nature portfolio

Peer Review File

Programmable production of bioactive extracellular vesicles in vivo to treat myocardial infarction

Corresponding Author: Professor David Leong

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
In this study, Authors propose a tissue-engineered approach based on an electroactive patch for macrophage-EV generation to support cardiac repair.
While this represents a significant and timely topic, there are concerns about the methodological soundness of the study which require major revision. Here are the main aspects that Authors should address in order to meet the standards of Nature Communication:
- it is not clear the rationale of using the murine microglia (BV2) cell line as source of M2 macrophages to be implanted in a rat model of myocardial injury; Authors should also used the indicated nomenclature in compliance with the minimal guidelines of the International Society of Extracellular Vesicles (ISEV) and do not use the term exosome, rather address nanoparticles as small EVs;
- Authors should provide details about the EV separation and concentration methods they used; moreover, it is mentioned that ePOWER device was cultured in EV-free medium: does this mean they used a serum(FBS)-free medium as it should be recommended?
- Authors do not provide the yield (numeber of EV particles / number of secreting BV2 cells) they obtained by NTA;ELISA may not be a reliable quantification method;
- EV characterization by WB should also include Syntenin-1 as reference marker and CD68 to monitor their M2 macrophage origin, and GRP94 to exclude contamination from apoptotic bodies in the EV preparation; an additional control group represented by secreting parental BV2 M2 cells should be also included in the analysis;
- H9c2 cells are not a reliable model to mimic cardiomyocyte behavious in vitro, especially when investigating mechanisms of myocardial renewal/proliferation since they represent a rat cardioblast cell line; Authors should use either murine neonatal ventricular cardiomyocytes or human iPSC-cardiomyocytes. Likewise, calcein and Hoechst staining is not a technically sound approach; Authors should use bona fide cell cycle indicators as markers and describe complete cytokinesis with expression of Aurora B kinase at mid bodies in the dividing cells;
- Authors should also characterize and profile the cardioprotective potential of the BV2 M2 cell-secretome (as for the in vitro cell-conditioned medium) from the ePOWER platform;
- English style and grammar should be revised.

Reviewer #3

(Remarks to the Author)
The manuscript describes a new technology called ePOWER, which is a bioelectronic interface that can produce bioactive extracellular vesicles (EVs) for localized treatment of myocardial infarction (MI). The technology uses wireless bioelectronics and a cardiac patch to stimulate embedded electrosensitive macrophages, which actively modulate EVs biosynthesis. The produced EVs have shown anti-inflammatory properties and improved cardiac function when implanted surgically. This technology holds promise as a therapeutic platform to treat post-MI dysfunctions and other human diseases. The work is novel and the idea is worth exploring.

My major concern is regarding the BV2 macrophages of brain origin used in the study. This point should be explained more thoroughly in the manuscript. A single line at the end of the discussion is not enough. Have you considered including other cell types in the patch, such as cardiac macrophages, cardiomyocytes (or iPS-derived cardiomyocytes), or MSCs?

- Title: why did you use “nanovesicles” in the title instead of “EVs”?
- The manuscript should provide details about the isolation and characterization of EVs, and not only in the supplementary section.
- After myocardial treatment, how do you ensure that all animals have the same degree of lesion?
- Regarding the BV2 macrophages-loaded ePOWER patches, how will the patch be implanted in humans? The translatability of the strategy is very important. Could you also comment on the scalability of this approach?
- Have you studied the biocompatibility of the ePOWER patch with cardiac tissue in vivo in rats?
- For the in vivo study shown in Fig. 6 “Implanted ePOWER therapy in mice with MI”, did you use mice or rats?
- Was there a statistically significant difference among groups in Fig 6c?
- What level of the heart is shown in Fig 6d?
- Could you comment on the combination therapy of ePOWER and APC groups? How was aspirin administered to rats?
- Please include a section in the supplementary materials that explains the statistics used.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
In this revised version of the manuscript, Authors have only partially addressed the previous comments that have been raised and that indicate critical weaknessess in this study. While I aprreciate that they made some effort, I am afraid there are still major concerns affecting this work which should be addressed by more robust methods and technique.

1. As indicated before, the use of BV2 microglia does not fit very well the idea of using the device to stimulate macrophages within the cardiac tissue following injury, as those final target cells may be represented by circulating monocyte-derived macrophages as we ass resident ones. I would again suggest the Authors to better address this aspect and to provide at least some preliminary data with macrophages isolated from the rat infarcted myocardial tissue.

2. Likewise, if Authors used FBS subjected to ultracentrifugation to get rid of the EV content within it, they must provide evidence that the EV-free FBS is devoid of any detectable EV-like nanoparticle by TEM, NTA and WB for canonical EV markers. Otherwise the contamination by residual FBS-derived EVs may affect data interpretation and the presented results. To overcome this aspect, Authors should have used FBS-free (also defined as serum-free) culture conditions in vitro. This does not only apply to EV separation and concentration but also to the additional experiments on target cells treated with EVs (i.e. rat cardiomyocytes) which must be performed in serum-free conditions.

3. I suggested Authors to evaluate EV yield, as the ratio of the number of particles measured by NTA on the million BV2/macrophage secreting cells. They provided EV concentration as measured by NTA (revised Figure 3, panels f and g) as EV million on ml. Besides, histogram showing the mean value for data at 5V and 20 min in the 2 graphs seems to be the exactly the same.

4. Neonatal rat cardiomyocytes should be isolated from pups within the first 72h from birth. The methodological protocol described is not appropriate (supplementary methods) as based on the use of trypsin alone, whereas the main enzyme used for this kind of method in order to release cardiomyocytes from the minced cardiac tissue is Collagenase type II. Indeed, the representative image of cardiac Troponin T (cTnT) immunostaining (suppl Figure 18) is not convincing: the Troponin T signal is very weak and not showing the canonical stripy pattern given by sarcomeric arrangement of the marker. In addition all cells seem to be expressing cTnT, whereas it is expected to have also fibroblasts within the primary culture which should be negative for sarcomeric markers. I suggest Authors to repeat this set of experiments by applying a bona fide protocol to obtain primary neonatal rat cardiomyocyte for in vitro culture.

5. Likewise, Aurora B kinase signal by immunostaining is not reliable (suppl Figure 19, panel a) as it should localize in between the dividing cells as a marker of mid bodies/cleavage furrow during cytokynesis. Besides, this analysis should be based on a double immunostaining for Aurora B kinase and a sarcomeric marker (cTnT or alpha sacomeric actinin) to identify bona fde cardiomyocytes. This analysis provides proof of cell division not cell cycle progression.

Reviewer #3

(Remarks to the Author)
The authors have revised their paper, addressing all feedback. With these improvements, the paper has met the necessary standards and is ready for publication.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

I appreciate the Authors' effort to address my comments in this round of revision. I have no further concerns at this point and I think the manuscript has now been improved to the point that it can be accepted for publication.

Reviewer#2

In this study, Authors propose a tissue-engineered approach based on an electroactive patch for macrophage-EV generation to support cardiac repair.

While this represents a significant and timely topic, there are concerns about the methodological soundness of the study which require major revision. Here are the main aspects that Authors should address in order to meet the standards of Nature communications:

1. It is not clear the rationale of using the murine microglia (BV2) cell line as source of M2 macrophages to be implanted in a rat model of myocardial injury.

