Responses to editors and reviewers

Manuscript title: Evaluation of the genetic response of mesenchymal stem cells to nanosecond pulsed electric fields by whole transcriptome sequencing

Dear Editors and Reviewers:

Thank you for your letter and the reviewers' comments concerning our manuscript "Evaluation of the genetic response of mesenchymal stem cells to nanosecond pulsed electric fields by whole transcriptome sequencing". All your valuable comments are very helpful for revising and improving our manuscript. We have already studied the comments and made our best efforts to rectify the article paper. Therefore, we are very hopeful for your kind approval in this regard. Revised portions are marked yellow in the paper. The main corrections and the response to the review's comments are mentioned below.

Response to reviewer's comments:

Reviewer #1:

Dear authors The manuscript entitled "Evaluation of the genetic response of mesenchymal stem cells to nanosecond pulsed electric fields by whole transcriptome sequencing" is important for the area of regenerative medicine as it contributes to the construction of knowledge about the biology and function of MSCs, which are considered one of the most promising cells for regenerative therapies. The manuscript brings, through usual methods, results on the transcriptome of bone marrow MSCs after electrical stimulation, which has the potential to contribute to less invasive strategies in regenerative medicine. The study briefly presented conclusions appropriate to the data it provided. The study contributed to filling gaps in knowledge about the effect of nsPEFs on MSCs at the entire transcriptomic level. Furthermore, the study is innovative for bringing insights into the effect of pulsed electric fields on a nanosecond scale on the transcriptome of bone marrow MSCs, bringing new

mechanistic information on the transcriptome of MSCs pretreated with nsPEFs, such as, for example, in the exosome pathway, in migration/proliferation and in the cellular differentiation pathway, fundamental properties for the repair/regeneration of tissues and organs. Therefore, the work brings interesting insights into the therapeutic potential of nanosecond pulsed electrical fields in tissue repair and regeneration. However, I highlight the concern of the number of MSC donors, whether it is sufficient and the species of the donor (specify the species further). And why didn't you use human MSC? Perhaps with these adjustments the conclusion about the effect of ndPRFs on MSCs would be more assertive. Also, MSCs present biological and functional differences according to the tissue and anatomical region, therefore, it would be interesting to evaluate whether the same results are repeated in MSCs from cartilage, skin and other tissues that are more accessible to nanosecond pulsed electric field therapy. Also, in the future it would be important to evaluate whether nsPEFs can affect chromatin accessibility and the cell fate of MSCs (as the authors themselves suggest in the discussion). Despite the excellent contribution for understanding the to the transcriptome of MSCs stimulated with nsPEFs, it would be essential to further investigate the proteome of these cells under the same conditions and cross-reference the results with transcriptome data obtained in this work. It would be important to determine whether nsPEFs are affecting the cellular senescence or neoplasia pathway, a normal cellular fate when the cell is under certain stress. I suggest that you expose the cell type used in the captions and change the "one million MSCs" methodology to cell density (cells/cm2). I congratulate the authors for presenting a concise and coherently organized work capable of impacting regenerative medicine.

Comment: 1) However, I highlight the concern of the number of MSC donors, whether it is sufficient and the species of the donor (specify the species further). And why didn't you use human MSC? Perhaps with these adjustments the conclusion about the effect of ndPRFs on MSCs would be

more assertive.

Author's Response: Thanks for the reviewer's comment. We previously found that nsPEFs can improve the stemness of MSCs and promote the osteochondral defect repair of rats (one million MSCs were suspended in 1 mL DMEM within a 0.4-cm gap cuvette (Bio-Rad, 165-2088, USA) and stimulated by 5 pulses of nsPEFs (100 ns at 10 kV/cm, 1 Hz), the time interval between two pulses was 1 s). In this study, nsPEFs with the same parameters were still applied to regulate the MSCs performance. We therefore believe that the number of MSC donors is sufficient according to the previous study.

Besides we applied the rat MSCs to promote the osteochondral defect repair of rats. To maintain homology, we chose rat MSCs. In another study, we used human MSCs to explore the effect of nsPFEs (Under review by Journal of Orthopaedic Translation) to make the conclusion about the effect of nsPEFs on MSCs more assertive. Thanks for the reviewer's comment. It is useful for our study.

