Peer Review File

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Reviewer A

In this study, the authors investigated gene expression changes after post-MI injection of alginate hydrogel in a mouse model. After pathway and signaling analysis, they focused on the gene B2M, which was unregulated after MI but not with MI plus hydrogel injection. In an in vitro study they then verified that hypoxia and glucose starvation of mouse cardiomyocytes caused a large increase in B2M expression that was not seen when cardiomyocytes were cultured on alginate.

I have the following comments:

1) Methods: The authors have a very incomplete description of the formation of alginate hydrogels. There are several typos (sodium alginate is listed twice) and the method used (just sodium alginate in water at room temperature) would not form a gel. At minimum, the authors needed to include some divalent ion. Additionally, the mixing of gels at 25 C and then setting at "room temperature" is confusing since, at least in my region, room temperature is defined as 25 C.

Reply 1: Thanks for your valuable comments. We have modified our text as advised.

Changes in the text: Page 7, line 183

2) Introduction: The manuscript gives results and repeats the abstract at the end of the introduction. This can be removed for conciseness.

Reply 2: Thanks for your valuable comments. We have modified our text as advised, and rewrote the abstract section

Changes in the text: Page 6, line 72-80

3) Methods: The methods for cell culture are incomplete without identifying the methods of plating cells on plastic or alginate.

Reply 3: Thanks for your valuable comments. We have modified as advised in the methods for cell culture.

Changes in the text: Page 12, line 319

4) Methods: The methods give a description of an experiment involving interfering RNA though results of this experiment are not given.

Reply 4: Thanks for your valuable comments. We have modified as advised in the methods for cell culture.

5) Results: The 1.5 page list of differentials expressed genes should be presented as a table.

Reply 5: Thanks for your valuable comments. We have modified as advised in Table 1.

6) Discussion: The authors overstate the involvement of B2M. Though they show an interesting

correlation, they present no evidence of a mechanism for upregulation of B2M by alginate and they show no evidence of downregulation of B2M aiding in heart recovery post-MI. The conclusions need to be greatly scaled back, or the authors need to investigate both mechanisms leading to B2M expression, including the cell types expressing this molecule in vivo, and the result of preventing the upregulation of B2M in the mouse model.

Reply 6: Thanks for your valuable comments. We have modified as advised in the Result and Discussion.

7) Results: The results text says 10 genes were randomly chosen for verification with RNA seq but only 4 results are shown. The authors need to identify and show the results of the other 6 genes evaluated.

Reply 7: Thanks for your valuable comments. In fact, we verificated 6 other genes from RNA seq, but we cannot detect the signals of these genes in most qRT-PCR detection. Meanwhile, the quantification of cycle (Cq) values are usually greater than 30, making it impossible to perform statistical analysis on the detection results of these genes.8) Figure 3B: Groups and sample identifications should be defined in a legend or the figure caption.

Reply 8: Thanks for your valuable comments. We have modified as advised in every figure caption.

9) Figure 5C: At least in the conversion to PDF shown here, the contrast is far too low to see the cell staining or even the legend in the figures.

Reply 9: Thanks for your valuable comments. We have modified as advised.

Reviewer B

Abstract should be rewritten in which reflect the all methods and results in an appropriate way.

Reply 1: Thanks for your valuable comments. We have modified our text as advised.

Changes in the text: Page 4, line 61-81

The volume and site injection of alginate hydrogel was not determined in the abstract.

Reply 2: Thanks for your valuable comments. We have modified our text as advised.

Changes in the text: Page 4, line 61-81

Statistically different outcomes should be indicated inside the abstract result using p values.

Reply 3: Thanks for your valuable comments. We have modified our text as advised.

Changes in the text: Page 4, line 61-81

The total volume of alginate hydrogel should be precisely addressed inside the manuscript.

Reply 4: Thanks for your valuable comments. We have modified our text as advised.

Changes in the text: Page 4, line 64

All catalog numbers of chemicals and reagent should be pointed inside manuscript.

Reply 5: Thanks for your valuable comments. We have modified as advised in Table 2.

There are several typos errors and grammatical issues inside manuscript that need further consideration.

Reply 6: Thanks for your valuable comments. We have modified our text as advised. Due to not being a native speaker, there may still be some grammatical issues.

The type of DMEM (either low or high glucose type) used for HL-1 cells should be indicated.

Reply 7: Thanks for your valuable comments. We have modified our text as advised.

Changes in the text: Page 12, line 319

In result section, significant and nonsignificant values were missed.

Reply 8: Thanks for your valuable comments. We have modified our text as advised and put significant and nonsignificant values in Figures.

The selection of LV slice for each analysis is an important issue. Which slice numbers were used for different analyses. Please be clear

Reply 9: Thanks for your valuable comments. We have modified our text as advised in figure caption of Figure 1B and Figure 5C.

In western blotting, the molecular weights are needed to indicated along with each immunoblot.

Reply 10: Thanks for your valuable comments. We have modified our text as advised in Figure 5D.

The intensity of fluorochromes is not bright. Please use representative samples for presentation.

