Notes for Reviewers

Reviewer #1:

This manuscript describes a study to determine whether decellularized sheets of ECM generated by deer antler stem cells can facilitate osteochondral defect repair by serving as a cell-free scaffold. The significance lies in the introduction of a new xenogeneic ECM cell scaffold. Experimental designs, sample sizes, and methods generally appear to have been sound. Sheets of ECM were grown from rat adipose-derived stem cells (aMSC, allogeneic), deer antler periosteal cells (APC, xenogeneic), and deer antler reserve mesenchymal cells (RMC, xenogeneic). They were characterized with respect to the support of rat bone marrow stromal cell attachment and proliferation and also with respect to their capacity to promote osteochondral chondral defect healing in rats. RMC supported the highest cell attachment and proliferation. The sheets were decellularized and residual DNA, GAG, and total collagen quantified. Collagen and GAG were substantially retained. The decellularized sheets were then packed into osteochondral defects surgically created in the distal femurs of rats.

General questions

1. A limitation of the study that should be mentioned is the size of the defect. While 1.4mm is considered to be the critical size of a rat OCD, defects of 2.0mm are commonly used to avoid obfuscation of results by spontaneous healing.

Response: We agree with the Reviewer's comments that defects cannot properly repair if its size at 1.4 mm or above without artificial intervention, this claim is also supported by Katagiri et al (2017). In our study, we selected the defect size at 1.5 mm based on the two facts: 1) We found rat articular cartilage defects at 1.5 mm all failed to properly repair (no hyaline cartilage) no exception in our pre-trial (Fig. S1A). 2) The actual surface area of the articular cartilage of 8-week-old rats really stretched to its limitation for making defects bigger than 1.5 mm (Fig. S1B). In order to make this clear, we have added this part of information in the M+M section.

2. ICRS scores and histology support the claim of superior defect filling and more seamless lateral integration of regenerated tissue with native cartilage in the RMC group. However, results do not support the claim of almost perfect restoration with articular hyaline cartilage. Saf-O staining suggests the repair tissue was far less rich in GAG than native cartilage in all experimental groups, and collagen immunostaining does not demonstrate an abundance of Col II positive staining in any experimental group. Although the study is interesting and the RMC may indeed hold promise as a cell scaffold for osteochondral tissue repair, the conclusions must be tempered to reflect the actual results.

Response: We are pleased to know that the Reviewer think RMC-ECM sheets hold the promise for the proper repair of osteochondral defects. The problems of the Reviewer encountered are 1) the repair tissue was far less rich in GAG than native cartilage in our best repair group, the RMC-ECM, judged by observing the results of Saf-O staining. In fact, in the study we used both alcian blue and Saf-O staining to demonstrate the GAG content in the repaired tissue. In the alcian blue staining, the cartilage of the repaired tissue is almost identical to the marginal native cartilage (Fig. 4). In terms of Saf-O staining: yes, we admit that the repaired cartilage was not deeply stained by Saf-O and even with patch staining, but the native cartilage was not deeply stained either. Staining status of the repaired cartilage was comparable to that of its native counterpart (Fig. S3). Therefore, the impression of the Reviewer's perceived may be due to Saf-O staining itself, i.e., not been done properly (staining time may not be long enough), rather than that real GAG content in the repaired cartilage is actually less than the native counterpart.

Likewise, Col II falling the same problem of GAG content above. Really, the shallow staining may be solely caused by IHC staining technique itself, rather than that Col II content in the repaired cartilage is actually less than the native counterpart. Consequently, we believe the repaired cartilage in the RMC-ECM group is truly hyaline cartilage-like in nature.

Additional concerns and comments are enumerated below.

1. In the Introduction, “the osteochondral interface” is referred to as a type of tissue. The type of tissue is calcified cartilage.

Response: We believe whether “osteochondral interface” can be considered as a type of tissue or not depends on the context. Here, we think this interface is the transzonal zone between bone and cartilage.

2. References 1 and 2, cited to support the opening statement, do not address the natural progression of osteochondral defects (see, for example, Knee . 2002 Feb;9(1):7-10. doi: 10.1016/s0968-0160(01)00133-8.). Please check that all cited references are appropriate.

