Dear Editors and Reviewers,

Thank you for your letter and for the reviewers' comments concerning our manuscript entitled "**Extracellular vesicles' miR-224-5p from hypoxia preconditioned mesenchymal stem cells alleviates myocardial injury by targeting TXNIP-Mediated HIF1α Pathway**" (**ID: 72372**). Those comments are all valuable and very helpful for revising and improving our manuscript. We have studied the comments carefully and have made corrections which we hope that our revised manuscript will meet approval. The main corrections and the replies to the editors' and reviewers' comments are replied point-by-point as follows (all the editors' and reviewers' comments are marked in red):

ETHICAL CORRECTIONS

The manuscript has been edited by Filipodia publishing(Filipodia ID: FP40009)

(1) Science editor:

The questions addressed by Mao and colleagues are highly relevant and their observations of great interest since they shed light on the mechanisms that are involved in the increased survival and resistance of cardiomyocytes to myocardial infarction in vivo and hypoxia in vitro following exposure to ADSC-derived EVs. Moreover, they identify miR-224 as a key player in these processes and show that expression of this factor is upregulated in ADSC-derived EVs following culture of these cells under hypoxic conditions, as compared to ADSCs grown in normoxic conditions. There are, however, a series of points that the authors must address before this manuscript can be considered for its publication in WJSC. Firstly, there are several questions that the reviewers have raised that must be answered, such as adding additional information to the Methods section and the graphs, demonstrating the knocking-out of miR-224 in ADSCs, editing of the English language, etc. Their queries must be answered. In addition, there are other requirements that should be met: - Many of the guidelines provided by WJSC for submitted

manuscripts have been ignored: The title is over 18 words-long, the font employed is not Book Antiqua, co-first authors and co-corresponding authors have been designated, although it is clearly specified that this is not allowed by the journal guidelines, names of the authors and affiliations have not been indicated accordingly, authors have not provided their ORCID numbers, supporting information and author contributions have not been indicated in the title page, the ARRIVE guidelines statement is missing, as is the core tip, the abstract is shorter than 350 words and it is not structured as indicated by the guidelines to authors, the references are not indicated within square brackets in the text, nor are their PMID or DOI numbers provided, nor the style adecuate, etc. - Besides English editing being required, the text is also difficult to read because it is plagued with initials that are not explained upon their first mention in the text but much later - Regarding the figures, these must be improved. Their legends are often unclear, not well explained (e.g. arrows in Figure 3: do these mean significant differences? what level of significance?), and not well-structured (e.g. Figure 3-A1, A2?). Not all the figures have been introduced in the text (e.g. Figure 1A). Importantly, when it comes to photographs of cell cultures and to some of the graphs, these are far too small for the results to be properly interpreted. The Graphical Abstract shown at the end of the manuscript has not been introduced in the text and it is not clearly explained: there are elements in the figure that do not correspond to what has been discussed in the legend.

Language Quality: Grade B (Minor language polishing)

Scientific Quality: Grade B (Very good)

Response to science editor:

Thank you very much for your positive feedback and meaningful suggestions for our manuscript, below are the point-by-point responses to each of your comments. The title has been refined to “Extracellular vesicles’ miR-224-5p from hypoxia preconditioned mesenchymal stem cells alleviates myocardial injury by targeting TXNIP-mediated HIF1α pathway”. The font has been

employed to Book Antiqua. Relevant information and correction in title page has been indicated according to journal guidelines. The abstract has been revised accordingly. We have made English editing of the language. The figures and relevant legends have been improved.

(2) Company editor-in-chief:

I have reviewed the Peer-Review Report, the full text of the manuscript, and the relevant ethics documents, all of which have met the basic publishing requirements of the World Journal of Stem Cells, and the manuscript is conditionally accepted. I have sent the manuscript to the author(s) for its revision according to the Peer-Review Report, Editorial Office's comments and the Criteria for Manuscript Revision by Authors. Before final acceptance, uniform presentation should be used for figures showing the same or similar contents; for example, "Figure 1Pathological changes of atrophic gastritis after treatment. A: ...; B: ...; C: ...; D: ...; E: ...; F: ...; G: ...". Please provide decomposable Figures (in which all components are movable and editable), organize them into a single PowerPoint file. The title of the manuscript is too long and must be shortened to meet the requirement of the journal (Title: The title should be no more than 18 words).