Reply: Thanks for the reviewer's concern and the opportunity to clarify the rationale behind using the murine microglia (BV2) cell line in our study. The choice of BV2 cells as the source of M2 macrophages was primarily guided by their well-characterized electrosensitivity and the presence of voltage-gated ion channels, which align with the mechanism of our ePOWER technology. Furthermore, these M2-typed BV2 cells demonstrate anti-inflammatory and regenerative properties. The ePOWER patch leverages wireless bioelectronics to control and stimulate these electrosensitive macrophages, enabling localized production of bioactive extracellular vesicles that promote anti-inflammatory and regenerative effects directly at the site of myocardial injury. Previous studies have demonstrated that glial cells, including BV2 microglia, possess these voltage-gated ion channels, allowing them to respond to electrical stimulation by modulating their behavior (*BMC Biomed Eng. 4(1), 7, 2022).* This electrosensitivity is critical for our approach, as the electrical signals delivered through the ePOWER patch are designed to enhance intracellular calcium signaling pathways, which are closely associated with EV biogenesis (*J Cell Biol 217(8), 2877-2890 (2018); Nat Biomed Eng 4(1), 69-83 (2020*)). Given that calcium signaling is a key pathway modulated by electrical stimulation in BV2 cells, these cells were particularly suited to our bioelectronic interface, enabling effective and controlled EV production.

In conclusion, the strategic selection of BV2 cells was based on their compatibility with the ePOWER technology, ensuring high responsiveness to electrical currents and precise control over EV production. We have expanded the discussion in revision version to clarify this rationale more thoroughly (Main text, page 11).

2. Authors should also use the indicated nomenclature in compliance with the minimal guidelines of the International Society of Extracellular Vesicles (ISEV) and do not use the term exosome, rather address nanoparticles as small EVs.

Reply: Thank you for pointing out the importance of adhering to the ISEV guidelines. The term “exosome” has now been replaced with “EVs” throughout the text to comply with the ISEV.

3. Authors should provide details about the EV separation and concentration methods they used; moreover, it is mentioned that ePOWER device was cultured in EV-free medium: does this mean they used a serum (FBS)-free medium as it should be

recommended?

Reply: Thank you for pointing out this for us. For the details of EV separation and concentration, we used differential centrifugation to isolate and concentrate EVs. First, all the culture supernatant was collected and centrifugated at 3,000g for 20 minutes to remove cell debris and larger particles. Then the cleared supernatant was subjected to 10,000g for 30 minutes at 4ºC. After that, the obtained solution was subjected to ultracentrifugation at 100,000g for 2 hours at 4ºC. The concentration of the obtained EVs was determined using nanoparticle tracking analysis (NTA, NanoSight NS300). The vesicle concentration was then fine-tuned to approximately 50 vesicles per field of view to ensure optimal counting.

For the use of EV-free medium, we utilized vesicle-depleted FBS. This was prepared by ultracentrifugation at 120,000g for 18 hours at 4ºC to remove bovine-derived EVs. This approach ensure the EVs analyzed were exclusively derived from the cells of interest and not contaminated with serum-derived vesicles.

The detailed methods has now been added in Methods (SI, page 3).

4. Authors do not provide the yield (numeber of EV particles / number of secreting BV2 cells) they obtained by NTA; ELISA may not be a reliable quantification method.

Reply: Thank you for your good suggestion. In the revised version, we have included the yield of EV particles in Fig. 3f, g, represented as the number of EV particles per number of secreting BV2 cells, as determined by NTA. We agree that ELISA provide only relative data and not be reliable for quantifying EVs. The new data has been added as Fig. 3f,g (Main text, page 19).

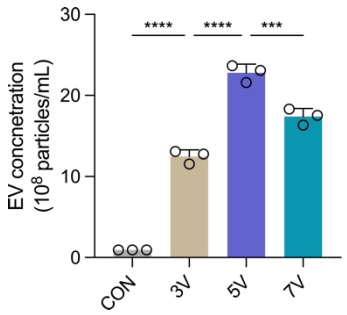


Fig. 3f, The amount of EVs generated at different applied voltages. Statistical differences were analyzed using one-way ANOVA with a Tukey multiple comparisons test. n = 3 per group; significances are presented by ***$P < 0.001$ and ****$P < 0.0001$. The data was represented by an average of ± SD.

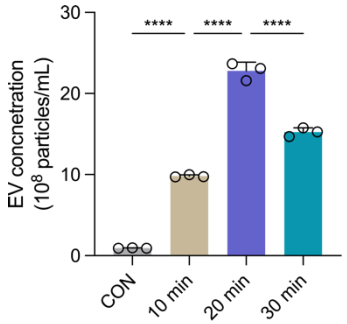


Fig. 3g, The amount of EVs generated at different durations of the stimulation. Statistical differences were analyzed using one-way ANOVA with a Tukey multiple comparisons test. n = 3 per group; significances are presented by ****$P < 0.0001$. The data was represented by an average of ± SD.

5. EV characterization by WB should also include Syntenin-1 as reference marker and CD68 to monitor their M2 macrophage origin, and GRP94 to exclude contamination from apoptotic bodies in the EV preparation; an additional control group represented by secreting parental BV2 M2 cells should be also included in the analysis.

Reply: We heeded the reviewer's advice and added Syntenin-1 as a reference marker and CD68 to confirm the M2 macrophage origin of the EVs in our WB analysis. Additionally, we included GRP94 to rule out any contamination from apoptotic bodies in the EV preparation. Furthermore, additional control group consisting of secreting parental BV2 M2 cells has also been added in the analysis to enhance accuracy of our findings. The new data has been added as Fig. 3f,g (Main text, page 19).

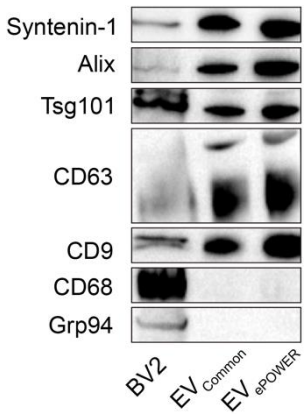


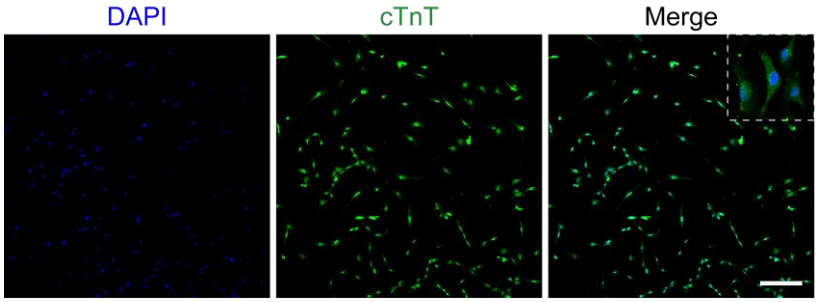
Fig. 3d, Western blot analysis of specific vesicular marker proteins (Syntenin-1, Alix, Tsg101, CD63, CD9, CD68, Grp94) in BV2-derived EVs generated with and without ePOWER stimulation..

6. H9c2 cells are not a reliable model to mimic cardiomyocyte behaviors in vitro, especially when investigating mechanisms of myocardial renewal/proliferation since they represent a rat cardioblast cell line; Authors should use either murine neonatal ventricular cardiomyocytes or human iPSC cardiomyocytes. Likewise, calcein and

Hoechst staining is not a technically sound approach; Authors should use bona fide cell cycle indicators as markers and describe complete cytokinesis with expression of Aurora B kinase at mid bodies in the dividing cells.

Reply: Thanks for the reviewer's advice. We recognize the limitations of using H9c2 cells for assessing the cardiomyocyte behaviors. According to the reviewer's good suggestion, we established a primary rat cardiomyocyte culture, which was confirmed by immunofluorescence staining for cardiac troponin T, a specific marker for cardiomyocytes. As shown in Supplemental Figure 18, the expression rate of cardiac troponin T exceeds 95%, indicating the successful establishment of the primary rat cardiomyocyte model.