Comment: 2) Also, MSCs present biological and functional differences according to the tissue and anatomical region, therefore, it would be interesting to evaluate whether the same results are repeated in MSCs from cartilage, skin and other tissues that are more accessible to nanosecond pulsed electric field therapy.

Author's Response: Thanks for the reviewer's suggestion. In our previous study, we explore porcine bone marrow MSCs (Stem Cell Res Ther. 2019 Jan 24;10(1):45. Stem Cell Res Ther. 2020 Jul 22;11(1):308. J Tissue Eng Regen Med. 2020 Aug;14(8):1136-1148.), human bone marrow MSCs (Under review by Journal of Orthopaedic Translation) and rat bone marrow MSCs (Sci China Life Sci. 2022 May;65(5):927-939.) pretreated with nsPEFs (100 ns, 10 kV/cm, 1 Hz, 5 pulses) . However, they all come from the bone marrow, the reviewer's opinion is very instructive. In the future, we will study the impact of nsPEF on MSCs from different tissue sources.

Comment: 3) Also, in the future it would be important to evaluate whether nsPEFs can affect chromatin accessibility and the cell fate of MSCs (as the authors themselves suggest in the discussion).

Author's Response: Thanks for the reviewer's suggestion. In the future we would evaluate whether nsPEFs can affect chromatin accessibility and the cell fate of MSCs. Thanks a lot. This is critical to improving the depth and impact of our research.

Comment: 4) Despite the excellent contribution for understanding the to the transcriptome of MSCs stimulated with nsPEFs, it would be essential to further investigate the proteome of these cells under the same conditions and cross-reference the results with transcriptome data obtained in this work.

Author's Response: Thanks for the reviewer's suggestion. In fact, we did a series of studies of nsPEFs, not only on the function of MSCs from different sources, but also on MSC-derived Extracellular Vesicles (EVs) and explore the potential mechanism of miRNA-seq analysis of EVs pretreated by nsPEFs. However, there are still gaps in our research, According to the reviewer's suggestion, we will investigate the proteome of these cells under the same conditions and cross-reference the results with transcriptome data obtained in this work in the future. Thank you for your scientific advice, which helps us so much.

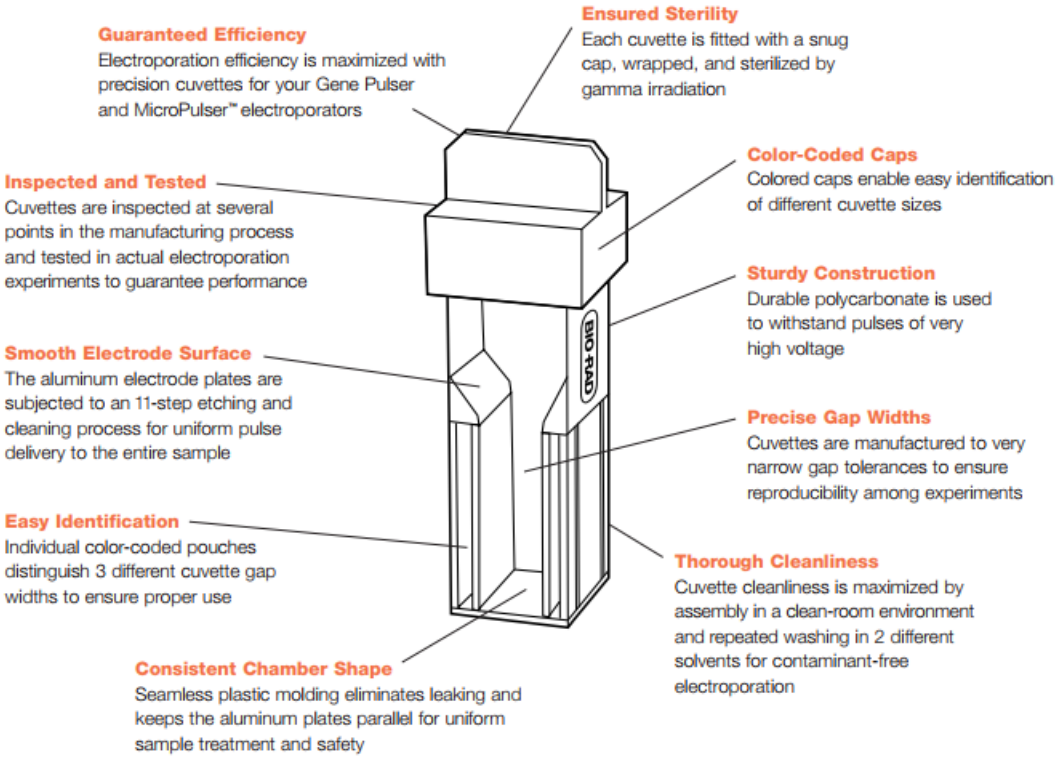
Comment: 5) It would be important to determine whether nsPEFs are affecting the cellular senescence or neoplasia pathway, a normal cellular fate when the cell is under certain stress.