Response to company editor-in-chief:

Thanks for your constructive comment. Basic publishing requirements have been completed and the revision according to the Peer-Review Report has been finished in a point-to-point form. However, we combined figures by using Photoshop. Thus, we submit tiff-format original figures with all components. Photoshop can be used to edit all the figures.

Reviewer #1

Specific Comments to Authors: My observations during the review of the manuscript are as follows: 1- Isolation and culture of mouse ADSCs in the phrase "ADSCs were isolated from adipose tissue from C57BL/6 mouse adipose tissue as previously described (?)– authors should add the reference here. 2- Methods Section: Isolation and characterization of EVs Authors should add the total number of cells used to extract EVs 3- Please review this paragraph: On groups 3 and 4, EVs were administered at a dose of 1μg/1g of mice body weight via injecting into the border zone of infarcted heart at three sites. (???? lack of a phrase) post MI surgery immediately. Authors should clarify this phrase. 4- Results Section: In Figure 3 B, authors should add a better description of x and y axis of each graph to clarify which one corresponds to CCK8 assay. In conclusion, I suggest minor corrections.

Response to reviewer#1:

Thanks for your constructive comments.

1.Reference has been added in the phrase of isolation and culture of mouse ADSCs. 2.Number of ADSCs are approximately 10^7 per dish to extract EVs which has been added in phrase of isolation and characterization of EVs.

3.The phrase has been revised in this correction version.

4. Your comments are pretty helpful to improve the quality of the manuscript. I have added description of x and y axis to clarify CCK8 assay in Figure3 legend.

Reviewer #2:

Specific Comments to Authors: Mao et al. aim to analyze the cardioprotective mechanism of extracellular vehicles (EVs) generated by hypoxia-preconditioned mesenchymal stromal/stem cells (MSCs) in an in vivo myocardial injury (MI) model and in an in vitro hypoxia model. They hypothesize that hypoxia-preconditioned extracellular vehicles (HP-EVs) could be more effective against myocardial injury-associated cardiomyocyte death than EVs derived from normoxic MSCs (NC-EVs). They analyze the

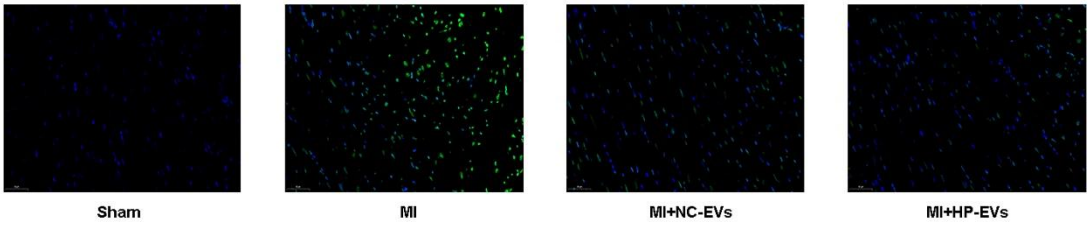
morphological and molecular changes associated with MI-induced apoptosis in cardiomyocytes and hypnotize a role for HP-EVs-associated miRNAs in cardiomyocyte survival. Although the paper is of interest several issues have to be addressed before considering it suitable for publication. Major issues 1. The paper needs to be revised by a native English speaker. 2. The Introduction paragraph lacks a proper definition of MSCs. Authors have to underlie their characteristics and function (see recent literature PMID: 34398443; PMID: 30001217). 3. It is well known that oxidative stress in the heart is increased in response to ischemia/reperfusion and heart failure. Indeed, in pathological situations, ROS accumulate due to excessive production or insufficient degradation, leading to oxidative stress (PMID: 28861421). ROS are among the most harmful DNA-damaging agents. A major product of oxidative damage to DNA is 8-oxo-2′-deoxyguanosine. In addition to apoptosis assessment, authors must address this issue in their experimental plan by analyzing at least this DNA damage marker (see for experimental setting PMID: 19804370; 20697355). 4. Author state that adipose-derived mesenchymal stem cells (ADSCs) were isolated from adipose tissue from C57BL/6 mouse adipose tissue as previously described without providing the reference. In addition, authors have to indicate at which culture passage they performed the experiments. To obtain the required number of cells, MSCs need to be cultured for several passages. Although in vitro expansion is a necessary procedure to guarantee the required number of MSCs, it is also considered to pose important issues. It has been demonstrated that in vitro growth of MSCs can give rise to replicative senescence. Have the authors addressed this issue? (see PMID: 32223893). 5. Figure 1A is not mentioned in the text.

