Peer Review File

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Reviewer A

It is an honor to review the great study title “TLR-activated mesenchymal stromal cell therapy and antibiotics to treat multi-drug resistant Staphylococcal septic arthritis in an equine model” by Lynn M. Pezzanite et el. This study focused on the antimicrobial role of MSCs and performed well from study design to the summary of results. If the following points are supplemented, it will be a better study.

Comment 1: in abstract section line No.58: please remove reference number 8,9,16. In the abstract section, the references should be removed.
Reply 1: This has been removed.
Changes in the text: References 8,9,16 have been removed from line 58 in the abstract section.

Comment 2: Line No. 89-91 MSCs not only stimulate anti-microbial activation including neutrophils and monocyte but also decrease anti-inflammatory reactions. In this study, the authors focused on anti-microbial activity with TLR-3 activation, however, the anti-inflammatory and immunomodulatory response should also be mentioned so that readers will not be confused. Please add one sentence on the anti-inflammatory and immunomodulatory function of MSCs with references.

Ref.
Shannam. S, Bouffi C, Djouad F et al. Immunosuppression by mesenchymal stem cells: mechanisms and clinical applications. Stem Cell Res Ther. 2010;1(1):2.

Kwon DG, Kim MK, Jeon YS, et al. State of the art: the immunomodulatory role of MSCs for osteoarthritis. Int J Mol Sci. 2022;23(3):1618.

Reply 2: Thank you for these suggested references. A sentence has been added to expand on the anti-inflammatory and immunomodulatory function of MSCs and these references have been added.
Changes in the text: Addition of text and references at line 91 as requested.

Comment 3: Line 135-136. Since the sample size of 4 animals per group is small and the statistical power may be low, it may be difficult to determine that the difference between the two groups is significant. Please suggest the statistical power of the authors' research results or the rationale for prior sample size selection based on the results of previous studies.
Reply 3: Thank you for bringing up this point. The fact that the numbers were based on previous studies was noted in previous line 137. While the reviewer is correct that this sample size is small, an underpowered study generally results in few significant differences – the SD or SEM tends to be large and therefore the overlap in confidence intervals is large. This study demonstrated a large number of significant differences in the outcome parameters, something that mathematically would be likely to continue with large sample sizes. Previous lines 493-497 and a sentence was added to clarify further.

Changes in the text: Further clarification has been added to lines 498-490.

Comment 4: Line 144-145 Although the process of producing bacterial arthritis in horses is well known, the degree of infection may vary from individual to individual, even with the same number of MRSA injections. If the degree of infection is different, the treatment efficacy may be different. How can it be judged that a similar degree of bacterial arthritis has occurred in each horse?
Reply 4: The reviewer raises a good point. As demonstrated in figure 2, pain and inflammation scores achieved with bacterial inoculation were similar across all horses, as determined by a grading scale that included physical examination, lameness evaluation, distal limb edema, joint circumference and synovial heat. Furthermore, this technique of septic synovitis induction was recently published by another group with similar and repeatable results in consistent induction of clinical findings of septic synovitis. Reference to this recently published manuscript has been added here.
Changes in the text: Citation to recently published work has been added in the methods section line 167 to support the model used and further emphasis to the fact that pain scores achieved were consistent between horses and did not differ between groups on the day following inoculation has been added.

Gilbertie JM, Schaer TP, Engiles JB et al. A platelet rich plasma-derived biologic clears staphylococcus aureus biofilms while mitigating cartilage degeneration and joint inflammation in a clinically relevant large animal infectious arthritis model. Front Cell Infect Microbiol 2022. https://doi.org/10.3389/fcimb.2022.895022.

Comment 5: Line 145, MRSA Please present full words when referring to it for the first time in the text.
Reply 5: Thank you for this comment. This abbreviation has been clarified.
Changes in the text: This abbreviation has been clarified in the text in previous line 145.

Comment 6: Line 147. The authors presented IV gentamicin for systematic use. However, gentamicin mainly acted as bacteriostatic, not bactericidal. Please, please explain why you chose this antibiotic instead of 1st generation cephalosporin or IV vancomycin.
Reply 6: Thank you for bringing up this important point. Gentamicin was selected as the bacterial isolate injected was sensitive to gentamicin and due to its frequency of use in equine patients and inexpensive cost. The bacterial isolate injected was resistant to cephalosporins tested. The reviewer brings up the point of using systemic vancomycin; however, intravenous systemic vancomycin is rarely administered in adult horses due to its prohibitive cost and concerns regarding limiting its widespread use in veterinary species as a reserved drug for human usage. The reviewer's point has been addressed in the methods section mentioning the sensitivity of this bacterial isolate to gentamicin and mentioned in the limitations section for discussion.
Changes in the text: Methods lines 149-150 have been altered to reflect the culture/sensitivity findings supporting use of gentamicin. The limitations section in the discussion has been altered to discuss this point and include the rationale for antibiotic selection in lines 513-518.

