Single dose of synthetic microRNA-199a or microRNA-149 mimic does not improve cardiac function in a murine model of myocardial infarction

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Abstract
Intramyocardial injection of synthetic microRNAs (miRs) has recently been reported to be beneficial after myocardial infarction (MI). We conducted a randomized blinded study to evaluate the efficacy and reproducibility of this strategy in a mouse model of reperfused MI using rigorous methodology. Mice undergoing a 60-min coronary occlusion followed by reperfusion were randomly assigned to control miR, hsa-miR-199a-3p, hsa-miR-149-3p, or hsa-miR-149-5p mimic treatment. Intramyocardial injections of miRs were performed in the border zone right after reperfusion. At 8 weeks after MI, there were no significant differences in ejection fraction (EF) among groups (EF = 27.1 ± 0.4% in control group [n = 6] and 25.9 ± 0.5%, 26.0 ± 0.8%, and 26.6 ± 0.6% in hsa-miR-199a-3p, hsa-miR-149-3p, or hsa-miR-149-5p groups, respectively [n = 9 each]). Net change (delta) in EF at 8 weeks compared with day 3 after MI was −4.1% in control and −3.2%, −2.4%, and −0.4% in the miR-treated groups (P = NS). Assessment of cardiac function by hemodynamic studies (a method independent of echocardiography) confirmed that there was no difference in left ventricular systolic or diastolic function among groups. Consistent with the functional data, histological analysis showed no difference in scar size, cardiomyocyte area, capillary density, collagen content, or apoptosis among groups. In conclusion, this randomized, blinded study demonstrates that intramyocardial injection of a single dose of synthetic hsa-miR-199a-3p, hsa-miR-149-3p, or hsa-miR-149-5p mimic does not improve cardiac function or remodeling in a murine model of reperfused MI. The strategy of using synthetic miR mimics for cardiac repair after MI needs to be evaluated with rigorous preclinical studies before its potential clinical translation.

Keywords microRNA · Myocardial infarction · Ischemia · Reperfusion · Cardiac function · Repair

Abbreviations
miR MicroRNA
MI Myocardial infarction
LCA Left coronary artery
I/R Ischemia/reperfusion
ECG Electrocardiography
BrdU 5-Bromo-2'-deoxyuridine
LV Left ventricle
EDV End-diastolic volume
ESV End-systolic volume
SV Stroke volume
EF Ejection fraction
PV loop Pressure–volume loop
Ees End-systolic elastance
Tau Relaxation time constant
IZ Infarct zone
BZ Border zone
RZ Remote zone
EdU Ethynyl-29-deoxyuridine

Introduction
MicroRNAs (miRs) are a class of highly conserved, endogenous, short non-coding RNAs that negatively regulate post-transcriptional gene expression by inhibiting the translation of protein-coding genes [1–3]. Numerous miRs demonstrate characteristic changes in expression during the pathogenesis of heart disease and are considered prognostic markers and potential therapeutic targets for pathological conditions such as myocardial infarction (MI) [4–7]. One of the methods to modulate miRs in the heart is to use synthetic miR mimics...
administered by direct intramyocardial injection. This strategy has recently been reported to improve cardiac function after MI with just one injection in mice [8, 9], and appears to be a promising approach with translational potential.

However, certain methodological aspects of these studies [8, 9] may undermine the validity of the functional results. For example, these studies did not mention randomization or blinding in the analysis of cardiac function, and they used only M-mode echocardiography for all functional assessments [8, 9]. Furthermore, it is unknown whether the results of these studies [8, 9] can be reproduced by other groups. Confirmation from rigorous preclinical studies by independent labs is required before this strategy can be moved towards clinical translation.

Methodological shortcomings, such as lack of randomization and blinding, are prevalent in preclinical cardiovascular research [10–17]. The resultant risks of bias and threats to study validity are major causes of irreproducibility in preclinical research and hinder clinical translation [10–17]. Starting almost two decades ago, our group has been among the first, if not the first, to call attention to this problem and suggest solutions that would enhance scientific rigor [11–17]. In particular, we have developed CAESAR, the first publicly available, multicenter consortium for rigorous preclinical studies of cardioprotection [15, 16]. CAESAR was based on the cardinal principles of randomization, investigator blinding, a priori exclusion criteria, strict protocol adherence, appropriate statistical analyses, and assessment of reproducibility [11, 15, 16]. After CAESAR, several guidelines and recommendations have been published regarding animal models of MI [18] and cardiac function measurement [19] and analysis [20]. Thus, CAESAR has promoted increased awareness of the need for rigor and reproducibility in designing and performing scientific research and a shift of the cardiovascular community toward more rigorous research methods [11]. The experience acquired with CAESAR, coupled with adherence to preclinical animal experiment guidelines, has enabled us to provide rigorous evaluation of putative therapies.