Response to reviewer#2:

Thanks for your insightful comments, which are pretty helpful to improve our study. 1. The manuscript has been sent to English editing as introduced by journal editor.

2. I have read two literatures carefully (PMID: 34398443; PMID: 30001217), which give me a deeper understanding of mesenchymal stem cell. We have added definition of MSCs in the corrected MS with citing of above two excellent literatures.

3. 8-oxo-2′-deoxyguanosine assay is a wise mean to detect the DNA-damage caused by ROS generated during the stage of ischemia/reperfusion. We tested the heart tissue subject to ischemia/reperfusion injury with 8-oxo-2′-dG antibody with FITC-conjugated secondary antibody. It is obvious that HP-EVs and NC-EVs alleviate the ROS damage in the period of ischemia/reperfusion



8-oxo-2′-dG assay of oxidative damage to DNA caused by ROS
Heart suffered from MI showed a significant DNA damage detected by 8-oxo-2′-dG assay. NC-EVs contributed a cardioprotective effect to alleviate ROS induced DNA damage. The cardioprotective effect of HP-EVs against ROS induced DNA damage was more significant than that of NC-EVs.
(unpublished content).

However, the potential mechanism of EVs derived from MSCs in relieving DNA-lesion caused by excessive ROS accumulate in the period of ischemia/reperfusion is still in the research of our group, which may not generate such DNA protective effects by targeting TXNIP. Your farsighted opinion opens up the future research direction for us.

4. The reference of isolating MSCs from adipose tissue from C57BL/6 mouse is provided in corrected manuscript.

I felt the same way about this problem of replicative senescence in the initial stage in this research. Initially, we cultured bone marrow mesenchymal stem cells (BMSCs) to generate EVs to verify our hypothesis, but in 4^{th} -5^{th} passage，BMSCs demonstrated a significant senescent trend and the amount of cells were relatively low. Thus, we isolated ADSCs from adipose tissue

from C57BL/6 mice, which demonstrated a higher extraction efficiency and amount of cells/mouse. For the cultivating condition, because we aimed to extract EVs from cell supernatant, FBS-free medium was necessary. So, we chose commercial FBS-free medium special to ADSCs (Oricell MUXMD-90011, cyagen, GuangZhou, China), which provided a none heterogeneous EVs and nonessential cytokines condition and keep ADSCs cultivated in a relatively stable environment.

5. In revised version, we have added index and explanation of Figure 1A which was the graphic abstract of extracting cardiomyocytes of mice and EVs form ADSCs.

Reviewer #3:

Specific Comments to Authors: Although many attempts have been made to use EVs derived from ADSCs to alleviate cardiac damage such as MI and to promote repair, the present study is unique in that it prepares ADSCs that have been exposed to hypoxia beforehand and shows that EVs released from these cells have a more pronounced cardioprotective effect against myocardial infarction. This effect was observed in normoxic ADSCs, however, since this effect is stronger than that of EVs generated from normoxic ADSCs, these differences led to the extraction of important miRNA factors. To further elucidate the molecular mechanism, they analyzed the regulation of target proteins by miRNAs and the nuclear export of HIF1a in cultured strain cells and primary cultured cells. Although the molecular mechanism of miR224-TXNIP-HI1a has already been clarified before such as in pancreatic cancer cells, the present study was able to verify the existence of a similar mechanism using cultured myocardium. On the other hand, in order to prove that the cardioprotection of ADSC-derived EVs against MI is due to the above molecular mechanisms, it may be necessary to verify the molecular dynamics in the myocardium after MI. 1. For the ADSC-cas9-miR224 used in the article, the method of cell establishment and data confirming KO should be presented. 2. The amount of TXNIP and HI1a in the nucleus and cytoplasm, as well as their ubiquitination, should be verified in mouse hearts after MI.

