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Editor-in-chief
Sub: Optimal concentration of mesenchymal stem cell to promote fracture healing in a rat model of long bone fracture

We wish to re-submit our revised manuscript titled "Optimal concentration of mesenchymal stem cell to promote fracture healing in a rat model of long bone fracture" for publication in the *World Journal of Stem Cells*. The previous manuscript ID is 80209.

We have thoroughly revised the manuscript according to the reviewers' constructive suggestions. This was a great opportunity to improve our study on the basis of the constructive advice of the reviewers. We wish to thank you and the reviewers for the time and efforts dedicated toward the review of our manuscript. The following are our detailed responses (in **bold and yellow highlight**) to the reviewers' comments and suggestions regarding our manuscript. The changes in the revised manuscript are indicated with yellow highlights.

Thank you for considering our manuscript for publication in the *World Journal of Stem Cells*.

Comments from editors and reviewers

Reviewer #1:

Scientific Quality: Grade C (Good)

Language Quality: Grade B (Minor language polishing)

Conclusion: Major revision

Specific Comments to Authors: In this study, the Authors aimed at defining the proper and optimal concentration range at which mesenchymal stem cell (MSCs) may prove effective in promoting fracture healing in a rat model of nonunion long bone fracture. They used three different MSC concentrations, referred to as Low (L) (2.5 × 106), Medium (M) (5.0 × 106), and High (H) (10.0 × 106), injected directly into the fracture site, and compared the healing outcome with reference to the control group (C), injected with saline. An inter-group (M, H, and L) comparison was also performed. Micro-computed tomography (CT) was used to assess new bone formation, in terms of bone volume (BV) and percentage bone volume (PBV). Histological analysis was performed to evaluate a fracture healing score. The protein expression of factors related to MSC migration (stromal cell-derived factor 1 [SDF-1], transforming growth factor-beta 1 [TGF-β1]) and angiogenesis (vascular endothelial growth factor [VEGF]) was evaluated using western blot analysis. Real-time PCR was used to investigate the gene expression of bone morphogenetic protein-2 (BMP-2), TGF-β1 and VEGF. The Authors found that: (i) BV and PBV were significantly increased in groups M and H, as compared to group C at 6 weeks post-fracture, (ii) Significantly more cartilaginous tissue and immature bone were formed in groups M and H than in group C at 2 and 6 weeks post-fracture, (iii) at 2 weeks post-fracture, SDF-1, TGF-β1 and VEGF expression were significantly higher in groups M and H than in group L, (iiii) BMP-2 and VEGF expression were significantly higher in groups M and H than in group C at 6 weeks post-fracture, (iiiiii) There were no significant differences in expression levels of chemokines related to MSC migration, angiogenesis and cytokines associated with osteogenesis between M and H groups at 2 and 6 weeks post-fracture. The Authors conclude that a concentration of 5.0 × 106 MSCs was optimal to promote fracture

healing in a rat model of long bone fractures.

The issue approached by the Authors is no doubt of relevance within the field of regenerative medicine. A major point is the observation that there is a clear-cut "watershed" among the MSC concentrations used, where L MSC started a rescuing, but incomplete repair, which was fully executed at M and H MSCs. In fact, it appears that L-related improvement at 6 weeks post-fracture didn't yield union, while union of fracture fragment with immature or mature bone was evident in a concentration-dependent fashion, in the presence of M, and even more H MSCs, as it is suggested by the histological analyses, and by the calculation of the respective histological scores. As an important point, the Authors should clearly report whether, in spite of the histological scores, they observed a complete ossification at the fracture site, an observation of major clinical implication. There are some major points that are not addressed in this study, which should be addressed in a revised version: The Authors did not assess at what extent the injected MSCs were retained within the recipient tissue. For instance, Huo Z et al., used MSCs carrying a reporter gene to detect engrafted donor cells in recipient mice tissues and fractured bone. In the absence of data showing the putative concentrations of injected MSCs within the fractured recipient tissue, it's difficult to correlate the observed outcome, such as fracture healing, to the concentration of MSC in the delivery buffer. This point should be addressed in the Discussion section, and listed among the limitations of this study.

Response: Thank you for your comment. We agreed with the reviewer's opinion, and did not evaluate whether the injected mesenchymal stem cells were retained in the fracture area through fluorescence imaging analysis. We have already described this as our third limitation, supplementing the reviewer's comments.

Page 15, line 425-427: Third, in the case of direct injection by mixing MSCs with normal saline, it may be difficult to retain MSCs at the fracture site for a long time. In addition, we did not evaluate the retention of the implanted MSC at the fracture site through fluorescence imaging analysis.

While the protein expression level of SDF-1, TGF- β1, and VEGF, and the relative western blots were shown from specimens at 2 weeks post-fracture, the Authors did not show similar protein expression analyses from specimens at 6 weeks post-fracture. For this time point, only mRNA expression data are provided. This is a relevant point. In order to attribute the major rescuing effect of M and H MSCs to the indicated growth factors, since the highest histological scores with these cell concentrations were achieved at 6 weeks (at 2 weeks, with both M and H MSCs union had not occurred yet), the Authors need to show protein expression data at the later observational point of 6 weeks. Providing gene expression results at this time point is interesting, but it is well known that quite often changes in gene expression are not matched by concomitant changes in protein expression levels.

Response: Thank you for your comment. We added the protein expression levels of BMP-2, TGF-β1 and VEGF as supplementary data 4 according to the reviewers' opinions. The protein expression levels showed similar statistical results to those of the mRNA expression data.