Response: We thank the Reviewer for the suggestion to properly check whether all the references are properly cited, and we have done that. In addition, we have cited the reference (Knee. 2002 Feb;9(1):7-10) recommended by the Reviewer.

3. While certain scaffolds may circumvent the need for exogenous cells, they do not undoubtedly do so (“scaffolds… can undoubtedly…”). The sentence in question should be revised.

Response: The sentence has been revised to “Certain type of engineered cell-free scaffolds capable of recruiting host endogenous MSCs can undoubtedly circumvent the problems…” following the suggestion by the Reviewer.

4. The Introduction is a bit too long. It should focus on cell-free ECM scaffolds that support osteochondral tissue regeneration. It should also mention any limitations thereof that antler ECM may address.

Response: The Introduction looks a bit long, but in this particular case, we need to introduce osteochondral defects and the current availability of treatment means; need to tell people cell-based treatment, mixture of cell and matrix treatment; lead to allogenic and xenogeneic cell-free sheet treatment, and eventually to introduce deer antlers and different types of antler cells. This is a really word-consuming process. We have asked our colleague/mentor Dr Peter Fennessy to help to shorten the section, but

he eventually came back to us by saying that deletion of any part of the Introduction would harm the flow of logic. Therefore, we may have to stay like this, unless the Reviewer pinpoints out to us where exactly should be trimmed.

With regard to "any limitations thereof that antler ECM may address", in the introduction, we believe we have clearly address this: application of antler cell ECM will solve the problem of material shortage in the clinical setting (this is another reason why the introduction is a little bit long).

5. The last sentence of the Introduction does not make sense. It seems to say that cell-free MSC-ECM will provide an unlimited source of cells. Please clarify.

Response: We have rewritten the sentence to solve the problem of confusing.

6. Cells isolated from adipose tissue should not be referred to as MSCs unless they were demonstrated to be capable of differentiating into multiple phenotypes (e.g. bone, fat, cartilage). The same is true for bone marrow-derived cells, which are more accurately termed bone marrow stromal cells.

Response: Following the Reviewer's suggestion, we have changed the term "mesenchymal stem cells" to "mesenchymal stromal cells", and the abbreviation is still "MSCs". The reason for using this term: 1) to accommodate the Reviewer's suggestion, i.e., to use stromal; 2) currently, more and more people in the field start adopt the term "mesenchymal stromal cells" for this type of cells to encompass more broadly.

7. Ascorbate is very unstable and rapidly oxidizes in aqueous systems. Therefore, it is typically replenished daily. Was it? Ascorbic acid 2-phosphate, an oxidation resistant analog of ascorbate, can be used to avoid the need for constant replenishment.

Response: We appreciate the Reviewer's recommendation to use ascorbate; and in hindsight, we should use ascorbate rather than ascorbic acid in our experiment. To us, using ascorbic acid didn't affect our science (produced a large quantity of ECM), although causing more hassles (add ascorbic acid more times to the culture medium (1 time per 2days) than using ascorbate). We chose ascorbic acid in our experiment mainly

based on the reference by Zhang et al (2021), and strictly conducted following the manufacturer's description (stored at -20 ^{0}C and used up within 15 days).

8. It would be nice to see the cross-sectional images of the ECM sheets before and after decellularization.

Response: We wish we can do what the Reviewer suggested, but these sheets were very thin and almost impossible to manipulate it for adjusting the cutting surface, particularly after the decellularization. Having said that, we believe through the whole-sale cut, we have got sufficient surfaces to allow us to evaluate the decellularization degree (Fig. 1).

9. Specify the "nucleic acid scavenging solution containing DNA enzyme." Were the sheets treated with RNase?

Response: Yes, the solution contained RNase (1 U/ml). We have added this information in the M+M section.

10. Report the density of bMSC cell seeding in cells/sq. cm.

Response: 2 x 10^4 cells/sq.cm, we have added this information in the M+M section.

11. Approximately what area of ECM sheet was pressed into each osteochondral defect?

Response: We divided each sheet evenly into 4 parts, and pressed each part into each defect hole (essentially the hole was fully filled). We have added this in the M+M section.