Response to reviewer#3:

Thanks for your insightful comments, which are all pretty useful to improve quality of our research.

1. To establish ADSC-cas9-miR224 cells. We designed sgRNA, the sequence was as follows：TCTGGGGCTTTGTAGTCACT

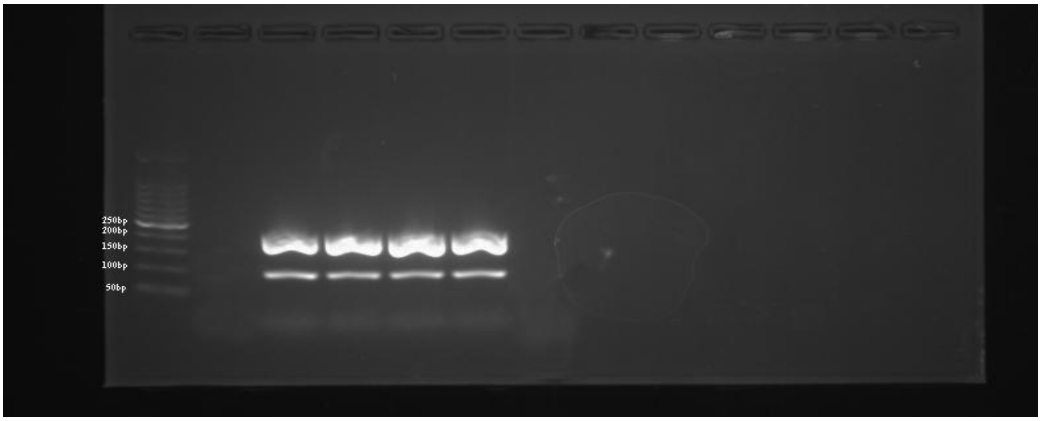
   The sgRNA context sequence was as follows:

   TGGCTCTGGGGCTTTGTAGTCACTAGGGCA

CRSPR/Cas9-mediated genome editing of ADSCs was carried out as described with reagent specification (riboEDIT™ CRISPR-Cas9 RNP, Ribobio, GuangZhou, China). The p2 ADSCs were rested overnight in medium (Oricell MUXMD-90011, cyagen, GuangZhou, China), electroporation was carried out using an Amaxa P3 Primary Cell kit and 4D-Nucleofecter (Lonza). 50pmol of recombinant CRIPSR-Cas9 (riboEDIT™) and 100pmol of chemically synthesized tracr/crRNA (riboEDIT™) were incubated for 20 minutes prior to electroporation to generate Cas9-gRNA ribonucleoprotein (RNP) complexes. 2×10^{6} ADSCs suspended in working buffer were added to the pre-incubated Cas9-gRNA RNP complexes. ADSCs were transfected by using program EO-115. Then, ADSCs were re-seeded at 5×10 5 cells/mL in commercial FBS-free medium (Oricell MUXMD-90011, cyagen, GuangZhou, China) for further study to extract EVs. T7E1 endonuclease was used to confirm KO. The data was as follow

Forward primer: tggcttggaattcttgctct

Reverse primer：ctccacaggaagagatgttgc



The data was submitted as supplementary material (Figure S2).

2. Thank you for your insightful comments. We have conducted immunofluorescence to detect the expression and distribution of TXNIP and HIF-1 in infarction tissue of hearts suffered MI. The results demonstrated that expression of TXNIP upregulated in the stage of MI，which was alleviated by NC-EVs and AP-EVs. Accordingly, expression of HIF-1 in the infarction tissues revealed an inversed trend to TXNIP. The results have added in revised MS supplementary material (Figure S2).