According to reviewer's recommendation about the use of more accurate cell proliferation markers, we also examined the expression of Aurora B kinase, a key marker of cytokinesis, in primary rat cardiomyocytes treated with EVs derived under different conditions. The revised Figure 5j presents immunofluorescence images demonstrating that EVs derived from the ePOWER platform effectively induce the expression of Aurora B kinase in a time-dependent manner (Supplemental Figure 18). This result suggests that the ePOWER-derived EVs promote cardiomyocyte proliferation. These new data have been added as Fig. 5j (Main text, page 22) and Fig. S18, 20 (SI, page 26 and 28).



Supplementary Fig. 18. Representative immunofluorescence staining of Cardiac Troponin T (cTnT) in the primary rat cardiomyocyte cells. Scale bar, 200 μm.

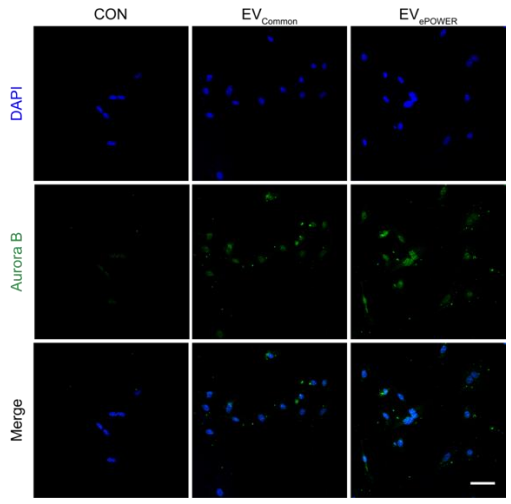
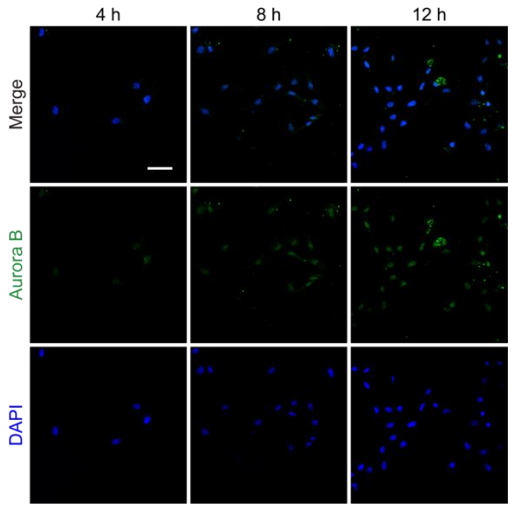


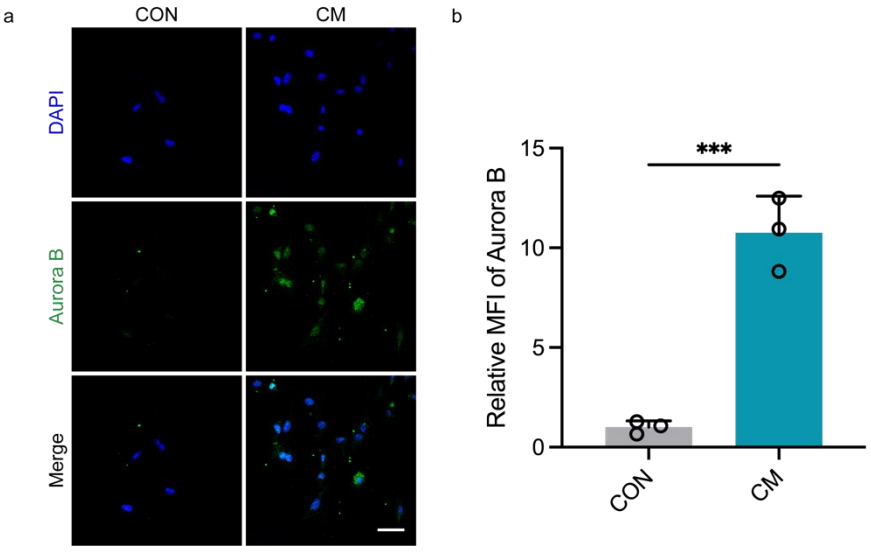
Fig. 5j. Proliferation of primary rat cardiomyocytes at 12 hours treated with EVs collected from common culture strategy and ePOWER stimulus. Scale bar, 50 μm.



Supplementary Fig. 20. Representative immunofluorescence staining of Aurora B in the primary rat cardiomyocyte cells at different time points after co-incubation with EV_{ePOWER}. Scale bar, 50 μm.

7. Authors should also characterize and profile the cardioprotective potential of the BV2 M2 cell-secretome (as for the in vitro cell-conditioned medium) from the ePOWER platform.

Reply: Following the reviewer's good advice, we have undertaken additional experiments to analyze the evaluate the cardioprotective properties of the BV2 M2 cell-secretome. We collected conditioned medium from BV2 M2 macrophages that were cultured on the ePOWER platform under electrically stimulated conditions. This conditioned medium was then applied to primary cardiomyocytes to assess its impact on cardiomyocyte proliferation. As demonstrated in Fig. S20, the cells treated with conditioned medium (CM group) exhibited increased expression of Aurora B, a marker of cell division, indicating that the BV2 M2 cell-secretome effectively promotes cardiomyocyte proliferation. The new data have been added as Fig. S19 (SI, page 27).



Supplementary Fig. 19. (a) Immunofluorescence staining of Aurora B in the primary

rat cardiomyocyte cells treated with normal medium (CON) or BV2-derived conditional medium (CM) (n=3), along with (b) the corresponding quantitative analysis (scale bar, 50 μm).

8. English style and grammar should be revised.

Reply: Thank you for this suggestion. We have carefully checked the manuscript to improve the english style and grammar for more clarity and readability of our work. If there are any specific areas that still need attention, we would appreciate further guidance. Thank you for your valuable input.

Reviewer#3:

The manuscript describes a new technology called ePOWER, which is a bioelectronic interface that can produce bioactive extracellular vesicles (EVs) for localized treatment of myocardial infarction (MI). The technology uses wireless bioelectronics and a cardiac patch to stimulate embedded electrosensitive macrophages, which actively modulate EVs biosynthesis. The produced EVs have shown anti-inflammatory properties and improved cardiac function when implanted surgically. This technology holds promise as a therapeutic platform to treat post-MI dysfunctions and other human diseases. The work is novel and the idea is worth exploring.

1. My major concern is regarding the BV2 macrophages of brain origin used in the study. This point should be explained more thoroughly in the manuscript. A single line at the end of the discussion is not enough. Have you considered including other cell types in the patch, such as cardiac macrophages, cardiomyocytes (or iPS-derived cardiomyocytes), or MSCs?

Reply: Thank you for highlighting this very good point about the rationale of choosing BV2 microglia as the embedded cells in our study. Our selection of M2-typed BV2 cells was primarily guided by their well-documented electrosensitivity with the presence of voltage-gated ion channels and anti-inflammatory and regenerative properties. These channels can be modulated by weak electric currents, aligning perfectly with the ePOWER technology, which uses an electroactive patch for wireless and controllable EV generation. Previous studies have demonstrated that glial cells, including BV2 microglia, possess these ion channels, enabling them to respond to electrical stimulation by altering their behavior (*BMC Biomed Eng. 4(1), 7, 2022*). Additionally, recent research has highlighted the critical role of intracellular calcium ion pathways in EV biogenesis, which can be regulated through electrical signals (*J Cell Biol 217(8), 2877-2890 (2018); Nat Biomed Eng 4(1), 69-83 (2020)*). Given that calcium signaling is a key pathway modulated by electrical stimulation in BV2 cells, we anticipated that these cells would be particularly responsive to our bioelectronic interface, thereby enhancing the production of bioactive EVs in response to the controlled electrical stimuli provided by ePOWER.

