmental temperature was controlled at 24-25°C during exposure. This information can be found in paragraph 3 on Page 4.
2. Histopathology: how can the section be assessed by independent authors?

Answer: We are sorry that we did not make it clear. The Histopathology diagnosis of osteonecrosis was made blindly and independently by two authors. If the diagnoses differed between the two examiners, a consensus was reached by discussing the histologic findings without knowledge of the group from which the sample was obtained. The revised text can be found in paragraph 2 on Page 6.

3. It would be appropriate to have histological images of all groups of rats.

Answer: Thanks for the suggestion. We have added histological pictures of all the three groups in the revised manuscript (Fig. 2).

4. The Authors should hypothesize and discuss the mechanism of action of PEMF on serum markers.

Answer: Thank you for the good suggestion. We have added hypothesis and discussion of the mechanism of action of PEMF on serum markers. The underlying mechanism of PEMF in the living organism remains unclear. Some theories have been proposed: that the electromagnetic fields have the potential to regulate flow through cation channels, changing the steady-state concentrations of cellular cations and thus the metabolic processes dependent on cation concentrations. We therefore hypothesize that the biological effects of PEMF on serum lipids were associated with ion-channel gating on the cell membrane. The
5. Rats are exposed total-body, is this expected to have an influence on serum markers?

If the mechanism of action of PEMF in rats depends on total-body exposure then the relevance for osteonecrosis in humans is very limited.

Answer: In the present study, we hypothesized that PEMF stimulation could prevent steroid-induced osteonecrosis, and decreasing the serum lipid levels was one of the underlying mechanisms. Our data confirmed this hypothesis. Rats are exposed total-body in the PEMF in the present study, but the prevention of PEMF on osteonecrosis is not depends on total-body exposure. In the past three decades, many studies demonstrated that PEMF could affect living organism in a variety of ways, including up-regulate several cytokines that are important in promoting osteoblast differentiation, decrease adipogenesis, inhibit adipocyte specific mRNA and protein expression of PPARγ2, promote osteogenic differentiation of the stem cells and concurrently inhibited adipocyte formation [1,2]. Therefore, topical use of PEMF can also produce biological effects. In addition, several studies demonstrated that generating an electromagnetic field on the gluteofemoral area can prevent or treat osteonecrosis of the femoral head [3,4]. As a preventive therapy, PEMF could be used in combination with corticosteroid for treatment of many clinical conditions, such as AIDS and SLE, which require high-dose corticosteroid treatment. Daily treatment of PEMF is both time consuming and demanding; patients may find it preferable to perform
the treatment at night. During the treatment, the coils were installed separately on the sides of the bed to generate an electromagnetic field on the gluteofemoral area. We added a new paragraph to discuss how we would use this method in the clinic (paragraph 1, page 14).


6. In table 1 it is shown a progression in the incidence of osteonecrosis in MPSL rats and not in PEMF exposed rats. In my opinion this important finding should be discussed in more details.

**Answer:** Thank you very much for the suggestion. This finding was discussed in more details in the revised manuscript. We found that the incidence of osteonecrosis in the MPSL group displayed a progression, while that in the
PEMF group did not. In the MPSL group, the incidence of osteonecrosis increased to a peak at 4 weeks after the last MPSL injection and then declined at 8 weeks. It is interesting that the serum lipid levels in the MPSL group showed a similar trend, increasing to a peak at 2 weeks after the last MPSL injection and declining at 4 weeks. This finding might indicate that hyperlipidemia contributes to the pathogenesis of osteonecrosis. The revised text can be found in paragraph 2 on Page 12.

Replies to Reviewer Dr. Daniel Grana:

1. PEMF are not fully characterized. I would appreciate a screenshot of an oscilloscope showing the morphology of PEMF. Pulses are quadrangular, sinusoidal or triangular?

I would rather appreciate the field characterization of the magnetic fields in terms of frequency, sine wave/pulsed inputs, frequency response, field amplitude, and harmonics.

Answer: We have added a screenshot of an oscilloscope showing the morphology of PEMF in the revised manuscript (Fig. 1). The Helmholtz coils were connected to a signal generator which delivered repetitive, single, square-wave pulses with a pulse duration of 4.5 ms and frequency of 15 Hz. The frequency of the PEMF was 15 Hz. During each pulse, the magnetic field increased from 0 to 12 G in 4.5 ms and then decreased back to 0 in 20 ms. The detailed characterization of the
PEMF can be found in paragraph 1 on Page 6.

2. "…and diagnosis of osteonecrosis was established based on the presence of empty lacunae or pyknotic nuclei of osteocytes in the bone trabeculae, accompanied by surrounding necrotic bone marrow"

I cannot observe necrotic bone marrow in figure 1.

Answer: We agree that the image provided in previous version of the manuscript was not clearly shown marrow necrosis. A new histological picture from the MPSL group is now provided in the revised manuscript (Fig. 2B).