Comment 7: Line 149. / 269 Please explain why the observation period between the two groups was divided into 7 days and 14 days.
Reply 7: The control horses were euthanized on day 7 versus 14 following observations made during a pilot study. These data were supported by the final outcome that pain scores were significantly higher in VAN-treated control horses versus TLR-MSC-VAN horses by day 3

following inoculation. Therefore, humane euthanasia of VAN treated horses was performed on day 7 in accordance with IACUC protocols.
Changes in the text: A line has been added to clarify this in the methods section lines 151-152.

Comment 8: Line 199-204. Even in subjects of the same age, BM aspirated MSCs may differ in their ability to proliferate, such as CFU, or to produce cytokines. In this case, the results of the study may also be affected. Has there been any pre-evaluation for the functional uniformity of the MSCs from 3 donor horses?
Reply 8: Thank you for requesting this information. The antimicrobial properties of the MSCs from these three donor horses were previously assessed, as published in Pezzanite et al. Vet Surg 2021;50:858-871. This has been clarified and cited in the text.
Changes in the text: Citation to Pezzanite et al. Vet Surg 2021;50:858-871 has been made on line 203.

Comment 9: line 339.~ It was suggested that normality was tested through the Shapiro-wilk test, but it is difficult to confirm the data. Usually, it is difficult to say that there are 4 subjects in each group to satisfy normality, and the Mann-whitney U test may be more appropriate than the t-test. Also, the ultrasound imaging score and histologic score are composed of a semi-quantitative score, so the difference between the scores is not quantitatively the same. Comprehensively, it does not seem appropriate to apply the t-test.
Reply 9: Thank you to the reviewer for bringing up these important points, which we will address individually. For these data, visual inspection did not show any indications that the standard parametric test assumptions had been violated. In addition, this reference indicates why a t-test could be appropriate for these cases (Winter JCF de. 'Using the Student' t-test with extremely small sample sizes.' Accessed May 19, 2022. https://doi.org/10.7275/E4R6-DJ05. The authors would argue that this is indeed an appropriate test based on the data provided.
In regards to histology, grading of histology was determined on a continuous scale, therefore the authors would maintain that the t-test performed adequately in detecting differences. However, the authors acknowledge the reviewer's point regarding ultrasound and MRI scoring being composed of a semi-quantitative scoring system; therefore, these statistical analyses have been redone using non-parametric testing. The updated p-values have been added to the text, which have changed the MRI scoring to not statistically significantly different ($p=0.089$).
Changes in the text: The data analysis section has been revised with the addition of two references and statistical analysis of the imaging section has been updated with revised outcomes.

Comment 10
Line 421 administration of Toll-like-receptor-3 -> TLR-3
Line 440. Please represent full name when referring to it for the first time in the text.
Ex. Extracellular Vesicles (EVs)
Line 453 antimicrobial peptides (AMPs).
Line 457. neutrophil extracellular traps (NETs)
Reply 10: These changes have been made.
Changes in the text: Changes have been made to clarify abbreviations in previous lines 421, 440, 453, 457.

Comment 11: Line 493-495 In the case of bacterial joint arthritis, early treatment is surely important, but it is also very important to evaluate whether it recurs. In this study, only 14 days were evaluated, so it is recommended to add it to the limitation.
Reply 11: Thank you for this suggestion. This has been further clarified in the limitations section.

Changes in the text: A sentence has been added to the limitations section, at previous line 493.

Comment 12: Line 497, 498 Considering Q No. 9, Could the authors provide statistical power to support “sufficient to detect differences.’?
Reply 12: As previously noted, an underpowered study generally runs a high risk of Type I error – but even with these low numbers there were statistical differences found between groups. It is not just unlikely but statistically improbable that the differences noted here would not continue to be found in a larger sample given the large differences between groups. We are not attempting to say that statistical power was sufficient to detect differences in the variables where no differences was found, just that when a differences was found, it is highly likely that these are true differences between groups.
Changes in the text: see as above for Question 9.

Comment 13: Line 509-510 In the conclusion section, it is recommended to summarize only the important results of this study. Comments on the next or future study should be deleted.
Reply 13: This sentence has been removed at the reviewer’s request.
Changes in the text: Previous lines 512-513 have been removed.