We fully understand the reviewer's suggestion to consider including other cell types, such as cardiac macrophages, cardiomyocytes, or mesenchymal stem cells (MSCs), in our patch. While each of these cell types has unique advantages, we chose BV2 microglia for specific reasons aligned with the objectives of our study. For cardiac macrophages, while cardiac macrophages are directly relevant to the myocardial environment, their electrosensitivity and response to the type of electrical stimulation used in ePOWER are less well-characterized compared to BV2 microglia. Additionally, the complex isolation and culture requirements of cardiac macrophages pose challenges to the scalability and reproducibility of EV production in our system. For cardiomyocytes or iPS-derived cardiomyocytes, they are directly involved in cardiac repair and could theoretically enhance the regenerative capacity of the patch. However, integrating electrosensitive macrophages with cardiomyocytes would require careful

optimization of electrical stimulation protocols to ensure both cell types respond appropriately. This added complexity could potentially hinder the precise control over EV production that ePOWER is designed to achieve. For mesenchymal stem cells (MSCs), MSCs are indeed a promising alternative due to their regenerative properties and ability to secrete therapeutic EVs. However, similar to cardiomyocytes, their response to electrical stimulation would need to be finely tuned to ensure that the bioelectronic interface modulates EV production as intended. Moreover, MSCs are known for their heterogeneity, which could introduce variability in EV output.

A key innovation of our study is the ability to wirelessly and programmatically control EV production using the ePOWER patch, which integrates electrosensitivity and EV production. This technology leverages the specific electrosensitive properties of BV2 microglia, which have been shown to modulate their behavior, including EV biosynthesis with anti-inflammatory and regenerative properties, in response to electrical stimuli. Targeted stimulation of voltage-gated ion channels in BV2 cells via ePOWER directly correlates with enhanced calcium signaling pathways, crucial for EV release. Thus, BV2 cells were strategically chosen for their responsiveness to electrical currents, making them ideal candidates for this bioelectronic application. The electrosensitivity of other cell types is less well-defined, which could compromise the precision and efficacy of the EV production process. Additionally, focusing on a single, well-characterized cell type allows for better control and optimization of variables involved in EV production, ensuring high reproducibility and consistency across experiments.

To address the reviewer's concern, we have expanded the manuscript discussion to provide a more detailed rationale for using BV2 macrophages. We have also acknowledged the potential benefits and challenges of incorporating other cell types, such as cardiac macrophages, cardiomyocytes, or MSCs, into the patch. Future research could explore engineering these functional cells with the expression of electric sensors like Cav1.1 and genetic transducing modules to enhance their sensitivity to electrical signals generated by ePOWER, potentially accelerating the electric-genetic transduction into cellular activities related to EV biogenesis. These considerations are now discussed as potential directions for further research to enhance the therapeutic efficacy of the patch (Main text, page 11).

2. Title: why did you use “nanovesicles” in the title instead of “EVs”?

Reply: Thank you for correcting for us. We have replaced “nanovesicles” with "EVs" throughout the manuscript to ensure accuracy and clarity.

3. The manuscript should provide details about the isolation and characterization of EVs, and not only in the supplementary section.

Reply: Thank you for your kind advice. We have now expanded the methods to include detailed descriptions of the isolation and characterization to ensure clarity and reproducibility of our findings. This information was also incorporated into the caption

of Figure 3 (page 20).
"For the details of EV separation and concentration, we used differential centrifugation to isolate and concentrate EVs. First, all the culture supernatant was collected and centrifugated at 3,000g for 20 minutes to remove cell debris and larger particles. Then the cleared supernatant was subjected to 10,000g for 30 minutes at 4oC. After that, the obtained solution was subjected to ultracentrifugation at 100,000g for 2 hours at 4oC. The concentration of the obtained EVs was determined using nanoparticle tracking analysis (NTA, NanoSight NS300). The vesicle concentration was then fine-tuned to approximately 50 vesicles per field of view to ensure optimal counting."

The detailed methods has now been added in Methods (SI, page 3).

4. After myocardial treatment, how do you ensure that all animals have the same degree of lesion?
Reply: Thank you for your question regarding the consistency of myocardial lesions across the treated animals. To ensure that all animals had a similar degree of myocardial lesion after treatmen, we implemented standardized protocols for inducing myocardial infarction model (*Nat Biomed Eng. 2(5), 293–303, 2018; J Control Release. 147, 30-37, 2010*). Specifically, 10-week-old male Sprague Dawley rats were fasted for 12 hours before undergoing myocardial infarction surgery. During the procedure, the left anterior descending (LAD) coronary artery was ligated to induce myocardial infarction, with the duration of artery occlusion and release kept within 15 seconds. On the following day, we measured the left ventricular ejection fraction (LVEF) using echocardiography. As shown in Figure 6c, rats with LVEF between 50% and 60% at day 2 post-MI were included in the study and randomly assigned to one of four treatment groups. By employing these measures, we ensure that all animals have a comparable degree of myocardial lesion at the the start of the treatment, thereby allowing for a fair comparison of the therapeutic effects.

5. Regarding the BV2 macrophages-loaded ePOWER patches, how will the patch be implanted in humans? The translatability of the strategy is very important. Could you also comment on the scalability of this approach?
Reply: Thank you for this important question about the clinical translatability and the scalability of our BV2 macrophages-loaded ePOWER patches.
For clinical application in humans, the implantation of the ePOWER patch would likely be performed through minimally invasive surgical techniques. The patch could be applied directly to the epicardial surface of the heart during a procedure such as thoracoscopy or via a small thoracotomy. These approaches are already established in cardiac surgery for procedures like epicardial ablation and coronary artery bypass grafting. The BV2 macrophages could be replaced with autologous or allogeneic human macrophages, which would be loaded onto the patch before implantation. The patch would be designed to conform to the heart's surface, ensuring effective delivery

and retention of the therapeutic cells at the site of injury.
The scalability of this approach is promising. The production of the ePOWER patch can be scaled using established manufacturing methods such as electrospinning or 3D bioprinting, which are capable of producing patches in large quantities with consistent quality. For the cellular component, human macrophages can be expanded and differentiated in vitro under controlled conditions, making it feasible to produce patient-specific patches efficiently. Additionally, an allogeneic approach with pre-prepared macrophage banks could provide an off-the-shelf solution, further enhancing scalability.
In conclusion, the ePOWER patch could be optimized to be both translatable to human use and scalable in production, supported by existing surgical techniques and advanced manufacturing processes. This discussion has been added into the revised version (Main text, page 11).

6. Have you studied the biocompatibility of the ePOWER patch with cardiac tissue in vivo in rats?

Reply: Yes, we have conducted a comprehensive biocompatibility study of the ePOWER patch with cardiac tissue and blood samples in rats. The results from our study confirm that the ePOWER patch is biocompatible with cardiac tissue. Specifically, the Masson staining of heart tissue shown in Figure 6d demonstrates that ePOWER treatment did not increase myocardial fibrosis, indicating that the patch does not induce adverse fibrotic responses in the heart. Histological analysis further demonstrated no significant differences in cardiac tissue between the ePOWER-treated group and the control group (Supplementary Fig. 23a). Additionally, we assessed the biocompatibility of the patch with other major organs, including the liver, spleen, lungs, and kidneys (Supplementary Fig. 23b). The results revealed no significant differences between the ePOWER-treated group and the control group, which supports the systemic biocompatibility of the ePOWER patch.