Supplementary data 4. Comparison of the protein expression levels of BMP-2, TGF-β1 and VEGF at 6 weeks post-fracture

Another important issue is that the changes in VEGF mRNA expression are not substantiated by experiments showing whether increased VEGF gene expression was associated to an enhanced vascularization at the fracture site. Showing these data at the histological level is extremely relevant, as it may create an effective link between the concentration of the injected MSCS, and the extent of bone healing. This point again reminds the importance of assessing the protein expression level at 6 weeks, showing that the observed increases at 2 weeks were not a transient phenomenon.

Response: Thank you for your comment. As mentioned above, we added the protein expression level of VEGF as supplementary data 4 at 6 weeks post-fracture.

On the whole I believe that the manuscript may be reconsidered after a major revision process, taking into account the above reported criticisms and suggestions.

Reviewer #2:
Scientific Quality: Grade C (Good)
Language Quality: Grade B (Minor language polishing)
Conclusion: Major revision
Specific Comments to Authors: I would like to congratulate the authors for this manuscript. The study is interesting. I have some comments: Methods: Regarding the dose, how many volumes (with and without MSCs) was administered into the fracture site? Is the volume based on previously published study? If so, please refer the study.

Response: Thank you for your comment. We have already described that mesenchymal stem cells were injected by mixing with 0.3 mL sterile normal saline in page 7, line 184. In a previous study, it was reported that 0.5 mL of normal saline was used, but we did not add it as a reference because it was injected intravenous.

Regarding the model of purposely making the fracture of femur, was this model based on previously published study? if so, please refer the study.

Response: Thank you for your comment. We made a femur fracture model of rats using the previous published study as a reference, and added the reference to page 7, line 179 and page 19, line 516-519.

Page 7, line 177-179: After applying an oscillating thin saw at a depth of 1 mm, a fracture was generated in the femoral shaft using the 3-point bending technique[14].

Page 21, line 557-560: Furuta T, Miyaki S, Ishitobi H, Ogura T, Kato Y, Kamei N, Miyado K, Higashi Y, Ochi M. Mesenchymal stem cell-derived exosomes promote

fracture healing in a mouse model. Stem Cells Transl Med 2016; 5: 1620-1630 [PMID: PMC5189643 DOI: 10.5966/sctm.2015-0285

Following the fracture, were fixation and stabilization of fracture done or not? If they were done, please add in the methods. If not done, please give the reasoning.

Response: Thank you for your comment. We fixed and stabilized the fracture site using an 18 gauge needle, which was already described in the long bone fracture model session on page 7.

Page 7, line 173-174: An 18-gauge needle was retrogradely inserted into the center of the intercondylar groove to prevent significant displacement during the fracture.

Since saline was used as scaffold / carrier of MSCs, how did you manage the MSCs to remain at the fracture site and not leaked into the surrounding areas?

Response: Thank you for your comment. We agree with the reviewer's opinion, and mesenchymal stem cells were injected after repairing the muscular fascia to prevent leakage. We described this on page 7, lines 182-184.

Page 7, line 182-184: The muscular fascia was repaired before direct injection of the cell suspension to prevent AD-MSCs from flowing out.

For the western blot and RT-qPCR analysis, which group and how many animals were allocated for these evaluations?

Response: Thank you for your comment. We allocated 6 rats to each group, described in page 8, line 207-209.

Page 8, line 204-206: After breeding for one week, the rats were randomly divided

into four groups (n = 6 in each group): rats injected with normal saline (C), 2.5 × 10^6 (L), 5.0 × 10^6 (M), and 10.0 × 10^6 (H) groups.

Results: Regarding figure 1 and 2, please add arrows to point out, and re-aligned the figures in full (proximal-distally).

Response: Thank you for your comment. We added arrows to the fracture lines in figures 1 and 2 on page 30-31, according to the reviewer's comments. In addition, we cannot represent the entire femur because we analyzed a 6 mm long section with the fracture site as the center.

Regarding figure 3 and 4, please arrange the figure to be in the same direction and same magnification, and please point out where the fracture was located.

Response: Thank you for your comment. Figures 3 and 4 are both 200× magnification, which we described in figure legends 3 and 4 on page 28, respectively. We also indicated the fracture site with black arrows according to the reviewer's comments.

References: Please use the latest references. Please recheck and correct the mistyped words. Line 69: At 2 weeks post-fracture...; Line 85: osteogenesis and...

Response: Thank you for your comment. We rechecked mistyped words and corrected them according to the reviewer's comments.

Page 3, line 67: At 2 weeks post-fracture

,

Page 4, line 85: osteogenesis

Revision reviewer:

Scientific Quality: Grade B (Very good)

Language Quality: Grade B (Minor language polishing)

Conclusion: Accept (High priority)

Specific Comments to Authors: Thank you for the revision and improvement of the manuscript. However, please correct the mistyped words in the author contribution section: provised, and in the core tip section: osteogesis.

Response: Thank you for your comment. We rechecked mistyped words and corrected them according to the reviewer's comments. Page 1, author contributions: Kang-Il Kim provided the study material and designed the research study and performed final approval of manuscript , Page 4, core tip: Factors related to the homing effect of mesenchymal stem cells, osteogenesis and angiogenesis were analyzed by in vivo (radiographic and histologic evaluation) as well as in vitro (RT-qPCR and western blot analysis)

We truly appreciate the time and effort dedicated toward reviewing this manuscript. We the authors appreciate reviewer's insightful comments. We thank all reviewers for taking out of their time and effort to help improve our manuscript.

Thank you for your consideration. I look forward to hearing from you.