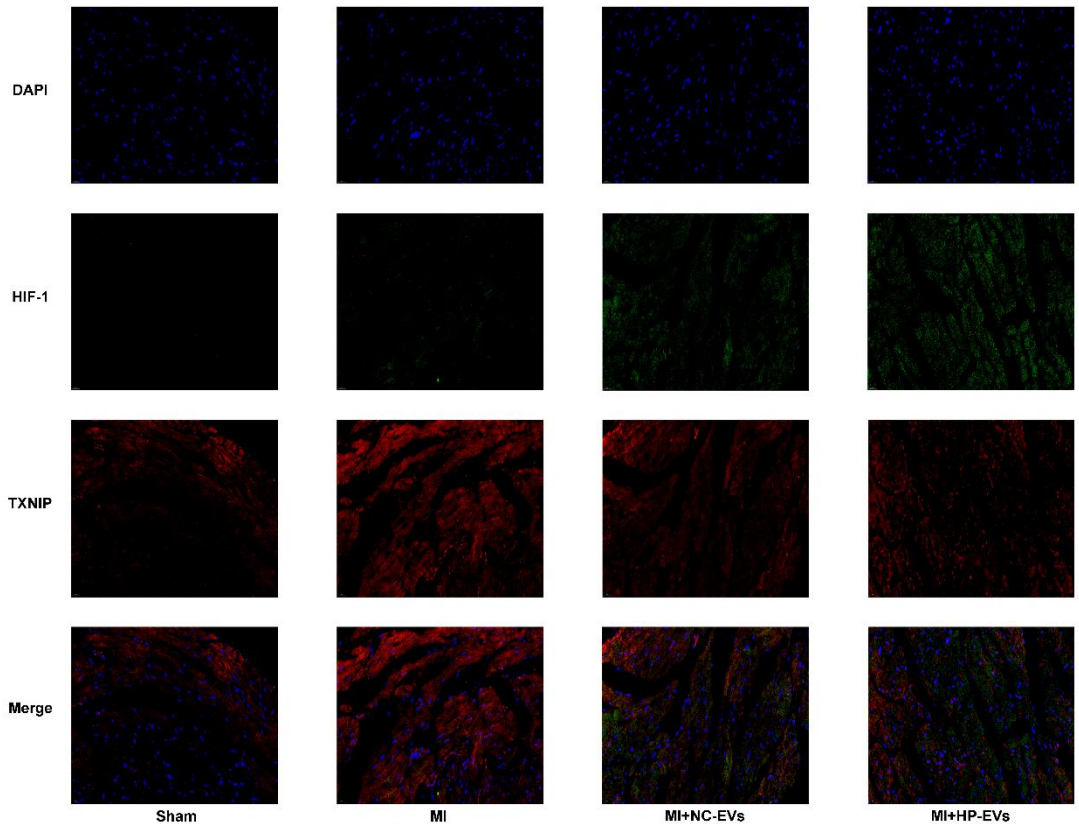


Figure S2 .Immunofluorescence results of HIF-1（green）and TXNIP（red）expression in the

infarction tissue. It was shown that TXNIP upregulated in the stage of MI， which was alleviated by NC-EVs and AP-EVs. Accordingly, expression of HIF-1 in the infarction tissues revealed an inversed trend to TXNIP.

However, for MG132 cannot inhibit proteasome-dependent degradation pathway in vivo, ubiquitination of HIF may be degraded rapidly. We tried several times to pulldown HIF but failed to detected ubiquitination of HIF with the absence of proteasome inhibitor in vivo, which was discussed in the corrected MS.

Reviewer #4:

Scientific Quality: Grade A (Excellent)

Language Quality: Grade A (Priority publishing)

Conclusion: Minor revision

Specific Comments to Authors: I would like to congratulate the authors for this study. I have some comments about the manuscript: Introduction: please include previous studies with correlation to this study. Material and method: There were total of 80 mice used, 20 mice per group. please clarify briefly your sample size determination. About the collection of neonatal mouse cardiomyocytes, please clarify how many mice used and the detailed criteria / specification. Result: where is figure 1A and 4D mentioned in the text? Please arrange the figures accordingly in the text. Discussion: please give the reasoning of this study compared to other study and provide the limitation of this study.

Response to reviewer#4:

Thanks for your insightful comments, which are all pretty useful to improve quality of our research.

1. Previous studies with correlation to our study were added in revised MS.
2. The sample size determination and neonatal cardiomyocytes extraction were clarified in revised MS. The identification was shown in the first phase of results section in revised MS.
3. Figure 1A and 4D were mentioned in the revised MS.
4. Limitation and the reasoning of this study compared to other study has been added in discussion in corrected MS

Once again, we sincerely thank all editors and the four reviewers very much for their wonderful suggestions!