In response to your query, we have also included post-treatment blood analysis of the rats in the revised manuscript. The newly added data on blood routine and blood biochemistry (Supplementary Fig. 25) show that all parameters in both the control and ePOWER treatment groups were within the normal ranges. Importantly, the levels of lactate dehydrogenase (LDH) and creatine kinase (CK), which are important markers associated with myocardial injury, were not elevated in the ePOWER-treated group, further confirming the safety and biocompatibility of the ePOWER patch. We have included these results in the revised manuscript to provide a comprehensive understanding of the biocompatibility of the ePOWER patch (SI, page 31-33).

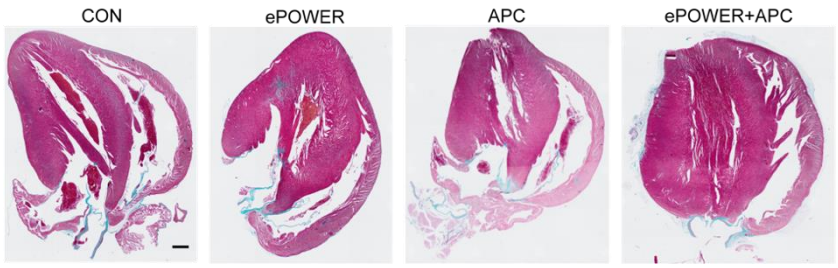
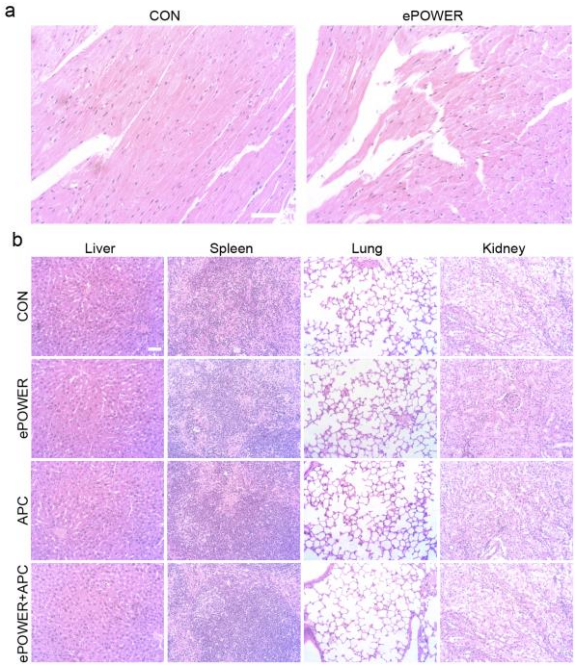
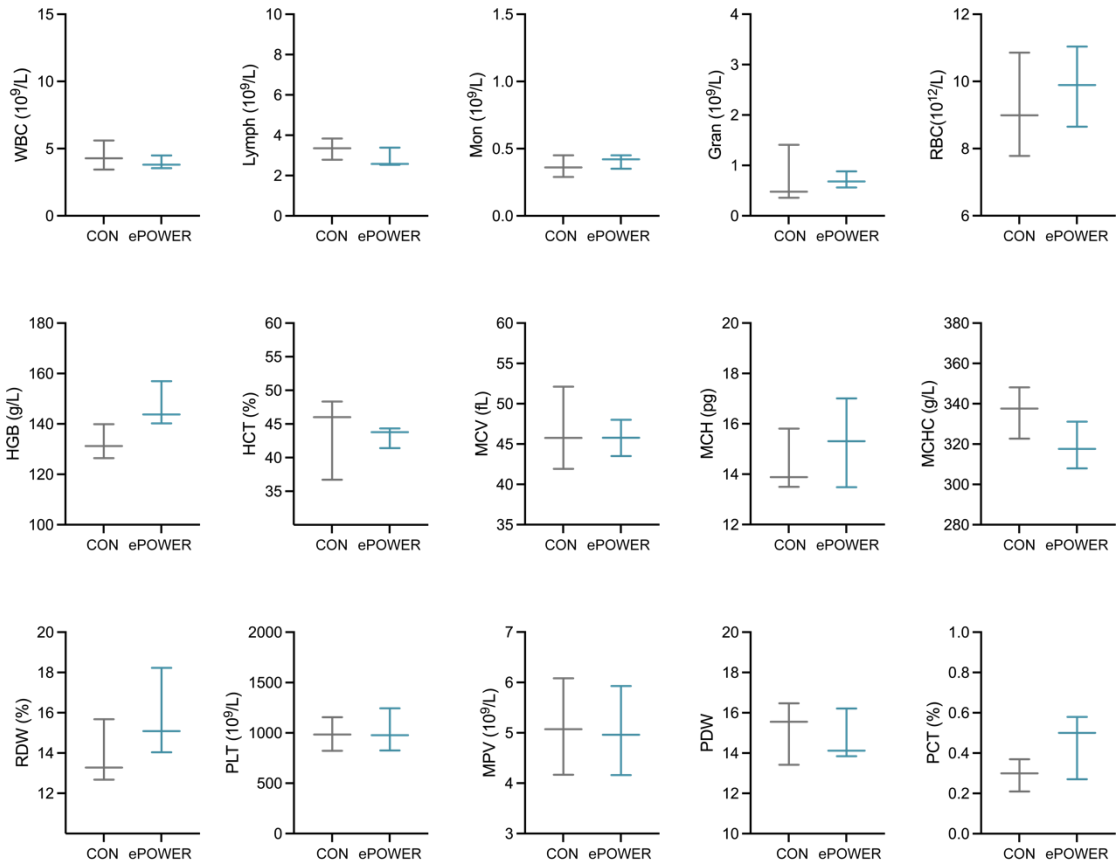


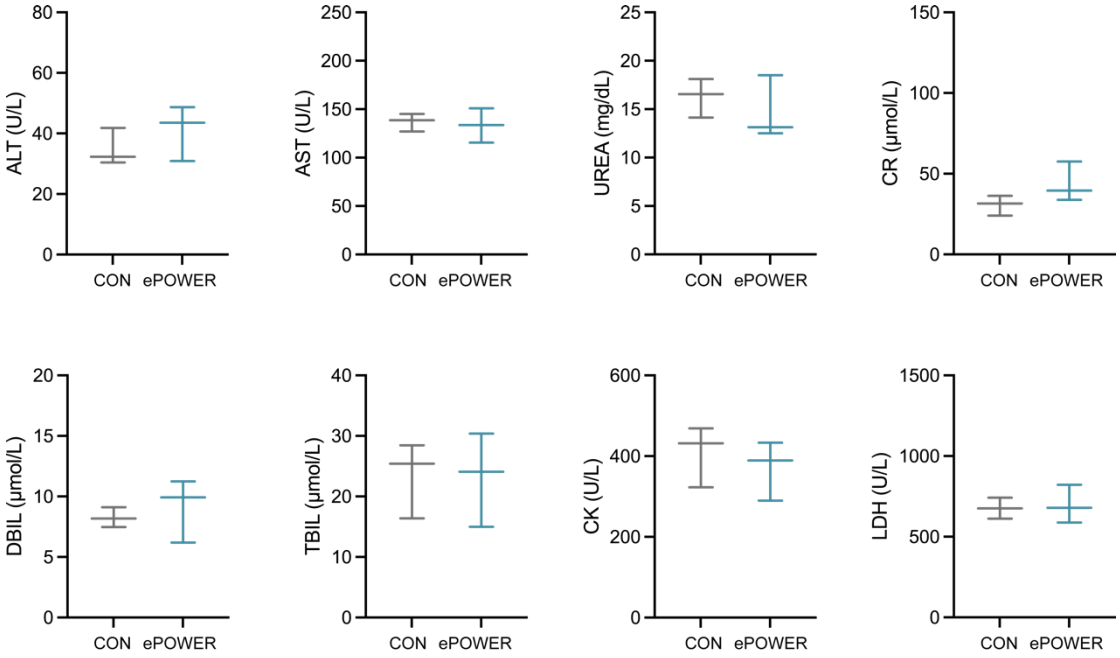
Fig. 6d, Representative MASSON staining of heart tissues in different groups (Scale bar, 1 mm).