Reply 11: Thanks for your valuable comments. We have modified our text as advised.

Data related to alginate hydrogel characterization and physicochemical properties were missed.

Reply 12: Alginate hydrogel is a mixture of sodium alginate and calcium alginate or calcium gluconate, which is a kind of polymer with a three-dimensional reticulation structure, and it can maintain a stable reticulation framework after water absorption and swelling.

(1) The final score of skin erythema and edema in all periods of polar and non-polar extracts of alginate hydrogel used in this study is less than 1.0, which is in line with the requirements of intradermal reaction test in the standard of GB/T16886.10-2017 "Biological Evaluation of Medical Devices Part 10: Irritation and Skin Sensitization Test".

(2) The corrected hemolytic index HI test result of alginate hydrogel used in this study is 0.08, and the content of hemolytic components is lower than the GB/T16886.4-2003 "Biological Evaluation of Medical Devices Part 4: Compatibility with Blood
Interaction Test Selection" specified index. ↔

(3) According to the results of the acute systemic toxicity test, no obvious clinical symptoms

were observed in the polar and non-polar immersion test groups of alginate hydrogel used in this study, which meets the requirements of GB/T16886.11-2011 "Biological Evaluation of Medical Devices Part 11: Systemic Toxicity Tests" regarding potential acute systemic toxicity. "

(4) According to the results of the cytotoxicity test, the alginate hydrogel samples used in this study are less cytotoxic and meet the relevant provisions and requirements of GB/T16886.5-2003. ←

In summary, the alginate hydrogel used in this study has more excellent comprehensive biological properties of high biosafety, and the following studies were carried out using this alginate hydrogel.

In regard to the fact that. The promotion of angiogenesis is a critical issue for the restoration of ischemic cardiac tissue. Previous data related to the importance of angiogenesis promotion after alginate injection should be debated in the discussion along with the reduction of fibrosis. For more information the previous findings should be cited and compared with the current data as follows; https://doi.org/10.1016/j.ijbiomac.2024.129633; https://doi.org/10.1016/j.cardiores.2007.03.028; https://doi.org/10.1186/s40824-023-00449-9;

Reply 13: Thanks for your valuable comments. Since we have not conducted any experiments about the promotion of angiogenesis, we did not have this convenient discussion.

Reviewer C

General Comments:

Intramyocardial injection of hydrogels into the area of myocardial infarct (MI) early post-MI has been shown to modulated post-MI remodeling. This study evaluated the transcriptome changes of infarcted murine hearts 4 weeks after intramyocardial injection of alginate-hydrogel in comparison to sham operated and control infarcted hearts with or without intramyocardial injection of saline. In this study, the authors examine the functional and molecular impact of hydrogels in reducing cardiac modeling following myocardial infarction. Specifically, the authors use bulk RNA-seq of the infarcted area of the left ventricle to compare gene expression changes in the murine heart after treatment with hydrogel vs. saline (control). Function was assessed with echocardiography. A gene ontology (GO) enrichment analysis was conducted to screen the differentially expressed mRNAs, which included those that were upregulated and those that were downregulated. The upregulated mRNAs were mostly related to the immune response, chemokine-mediated signaling pathway, and inflammatory response, while the downregulated mRNAs were mostly associated with signal transduction, chemokine-receptor

activity, C-C chemokine-receptor activity, C-C chemokine binding, and chemokine binding. They also applied Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis to analyze the RNA sequencing data. This KEGG pathway analysis revealed that the upregulated mRNAs mainly participated in the pathways associated with the cytokine-cytokine receptor interaction, chemokine-mediated signaling pathway, and NOD-like receptor signaling pathway, while the downregulated mRNAs mainly corresponded to the pathways associated with metabolic pathways. The study reports that Beta-2-microglobulin(B2m) contributes to the development of MI by influencing early inflammation or oxidative stress after MI.

The study explores an interesting question in defining alterations in signally pathways in relation to the beneficial effects of intramyocardial hydrogel delivery post-MI. However, the relevance of this murine study to post-MI remodeling in humans considering the model of permanent coronary occlusion and the timing and method of hydrogel delivery warrants further discussion.

Specific Comments:

Methods: It appears that subsets of mice were euthanized at different time points post-MI for different types of analysis. However, the numbers of mice used for each analysis is not clearly defined and the experimental timeline is confusing. A flow diagram would be helpful in better defining the numbers and timing of imaging and tissue analyses.

Reply 1: Thanks for your valuable comments. We have modified our text as advised in Figure 1A.

Methods, Line 255: Please clarify whether RNA was extracted and sequenced separately for each biological replicate or pooled across multiple mice within the same group.

Reply 2: Thanks for your valuable comments. We have modified our text as advised.

Results, Fig. 1: How did they control for comparability of the initial ischemic insult (infarct size) between the groups of mice with surgically induced MI?

Reply 3: Each mouse in the surgical group must be observed to have an infarct area (white ischemic tissue) of approximately 3 x 3 mm^2 half an hour after ligation.