Author's Response: Thanks for the reviewer's suggestion. We previously found that nsPEFs (100 ns, 10 kV/cm, 1 Hz, 5 pulses) can improve the performance of porcine bone marrow MSCs (Stem Cell Res Ther. 2019 Jan 24;10(1):45. Stem Cell Res Ther. 2020 Jul 22;11(1):308. J Tissue Eng Regen Med.

2020 Aug;14(8):1136-1148.), human bone marrow MSCs (Under review by Journal of Orthopaedic Translation) and rat bone marrow MSCs (Sci China Life Sci. 2022 May;65(5):927-939.), and promote the osteochondral defect repair of rats. In our another study ,we founed nsPEFs promoted MSCs migration and viability, particularly enhancing their viability temporarily in vivo. It also significantly inhibited the development of OA-like chondrocytes in vitro and prevented OA progression in rat models. (Under review by Journal of Orthopaedic Translation). In the future, we will explore whether nsPEFs are affecting the cellular senescence or neoplasia pathway according to the suggestion of the reviewer. This will greatly increase the depth of our research. Thanks again for the suggestion.

Comment: 6) I suggest that you expose the cell type used in the captions and change the "one million MSCs" methodology to cell density (cells/cm2).

Author's Response: Thanks for the reviewer's suggestion. According to our previous study, one million MSCs were suspended in 1 mL DMEM within a 0.4-cm gap cuvette (Bio-Rad, 165-2088, USA) and stimulated by 5 pulses of nsPEFs (100 ns at 10 kV/cm, 1 Hz), the time interval between two pulses was 1 s. The cuvette is shown below, there is a 0.4-cm gap between the electrodes. The inside of the cuvette is irregular, so we do not know the specific area and volume, it is difficult to calculate the cell density. So we described it as "one million MSCs were suspended in 1 mL DMEM within a 0.4-cm gap cuvette".



Reviewer #2: It has good quality… It does not need to be reviewed again.

Author's Response: Thank you for the reviewer's appreciation of our research, and we will continue to work hard and look forward to furthering exploration in this field. Look forward to cooperating with you again in the future, and once again express our sincere thanks.

Reviewer #3: I have carefully reviewed the manuscript entitled "Evaluation of the genetic response of mesenchymal stem cells to nanosecond pulsed electric fields by whole transcriptome sequencing." Overall, the study is well designed and intriguing; however, there are some minor points that require attention from the authors. Sincerely yours, Reza Soltani Department of Biology and Anatomical Sciences, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

Comment: 1) The summary of the article should be 250 words.

Author's Response: Thanks for the reviewer's suggestion. We have revised the Abstract in the revised manuscript to close to 250 words based on your

suggestion. Thanks again for the seriousness of you, I have benefited a lot.

Comment: 2) The introduction of the article is well written

Author's Response: Thanks for the reviewer's comment. The manuscript has undergone extensive literature research and repeated modifications. More efforts will be made to present better articles for everyone in the future. Thank you again for your appreciation.

Comment: 3) Why mice were not used in the extraction of mesenchymal stem cells?