Supplementary Fig. 23. The biocompatibility of the ePOWER patch. (a) Hematoxylin and Eosin (H&E) of the cardiac tissue on day 28 (n = 3; scale bars, 100 μm) and (b) Masson staining of the liver, spleen, lung and kidney on day 28 (scale bars, 100 μm).



Supplementary Fig. 25. Standard hematological safety tests for rats in CON group or ePOWER treatment group.



Supplementary Fig. 24. The blood chemistry for rats in CON group or ePOWER treatment group.

7. For the in vivo study shown in Fig. 6 “Implanted ePOWER therapy in mice with MI”,

did you use mice or rats?

Reply: We used Sprague-Dawley (SD) rats for in vivo study. We apologize for this confusion. We have corrected these inaccuracies in the revised manuscript to ensure consistency and accuracy in describing the animal model used in our experiments.

8. Was there a statistically significant difference among groups in Fig 6c?

Reply: Thank you for your question. We compared the groups using one-way ANOVA with a Tukey multiple comparisons test. The analysis revealed that there were statistically significant difference among the groups. We have now updated Fig 6c and included this statistic information in figure legend for clarity (Main text, page 24).

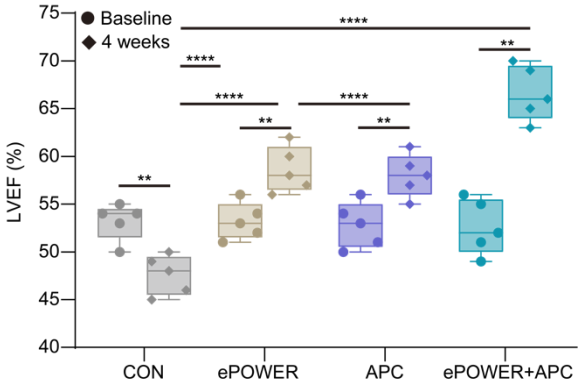


Fig. 6c, Quantification of LVEF percentage after 4 weeks of treatment (n = 5). **P < 0.01, ***P < 0.001, ****P < 0.0001 were considered statistically significant.

9. What level of the heart is shown in Fig 6d?

Reply: Fig. 6d shows the representative transverse cardiac sections from the mid-ventricular region. This level was chosen to provide a clear and comprehensive view of the myocardial tissue, allowing for an accurate assessment of the therapeutic effects of different treatments. We have included more detailed description of Fig 6d in the revised version (Main text, page 9)

10. Could you comment on the combination therapy of ePOWER and APC groups? How was aspirin administered to rats?

Reply: Thank you for your question about the combination therapy of the ePOWER and APC groups. The combination therapy group involving ePOWER and APC was designed to evaluate the potential synergistic effects of both treatments on myocardial infarction (MI) recovery. The ePOWER therapy was aimed at controllable producing therapeutic EVs to modulate homeostasis and promote tissue repair, while APC is known to provide cardioprotection through its anti-inflammatory and antithrombotic properties.

Aspirin was administered to the rats via gavage at a dosage of 100 mg/kg/day. The administration began one day after the induction of myocardial infarction and continued daily throughout the duration of the study. This method ensures consistent and

controlled delivery of the medication, allowing us to accurately assess the combined therapeutic effects of ePOWER and aspirin. We have included this experimental details in the revised version (SI, page 7).

11. Please include a section in the supplementary materials that explains the statistics used.

Reply: Thanks for pointing out this for us. The statistics have now been added as Statistical analysis and reproducibility in the supplementary materials (SI, page 8).

Statistical analysis was performed using GraphPad Prism software (v.8.0). Data were analyzed by one-way ANOVA (multiple comparisons) with Tukey's test and unpaired two-tailed t-test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 were considered statistically significant. All results were presented as mean ± s.d. Unless otherwise stated, biological replicates were used for all experiments.

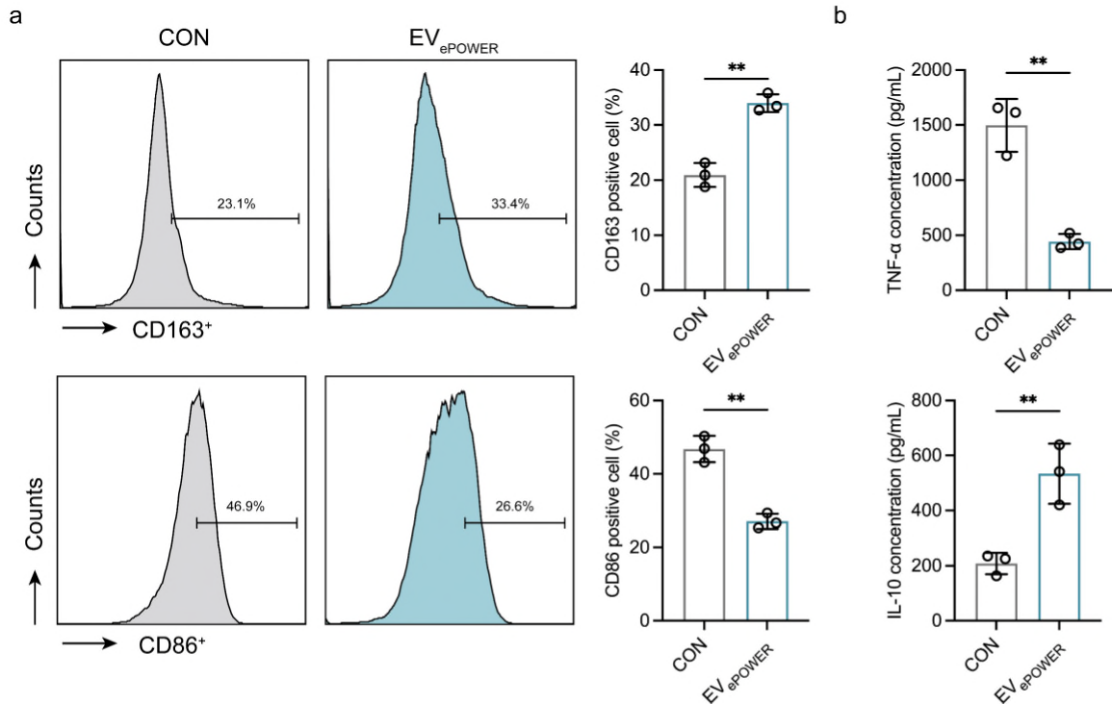
Reviewer#2

In this revised version of the manuscript, Authors have only partially addressed the previous comments that have been raised and that indicate critical weaknessess in this study. While I aprreciate that they made some effort, I am afraid there are still major concerns affecting this work which should be addressed by more robust methods and technique.

Response: We thank the reviewer for the thoughtful and thorough assessment of our work. We are sorry that we did not completely address the concerns that were mentioned in the first revision. In this revision, we have done all the suggested experiments and many thanks for giving us opportunities to improve on our manuscript.