Results, Fig 2: The authors need to explain the tremendous variability in the functional and structural indices in the two control MI groups, and low variability in the hydrogel group. How did this variability influence their findings?

Reply 4: First, the hydrogel group seem to also has greater variability in Figure 2b and 2C. In addition, although we have defined the initial infarct size of the MI mice enrolled, individuals have significantly different responses to the proximal LAD coronary artery ligation surgery. Finally, low variability in the hydrogel group may suggest that our current hydrogel surgery

scheme may have a upper limit of efficacy and needs further optimization.

Results, Fig. 3B: The biological replicates of Group B are highly variable. Please explain how this impacts the identification of statistically significant results.

Reply 5: The high variability of Group B leads to significant intra group differences, which seems to reduce the number of differentially expressed genes (DEGs) between Group A and Group B. In addition, the variability of the "blue gene" in Group B at the upper right of Figure 3B is small, which seems to indicate that the greater variability in Group B seems credible.

Results, Fig. 5C: The authors show that the B2M level is elevated in the infarcted area and that the expression of this inflammatory marker decreases with hydrogel intervention. To identify the possible immune cell types that could mediate this inflammatory response, they should perform co-staining with several immune markers, e.g. CD3 for T cells, and CD45 for macrophages.

Reply 6: Thanks for your valuable comments. Subsequent experiments will be supplemented and explored. In this study, we have demonstrated that change of B2m in cardiomyocyte respond to myocardial infarction induced ischemia.

Results, Fig. 6A: Please clarify how the Venn diagram of DEGs was constructed. It appears that the DEGs between the hydrogel and saline groups were intersected with two different MsigDB gene sets, and that only one DEG co-occurred with the hypoxia gene set while 10 co-occurred with the Inflammatory response gene set. However, there is no test for significance; this can be done with a Fisher's exact test.

Reply 7: Thanks for your valuable comments. We have modified our text as advised. Since we just want to look for some molecular clues for future research, we searched for common genes between our DEGs and the two known gene datasets closely related to myocardial infarction, inflammatory response (M5932) and hypoxia 447 (M5891) from MSigDB database. Therefore, we believe that statistical analysis is not necessary.

Results, line 444-451: This reviewer recommends either removing this section, or explicitly stating the absence of statistical significance. Qualitative lists of interesting genes should be presented in the presence of a statistically significant observation.

Reply 8: Thanks for your valuable comments. Please take a look at the content of Reply 7.

Reviewer D

1. Figure 1

Please define SEM in the legend.

Reply: Thanks for your valuable comments. We have modified our text as advised.
Changes in the text: Page28, line 691

2. Figure 2

a) Please check if here should be LV-ESD and LV-EDD as the figure.

ESV), left ventricular end-diastolic volume (LV-EDV), End-Systolic Diameter (ESD), and End-Diastolic Diameter (EDD) analyses (A–G). **, P<0.01, ***, P<0.001, ****,

Reply: Thanks for your valuable comments. We have modified our text as advised.
Changes in the text: Page28, line 691

b) Please define SEM in the legend.

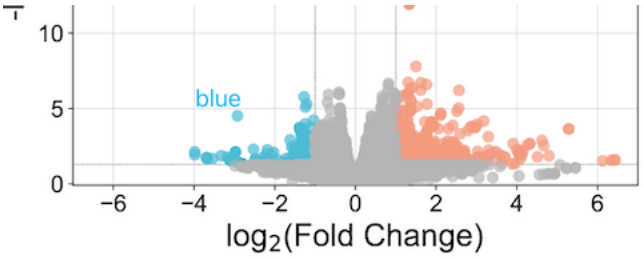
Reply: Thanks for your valuable comments. We have modified our text as advised.
Changes in the text: Page29, line 704-705

3. Figure 3

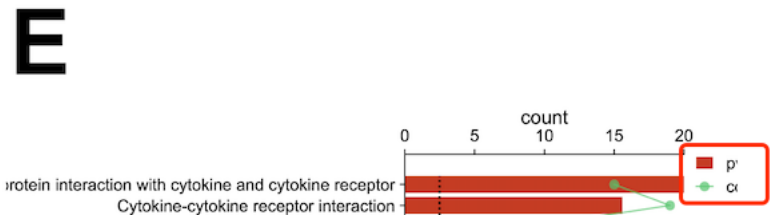
a) Here should be blue dots, please revise.

Reply: Thanks for your valuable comments. We have modified our text as advised.

735 Volcano plot of gene expression. The red dots represent upregulated expression, and the
736 green dots represent downregulated expression (fold change ≥ 2.0, P < 0.05, two-tailed



b) The words are not complete, please revise.



Reply: Thanks for your valuable comments. But our Figure 3E has the full text displayed.
Page31, line 707

4. Figure 5

a) Please provide the staining method of Figure 5C in the legend.

b) Please define DMEM and HBSS in the legend.

Reply:

a) Thanks for your valuable comments. We have added the staining method in the legend, and see the Page9, line 223-235 for the exact procedure.
b) We have modified our text as advised.
Changes in the text: Page34, line 749-750