The goal of this study was to harness the methodological rigor of CAESAR to evaluate the efficacy and reproducibility of the strategy of using synthetic miR mimics via direct intramyocardial injection after MI. To this end, we conducted a randomized blinded preclinical experiment in a mouse model in which we evaluated whether a single dose of synthetic hsa-miRNA-199a-3p or hsa-miRNA-149 mimics improves cardiac function after MI. We chose a model of ischemia/reperfusion (I/R) because it mimics the clinical setting [18], as most patients are reperfused after MI. The reason for choosing hsa-miRNA-199a-3p is that it is well studied in mice [8, 21] and larger animals such as pigs [22], and its mechanism is better understood than other miRs [23]. We used a protocol and model similar to those of a previous study [8] in order to examine the reproducibility of this strategy with the same hsa-miRNA-199a-3p mimic. In addition, we sought to evaluate the generalizability of this strategy in order to determine whether it can be used for other miRs. To this end, we chose hsa-miRNA-149 based on the fact that it is involved in myocardial differentiation [24] and apoptosis [25] and has been reported to be downregulated after MI [4, 26].

**Methods**

All animal procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Louisville Institutional Animal Care and Use Committee (protocol number:14034).

**Preparation of microRNA mimics**

Synthetic hsa-miR-199a-3p, hsa-miR-149-3p, hsa-miR-149-5p, and cel-miR-67 mimics were all acquired from Dharmacon (GE Healthcare Dharmacon, Inc.). *Caenorhabditis elegans* cel-miR-67, a nontargeting miRNA which has no homolog in vertebrates, served as control mimic. Detailed miR sequences are provided in Supplemental Table 1. Lipofectamine RNAiMAX was used as lipid buffer in this study. Similar to a previous report [8], 300 μmol of each miR mimic (in a volume of 15 μl) was freshly mixed with 15 μl lipid buffer, according to the manufacturers’ specific protocols, in a total volume of 30 μl before injection into the heart. In the current study, each 15 μl of miR mimic was loaded into an Eppendorf tube according to the randomized code kept by a third-party person. The tube was then labeled with a sequence number and kept frozen in a −20 °C refrigerator. After receiving the call from the animal lab announcing that the animal was ready for miR treatment, the lipid/miR mix was freshly prepared and then delivered to the animal lab.

**Ischemia/reperfusion mouse model**

The murine model was similar to that used in CAESAR [11, 15]. Adult (16 weeks old), female, C57BL6/J mice were subjected to 60 min of regional myocardial ischemia followed by reperfusion as previously described [15, 27–34]. In this model, an 8–0 nylon suture was passed under the left coronary artery (LCA) approximately 2 mm below the left auricle and a nontraumatic balloon occluder was applied on the artery. Ischemia/reperfusion (I/R) was induced by tightening and inflating the occluder and then deflating and removing it. To minimize the impact of blood loss, blood from a donor mouse was given at serial times during surgery.
Rectal temperature was carefully monitored and maintained between 36.8 and 37.2 °C throughout the experiment. Continuous electrocardiography (ECG) was monitored during the experiment. Typical ischemic ECG changes (including widened QRS and elevated ST segment) and pale color of the ischemic myocardial region were used to confirm successful coronary occlusion. Animals whose ECG changes were atypical or not persistent throughout the ischemic period were excluded [15, 32].

**Treatment protocol**

The experiments were conducted in a randomized and blinded manner [11, 15]. Mice were randomly assigned to four groups, labeled initially as Group A to D. The group code was kept blinded to the surgeons, sonographer, hemodynamic investigator, and pathology investigator. The code was broken in front of all parties by a third-party person after all the results were submitted to the principal investigator [11, 15].

Immediately following reperfusion, mice received intramyocardial injections of a mix of lipid buffer and either synthetic hsa-miR-199a-3p mimic, hsa-miR-149-3p mimic, hsa-miR-149-5p mimic, or control miR mimic according to experiment assignment. Similar to our previously reported methods of intramyocardial injections for cell therapy [27, 29, 35, 36] and gene therapy [37–40], using a 100 μl syringe attached with a 30-gauge needle, the lipid/miR mix (total volume of 30 μl) was filled into the syringe, and air bubbles were carefully removed. Subtracting the dead volume of the needle and the volume lost during air removal, a total volume of 20 μl was available for treatment. The 20 μl volume of lipid/miR mix (with 200 μmol miR mimic) was delivered via two 10 μl intramyocardial injections into the border zone of the infarcted region of the heart (one into the anterior septum and another into the posterior wall).