Author's Response: We previously found that nsPEFs (100 ns, 10 kV/cm, 1 Hz, 5 pulses) can improve the performance of porcine bone marrow MSCs (Stem Cell Res Ther. 2019 Jan 24;10(1):45. Stem Cell Res Ther. 2020 Jul 22;11(1):308. J Tissue Eng Regen Med. 2020 Aug;14(8):1136-1148.), human bone marrow MSCs (Under review by Journal of Orthopaedic Translation) and rat bone marrow MSCs (Sci China Life Sci. 2022 May;65(5):927-939.). Besides we applied nsPEF-pretreated rat and porcine MSCs to promote the osteochondral defect repair of rats and explore the mechanism. The use of large animal cells could make this conclusion more suitable and assertive for clinical applications in the future. So we seclected the rat MSCs not for MSCs extracted from in mice. Thank you for your scrupulous questions. I hope the reply is satisfacted.

Comment: 4) Can nsPEFs cause degenerative changes and cell death?

Author's Response: Previous study found Tca8113 cells showing early apoptosis after nsPEFs (60 ns, 20 kV/cm, 1Hz, 20 pulses) combining with radiotherapy (Bioelectrochemistry. 2017 Feb;113:35-41.). To optimize the parameters, cytotoxic effects of nsPEFs with 16 conditions (4 durations with 4 field strength) on the MSCs were evaluated. We found no significant apoptosis was observed, indicated by the presence of ~ 80% live cells, at 1 h after nsPEF

treatment in a majority of the 16 nsPEF conditions, except 300 ns at 20 kV/cm and 60, 100, and 300 ns at 30 kV/cm, compared with the non-nsPEF-preconditioned cells. However, a significant decrease in cell viability was detected at day 1 after nsPEFs with increased pulse duration and field strength, with the percentage of viable cells dropping to less than 40%. The percentage of viable cells decreased drastically at 20 and 30 kV/cm with longer pulse duration while nsPEFs at 5 kV/cm showed little toxic effects even with longer pulsing time. Ten conditions that had no toxic effects were utilized to evaluate their effects on chondrogenic differentiation of MSCs described in the next section (Stem Cell Res Ther. 2019 Jan 24;10(1):45.). Finally we found that nsPEFs (100 ns, 10 kV/cm, 1 Hz, 5 pulses) can improve the stemness of MSCs and promote the osteochondral defect repair of rats (Stem Cell Res Ther. 2019 Jan 24;10(1):45. Sci China Life Sci. 2022 May;65(5):927-939.). In another study, we found that nsPEFs pretreatment (100 ns, 10 kV/cm, 1 Hz, 5 pulses) promoted MSCs migration and viability, particularly enhancing their viability temporarily in vivo. It also significantly inhibited the development of OA-like chondrocytes in vitro and prevented OA progression in rat models. (Under review by Journal of Orthopaedic Translation). So in this study, nsPEFs with the same parameters were still applied to regulate the MSCs performance.

Comment: 5) What scientific justification do you have for the nsPEFs parameters?

Author's Response: Thanks for the reviewer's concern. We percious found that nsPEFs (100 ns, 10 kV/cm, 1 Hz, 5 pulses) can improve the stemness of MSCs and promote the osteochondral defect repair of rats (Stem Cell Res Ther. 2019 Jan 24;10(1):45. Sci China Life Sci. 2022 May;65(5):927-939.). Besides, nsPEFs pretreatment (100 ns, 10 kV/cm, 1 Hz, 5 pulses) promoted MSCs migration and viability, particularly enhancing their viability temporarily in vivo. It also significantly inhibited the development of OA-like chondrocytes in vitro and prevented OA progression in rat models. (Under review by Journal

of Orthopaedic Translation). So in this study, nsPEFs with the same parameters were still applied to regulate the MSCs performance and explore the mechanism.

Comment: 6) What software was used for statistical analysis? If it is SPSS, please mention it.

Author's Response: Thanks for the reviewer's careful check. In fact, we performed the statistical analysis using the statistical function that came with the Prism 8.21 software (GraphPad). The statistical significance level was set as $P < 0.05$. We have mentioned it in the "***Statistical analysis***" of the revised manuscript.