Reviewer B

Comment 1: Please correct the following sentences:
Lane 641: “remained above 1 μg/mL in both treatment and control horses at each of the time points following administration (days 4, 7, 14)”: Supplemental 1 E: Day 4 and 7 please explain the differences between VAN levels control vs TLR- MSC.
Reply 1: Vancomycin levels varied widely between individuals which was attributed to differences in joint size and effusion between VAN vs TLR-MSC-VAN treated groups; however, overall concentrations of vancomycin did not differ in the synovial fluid of VAN vs TLR-MSC-VAN groups and remained at therapeutic levels above MIC for the bacterial isolate targeted throughout the study duration.
Changes in the text: Additional information has been added to Supplemental 1E (lines 655-657) to clarify.

Comment 2: Lane 214: add MSC stimulation time.
Reply 2: MSC were stimulated for 2 hours.
Changes in the text: MSC stimulation time of 2 hours has been added to line 218.

Comment 3: P544, Figure 4 E: add key legend
Reply 3: The figure 4 has been altered to remove significances for MRI findings due to changes in statistical analysis and a legend has been added.
Changes in the text: Figure 4E has been altered to remove significances and to add legend to E.

Comment 4: Lane 440: “EV proteins” spell EV.
Reply 4: This has been changed.
Changes in the text: EV has been changed to extracellular vesicles in previous line 440.

Reviewer C

The manuscript described TLR-activated mesenchymal stromal cell therapy and antibiotics to treat multi-drug resistant Staphylococcal septic arthritis in an equine model. Paper is generally well written, but there is a problem with the experimental design. Questions and concerns are listed:

Comment 1. Line 145: Why did you set the number of cells like that? If there is an appropriate basis, it would be better to state it.
Reply 1: As described in the review article by Schnabel et al., studies to determine the optimal number of stem cells for treatment in horses have yet to be performed, but the range of 10 to 30 million cells per joint is commonly described. This citation to support the number used has been provided.
Changes in the text: This citation has been provided in the text as support of the cell number used.

Schnabel LV, Fortier LA, McIlwraith CW, Nobert KM. Therapeutic use of stem cells in horses: which type, how and when? Vet J 2013;197:570-577.

Comment 2. Line 147-149: Why did the timing of euthanasia differ between groups? As the euthanasia period changed, more antibiotics (Gentamicin) were applied to the experimental group. This may affect the experimental results, so what do you think? A reasonable explanation of the time of euthanasia and the period of antibiotic use is needed.
Reply 2: See reply to reviewer 1 comment 7. This has been further clarified in the methods section and this reviewer's point regarding additional antibiotic administration addressed further in the limitations section.
Changes in the text: Methods lines 151-152. The limitations in design have been expanded upon further in the discussion section lines 519-522.

Comment 3. Line 160: During the experiment, NSAID drugs were given during the experiment, and are you sure this does not affect the results? NSAID is used as an anti-inflammatory drug, but isn't it necessary to treat it to see the effectiveness of TLR-MSC to clearly demonstrate its effectiveness? Appropriate explanations are needed regarding the reasons for using NSAID and its impact on experimental results.
Reply 3: The reviewer brings up an interesting point. Nonsteroidal anti-inflammatory medications were required to be administered for pain in this study as dictated by the Institutional Animal Care and Use Committee. Furthermore, NSAIDs are routinely administered at the same time as MSC injection in clinical equine cases treated for osteoarthritis at the co-authors' institutions. However, the reviewer raises an important point that the co-administration of a single or multiple doses of NSAIDs concurrent to and following MSC injection on the immunomodulatory effects of MSC is unknown and should be addressed as a point of discussion. This has been added to the limitations section.
Changes in the text: Further discussion of this point has been added to the discussion lines 523-532.

Comment 4. Line 204-207: Did you just plant stem cells on a plate without the process of making a single cell suspension through trypsin treatment and filtering them into strainers? I think there must have been quite a lot of debris. A more detailed explanation of the cell culture method is needed.
Reply 4: Bone marrow aspirates were purified via ficoll density gradient centrifugation as previously described by Schnabel et al and Radcliffe et al. Appropriate citations have been added to support methods performed.
Changes in the text: The following citations have been added to line 232.

Radcliffe DH, Flaminio MJ, Fortier LA. Temporal analysis of equine bone marrow aspirate during establishment of putative mesenchymal progenitor cell populations. Stem Cells Dev. 2010, 19:269-282.