1. As indicated before, the use of BV2 microglia does not fit very well the idea of using the device to stimulate macrophages within the cardiac tissue following injury, as those final target cells may be represented by circulating monocyte-derived macrophages as we ass resident ones. I would again suggest the Authors to better address this aspect and to provide at least some preliminary data with macrophages isolated from the rat infarcted myocardial tissue.

Response: Thank you for this important point. We heeded your suggestions and isolated macrophages from infarcted heart tissue. We then treated them with IL-4 cytokines to obtain anti-inflammatory macrophages and loaded on our ePOWER platform. The EVs secreted under these conditions were collected and used to treat additional macrophages isolated from the infarcted myocardium. As shown in **Supplementary Fig. 27**, the ePOWER-derived EVs significantly enhanced the expression of the anti-inflammatory macrophage marker CD163 while reducing the pro-inflammatory marker CD86. This correlated with lower TNF-α levels and higher IL-10 levels. These preliminary results suggest that EVs produced on the ePOWER platform can favorably modulate cardiac-tissue macrophages by promoting an anti-inflammatory phenotype and potentially facilitating repair. We have added these new data in the revised section (**Supplementary Fig. 27**).



Supplementary Fig. 27 Phenotypic changes in macrophages isolated from infarcted myocardial tissue following treatment with EV_{ePOWER}. The ePOWER system was embedded with macrophages isolated from myocardial tissue. (**a**) Flow cytometry analysis of CD163 and CD86 expression on macrophages. (**b**) The analysis of TNF-α and IL-10 cytokine levels in EV_{ePOWER}-treated macrophages.

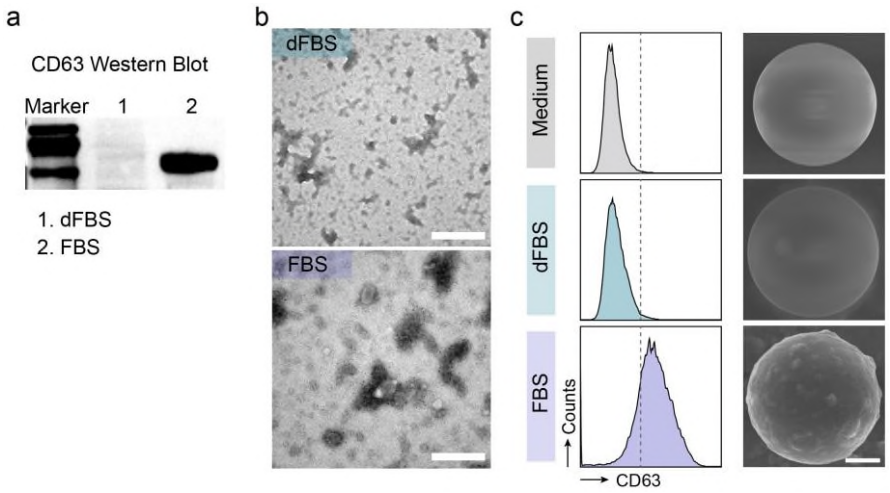
2. Likewise, if Authors used FBS subjected to ultracentrifugation to get rid of the EV content within it, they must provide evidence that the EV-free FBS is devoid of any detectable EV-like nanoparticle by TEM, NTA and WB for canonical EV markers. Otherwise the contamination by residual FBS-derived EVs may affect data interpretation and the presented results. To overcome this aspect, Authors should have used FBS-free (also defined as serum-free) culture conditions in vitro.

This does not only apply to EV separation and concentration but also to the additional experiments on target cells treated with EVs (i.e. rat cardiomyocytes) which must be performed in serum-free conditions.

Response: We appreciate your concern regarding potential FBS-resident EV contamination. Following your excellent suggestions, for this revision, we performed additional analyses to confirm the absence of EV-like particles in our depleted FBS (dFBS). As shown in **Supplementary Fig. 9a**, sensitive Western blot analysis could not detect any appreciable amounts of CD63, the common EV marker, in the dFBS sample. No apparent vesicle structures were observed in the TEM images of dFBS samples compared to FBS medium (**Supplementary Fig. 9b**). Furthermore, in **Supplementary Fig. 9c**, we used CD9 antibody functionalized-polystyrene microspheres to capture potential EVs from different media (basic medium, dFBS-containing medium, and FBS-containing medium), followed by flow cytometric analysis and SEM imaging of the microspheres. Compared with the FBS-containing group, the dFBS group did not

show detectable levels of EV-like structures of CD63 fluorescence. Based on these observations, we are confident that our dFBS is effectively depleted of FBS-resident EVs. All subsequent EV isolation and functional studies used this EV-depleted FBS. We hope these clarifications address your concerns and confirm that our results primarily reflect EVs generated by the ePOWER platform.

With respect to the suggested serum-free media culture, we fully agree that it would be a cleaner experiment, scientifically. However, we weighed this against the additional uncertain stress imposed onto the cells by culturing in serum free condition over rather long periods of time. Moreover, in vivo, even at the injured sites, cells are flooded with serum. Thus, with a bit of dFBS serum in the culture is a closer recapitulation of the healing process than without any serum.



Supplementary Fig. 9. The analysis of dFBS. (**a**) The western blot analysis of dFBS and FBS. (**b**) The TEM analysis of dFBS and FBS. The samples were negative stained with 1% uranyl acetate. Scale bar, 200 nm. (**c**) The representative flow cytometry analysis (left) and SEM images (right) of EVs immuno-captured by anti-CD9 functionalized beads. CD9 antibody beads were incubated with different mediums and then detected with CD63 antibody. Scale bar, 500 nm.

3. I suggested Authors to evaluate EV yield, as the ratio of the number of particles measured by NTA on the million BV2/macrophage secreting cells. They provided EV concentration as measured by NTA (revised Figure 3, panels f and g) as EV million on ml. Besides, histogram showing the mean value for data at 5V and 20 min in the 2 graphs seems to be the exactly the same.

Response: We apologize for any confusion in our previous presentation of EV yield. In the revised Figure 3, the EV yield is now expressed as a ratio of the number of particles per million secreting cells (10^6 EV particles per million cells) for clarity.

Regarding the identical value for the 5V/20 min condition in **Figures 3 f** and **g**, we confirm that this overlap reflects the intersection of our dual-variable (voltage + time)

experimental design in which we systematically tested time and voltage in parallel using the same batch of samples. Thus, the identical histogram values are the same because it is the same set of data plotted on the two segregated variables. To reduce the confusion, we have replotted the data into a dual variable plot.

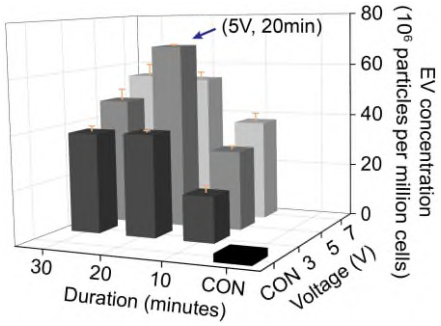


Fig 3 f, EV yield under varying applied voltages and stimulation durations of the ePOWER system. The optimal yield was achieved at the condition of 5 V and 20 min.