12. Provide additional details of the in vivo experiment. How were the wounds closed? Were the rats administered any pain medication? Any restrictions on animal activity or food post-surgery? If not, so state.

Response: We used absorbable threads to suture the muscle and connective tissue layers, and used silk threads to suture skin. After the operation, rats were monitored till their fully waking up, tramadol analgesia (50 mg/kg; Intramuscular injection; Once a day for 3 days) was given immediately after the surgery, and the rats could freely access to

water and food. This information has been added in the M+M section.

13. Please list the variables that were quantified for statistical analysis.

Response: Cell proliferation rate, ICRS score, and area of positive IHC staining

14. Regarding DNA, the result is either % DNA removed or % of original. As stated, the results should be 1.9%, 2.2%, and 2.8%, respectively, of the original level. DNA removal may have been efficient, but it should be determined whether residual DNA is less than the accepted upper limit of 50 nanograms per milligram dry weight.

Response: Thanks the Reviewer for pointing out this mistake, we have changed the numbers to "1.9%, 2.2%, and 2.8%". The residual DNA was 23.22 ng per mg in the RMC group. We have added this in Results section.

15. Correct the sentence presenting residual collagen and GAG contents. Is Table 1 missing? I could not find it.

Response: The sentence was rewritten by Dr Fennessy, and Table 1 is added to the paper.

16. ICRS scores are presented in the Results, but the methodology is not (e.g., how many raters? Were they blinded to the experimental groups?). ICRS scores may have been significantly higher in the RMC group, but only by a slim margin.

Response: The Detailed methodology is added, please refer to the M+M section.

17. The Col I and Col II immunostaining is not convincing. For example, there seems to be hardly any brownish staining in the bone to demonstrate Col I. And there is lack of positive Col II staining in the native articular cartilage on either side of the defect, and in the tissue which had filled the defect, regardless of group. Perhaps the antigen retrieval methods were ineffective. I don't see how collagen staining could be quantified from the representative images. And results are overstated with respect to Col II. There were clearly no differences in positive areas among the experimental groups. RMC may have facilitated defect filling, but safranin-O staining is lacking in

regenerated tissue, with the exception of a slight amount in the RMC 12W group. There is inadequate evidence of tissue rich in proteoglycan and Col II to support a claim of hyaline-like cartilage regeneration.

Response: We appreciate the Reviewer's strict scientific attitude, and agree with the Reviewer in that antigen retrieval procedure did not work as well as expected. However, we believe it is still discernable between the positive and negative in color staining from our side, particularly when these photos are enlarged (unfortunately, we don't have luxury space to do so in the paper). Alternatively, we applied Photoshop to enhance the color depth of these photos and we found the results have been increased in so doing. By the way, all these figures the Reviewer raised are supplementary figures. With regard to Col II and GAG, please refer to "General question response 2"

18. The second sentence of the Discussion is not supported by results, as successfully repaired would require clear demonstration of hyaline-like cartilage regeneration.

Response: We appreciate the Reviewer's strict scientific attitude, but we believe our results can sufficiently support the conclusion "successfully repaired". For the reason, please refer to "General question response 2".

19. Regenerated bone is characterized as well-vascularized. Was this vasculature observed? If not, then refrain from claims regarding vascularity.

Response: To us the vasculature is clearly discernable, to make this clearer we have labeled this vasculature on the relevant figures.

20. The overall Conclusion is not supported by results and must be tempered. The RMC-ECM facilitated a degree of restoration that was far from "almost perfect."

Response: For the safe side and to respect the Reviewer's concern, we changed term "almost perfect." to "high quality" in the paper, although we believe that "almost perfect." withstands testing.

21. What is meant by "barely detectable immune response?" Was any effort made to

characterize or measure the immune response? This claim should only be made if the tissue was examined macroscopically (e.g. osteophyte) and microscopically for signs of an immune response. In particular, the aMSC and BMS (allografts) should be carefully compared to APC and RMC (xenografts).

Response: We used the sentence “barely detectable immune response?”, because we didn’t detect any immune response in our repaired tissue or surroundings at both macroscope level (no osteophyte) and microscope level (no infiltration of immune cells).