Schnabel LV, Pezzanite L, Antczak DF, Felippe MJB, Fortier LA. Equine bone marrow-derived mesenchymal stromal cells are heterogeneous in MHC class II expression and capable of inciting an immune response in vitro. Stem Cell Res Ther 2014,5:13.

Comment 5. Line 214-215: Is there any other experiment that TLR-3 is active? It seems that the comparison results with MSC that has not been TLR-3 activated should be included.
Reply 5: Enhanced antibacterial activity with TLR activation of MSC has been demonstrated in both in vitro assays in multiple species including equine specifically and in mouse models of infection. Based on these findings and out of desire to reduce large animal number in this study, only TLR-MSC were compared to control. More specific reference has been made to these previous publications as justification for the lack of treatment group receiving MSC alone in this large animal model.
Changes in the text: Specific reference to previous studies demonstrating an improvement in antimicrobial activity with TLR activation of MSC and therefore justification for why an MSC alone group was not included in this study has been added on line 248.

Pezzanite LM, Chow L, Johnson V, Griffenhagen G, Goodrich L, Dow S. Toll-like receptor activation of equine mesenchymal stromal cells to enhance antibacterial activity and immunomodulatory cytokine secretion. Vet Surg. 2021;50(4):858-871.

Chow L, Johnson V, Impastato R, Coy J, Strumpf A, Dow S. Antibacterial activity of human mesenchymal stem cells mediated directly by constitutively secreted factors and indirectly by activation of innate immune effector cells. Stem Cells Transl Med. 2020;9(2):235-249.

Johnson V, Webb T, Norman A, Coy J, Kurihara J, Regan D, Dow S. Activated mesenchymal stem cells interact with antibiotics and immune responses to control chronic bacterial infections. Sci Rep. 2017;7(1):9575.

Comment 6. Line 145, 216: Correct the spacing of the numbers properly.
Reply 6: Spaces have been added between numbers as indicated.
Changes in the text: Previous lines 145 and 216 have had spaces added between numbers.

Comment 7. Line 217: Stem cells change their characteristics over time. No matter how stem cell markers are identified, low passage stem cells have better stemness. The use of stem cells from the 1st to 5th passages is too widespread. Do passages 1 and 5 have the same stemness? If so, I think we need a relevant basis (not only marker but it should also be included growth potential and cell viability). In addition, stem cells themselves have antimicrobials and immunomodulatory properties. Under the same conditions of the same passages, results are needed for differences between cells that have been TLR-3 activated in cells and those that have not.
Reply 7: Thank you for this comment. Our group has previously demonstrated that MSC from multiple species maintain consistent antibacterial activity against S. aureus bacteria and antimicrobial peptide cathelicidin up to passage nine. This has been included as supplemental for the reviewer.
Changes in the text: Supplemental information (unpublished data from the authors' laboratory has been included for this reviewer.

Comment 8. Line 450, 493 paragraph: This experiment has no MSC group as you wrote in the limitation. Experiments show that activating TLR-3 is effective. However, MSC itself has antimicrobials and immunomodulatory properties. Since there is no MSC group from the experimental design stage, it is questionable whether it is necessary to simply activate TLR-3 to produce better results. If you can do more animal experiments, you'd better do it, and if not, you'll have to prove it through related experiments in vitro.
Reply 8: As above, previous published studies both in vitro and in rodent models have demonstrated the enhanced antimicrobial and immunomodulatory properties of TLR-activated MSC. In an effort to reduce number of large animal equids that would be euthanized for this study, in accordance with ARRIVE guidelines, this group was removed from this study. Reference to previous work supporting this approach have been emphasized.
Changes in the text: Additional references to previous work have been emphasized here.

Comment 9. Line 493 paragraph: Is there any result of how much stem cells survive within the joint? The survival period of stem cells is thought to play a major role in explaining the results of this experiment. It is necessary to describe how much stem cells can survive and produce a therapeutic effect.
Reply 9: Intra-articularly injected MSC have been detectable up to one month post injection, engrafted within cartilage lesions (Satue et al. 2019). MSC tracking was not performed in this study as it was not the focus of this work; however, given the short time frame of this study MSC could have been expected to remain within the joint for the duration of the study. Further reference has been made to this and previous citations included to support these statement.
Changes in the text: Further reference to citations on engraftment of intra-articularly injected MSC has been made here in the limitations section.

Satue M, Schuler C, Ginner N, et al. Intra-articularly injected mesenchymal stem cells promote cartilage regeneration, but do not permanently engraft in distant organs. Sci Reports 2019;9:10153.