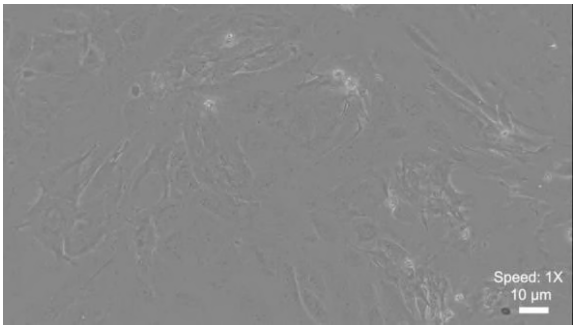
4. Neonatal rat cardiomyocytes should be isolated from pups within the first 72h from birth. The methodological protocol described is not appropriate (supplementary methods) as based on the use of trypsin alone, whereas the main enzyme used for this kind of method in order to release cardiomyocytes from the minced cardiac tissue is Collagenase type II. Indeed, the representative image of cardiac Troponin T (cTnT) immunostaining (suppl Figure 18) is not convincing: the Troponin T signal is very weak and not showing the canonical stripy pattern given by sarcomeric arrangement of the marker. In addition all cells seem to be expressing cTnT, whereas it is expected to have also fibroblasts within the primary culture which should be negative for sarcomeric markers. I suggest Authors to repeat this set of experiments by applying a bona fide protocol to obtain primary neonatal rat cardiomyocyte for in vitro culture.

Response: Thank you for your guidance and patience. We have learned a lot. In this revision, we have improved the protocol for isolating and staining rat cardiomyocytes in accordance with standard methods (*Frontiers in Bioengineering and Biotechnology 10 (2022): 902038.*). In detail, the primary rat cardiomyocytes were isolated from the heart tissue of rats aged 1 day. After anesthetizing the rats, the hearts were excised and finely minced. The tissue fragments were washed by Hank's Balanced Salt Solution (HBSS) and then subjected to digestion with 1 mg/mL of type II collagenase (Gibco) solution at 37℃ for 10 minutes. The digestion process was repeated 4-5 times. The supernatants were collected and centrifuged at 1,000 rpm for 5 minutes to collect the cells. The collected cells were pre-plate for 45 minutes twice to remove Cardiac fibroblasts for enrichment of cardiomyocytes. Finally, the isolated rat cardiomyocytes were plated on 1% gelatin-coated petri dishes and cultured in completed medium DMEM medium supplemented with 10% FBS for 4 days.

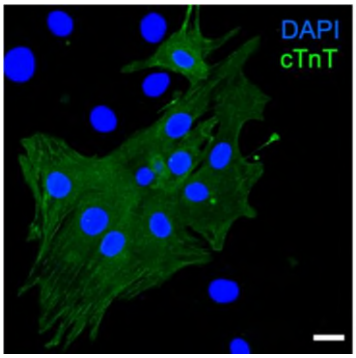
We provide new data in the supplementary materials:

(1) A video demonstrating the spontaneous beating of the isolated cells as an indication

of functional cardiomyocytes.
(2) Supplementary Fig. 19, showing improved immunofluorescence staining for cardiac Troponin T (cTnT), exhibiting the characteristic striated pattern.
We greatly appreciate your valuable guidance on this.



Supplementary Fig. 18. The isolated cardiomyocytes with beating behavior. Scale bar: 10 µm.



Supplementary Fig. 19. The immunofluorescence staining of the primary rat cardiomyocytes. Blue, DAPI; Green, Cardiac Troponin (cTnT). Scale bar, 10 µm.

New reference has been added:
52. Costa, Ambra, et al. Investigating the paracrine role of perinatal derivatives: human amniotic fluid stem cell-extracellular vesicles show promising transient potential for cardiomyocyte renewal. *Front Bioeng Biotechnol* 10, 902038 (2022).

5. Likewise, Aurora B kinase signal by immunostaining is not reliable (suppl Figure 19, panel a) as it should localize in between the dividing cells as a marker of mid bodies/cleavage furrow during cytokynesis. Besides, this analysis should be based on a double immunostaining for Aurora B kinase and a sarcomeric marker (cTnT or alpha sacomeric actinin) to identify bona fde cardiomyocytes. This analysis provides proof of cell division not cell cycle progression.
Response: Thank you for emphasizing the proper identification of cardiomyocyte proliferation. In this revision, we headed your suggestion and incorporated a double immunostaining protocol for Aurora B kinase (AuBk) and cardiac Troponin T (cTnT). As shown in our revised Figure 5j, we now observe AuBk signals at the mid-bodies of

dividing cardiomyocytes that co-express cTnT. These results support the conclusion that ePOWER-derived EVs can enhance bona fide cardiomyocyte proliferation. We hope this improved methodology addresses your concerns about confirming cytokinesis rather than merely indicating cell cycle progression.

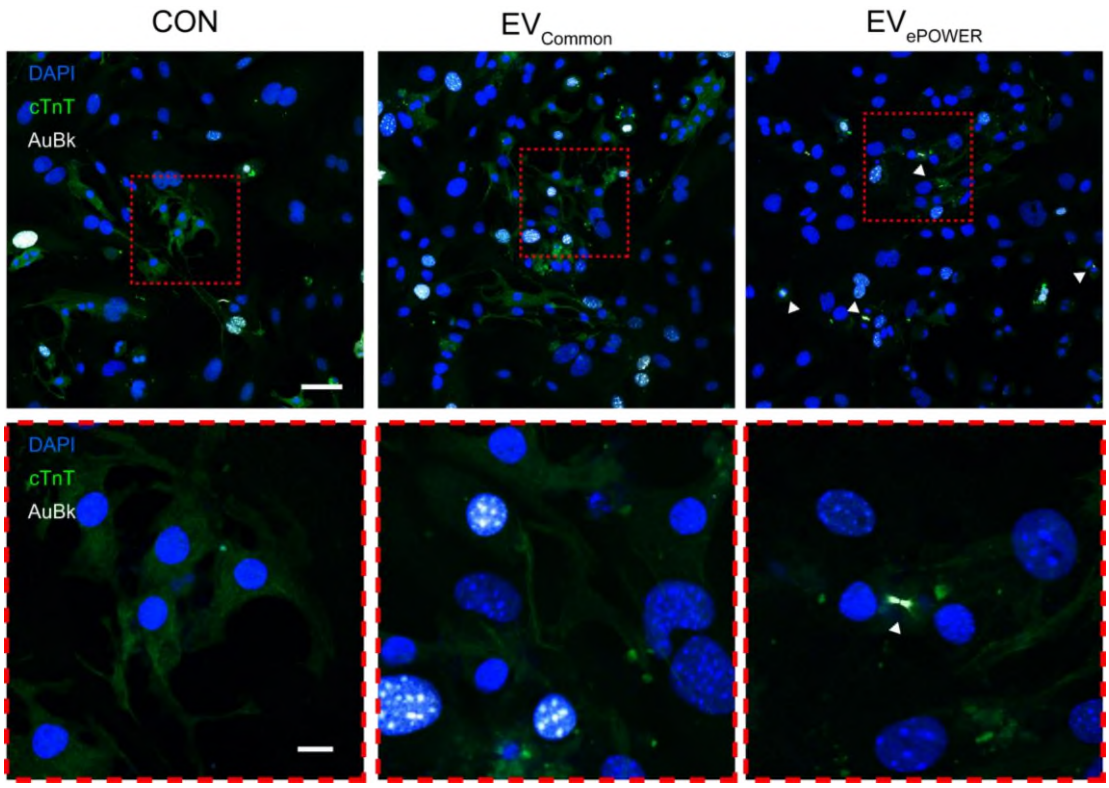


Fig. 5j, Proliferation of rat cardiomyocytes at 12 hours treated with various sourced EVs (Common culture strategy and ePOWER stimulus). Scale bar (top), 50 μm; scale bar (down), 10 μm. White arrows denote the presence of AuBK at cell mid-body.