After the injections, the chest was closed in layers and the mice were allowed to recover in cages with heat and oxygen supply. Animals were followed up for 8 weeks before euthanasia. Series echocardiographic studies were performed during the follow-up; a hemodynamic study was performed before euthanasia, and the heart was harvested at the end of the experiment (Fig. 1A). To monitor any potential formation of new cells, half of the mice received 5-bromo-2’-deoxyuridine (BrdU) 25 mg/kg/day s.c. for 5 weeks right after miR treatment and the other half of the mice received BrdU during the last 3 weeks of follow-up. All surgeons were blinded to treatment assignment.

**Echocardiography**

Transthoracic echocardiography was performed to assess cardiac function at 3 days (Post-MI) and 5 weeks and 8 weeks after reperfusion. As previously described [27–29,
41, 42], a Vevo 2100 Imaging System (VisualSonics, Inc.) equipped with a 30-MHz transducer was used in this study. All echocardiography measurements were performed under isoflurane anesthesia (3% for induction and 1% for maintenance). Body temperature was carefully maintained at 37.0 ± 0.2 °C throughout the study. The parasternal long-axis views and short-axis views were used to obtain 2D mode images for the measurement of left ventricle (LV) end-diastolic and end-systolic volume (EDV and ESV), stroke volume (SV), and ejection fraction (EF). At least three measurements were taken and averaged for each parameter. Digital images were analyzed by blinded observers using the Vevo 2100 workstation software. Measurements were performed according to the American Society for Echocardiography. Left ventricular EF was calculated by using the formula: 

\[
\frac{[(EDV - ESV)/EDV] \times 100}{100}
\]

The sonographer was blinded to group assignment.

Hemodynamic study

After the final echocardiographic study and right before euthanasia, mice were subjected to pressure–volume loop assessments, as previously described [27–29, 41]. Briefly, mice were anesthetized with sodium pentobarbital (60 mg/kg, I.P.), intubated, and ventilated. Rectal temperature was kept at 37 ± 0.2 °C. A 1.0 French pressure–volume (PV) catheter (PVR-1035, Millar Instruments) was inserted into the left ventricle via the right carotid artery. The position of the catheter was carefully adjusted until typical and stable PV loop signals were acquired. After 30 min of stabilization, the PV signals were recorded continuously with a MPVS ULTRA Pressure–Volume Unit (Millar Instruments). Inferior vena cava occlusion was performed with external compression to produce variably loaded beats for determination of the end-systolic PV relation and other derived constructs of LV performance. Parallel conductance from surrounding structures was calculated by a bolus injection of 5 µl of 30% NaCl through the jugular vein. Echocardiography-derived SV was used as outside reference in alpha calibration for LV volume. All hemodynamic data analyses were performed off-line, using LabChart 7.0 software, by an investigator blinded to the treatment.

Histologic analysis

The protocol for histologic analyses has been described [27–29, 33, 35, 36, 41]. Briefly, at the end of the hemodynamic study, the heart was arrested in diastole by an i.v. injection of 0.15 ml of CdCl₂ (100 mM), excised, and perfused retrogradely at 60–80 mmHg (LVEDP = 8 mmHg) with heparinized PBS followed by 10% neutral buffered formalin solution for 15 min. The heart was fixed in formalin for 24 h, sectioned into three transverse slices (~2 mm thick) from apex to base, and the samples were subsequently embedded, cut, and mounted. Later, the sections were deparaffinized and rehydrated as needed for the appropriate stain. Every heart that completed the protocol was subjected to histological analysis. For all analyses described below, the pathologist was blinded to group assignment.

Trichrome stain

Cardiac sections were stained with Masson’s trichrome stain to determine scar size according to published protocols [33]. The risk area was defined as the transmural area between the furthest outer lateral edges of the scar, which was identified by Masson’s trichrome staining. Viable myocardium in the risk area was determined as the difference between risk and scar areas. Images were acquired digitally and areas measured using NIKON software (NIS-Elements AR Analysis 4.13.05 64-bit).

Cardiomyocyte size

Cardiac sections were stained with wheat germ agglutinin (WGA; AlexaFluor 555 conjugate; Invitrogen) to identify cell borders and stained with DAPI to detect nuclei. WGA-stained cells were visualized using a Nikon TE-2000E2 microscope interfaced with a Nikon A1 confocal system. Cell areas were measured using Nikon Elements software [64-bit version 3.22.00 (Build 7100)]. Cardiomyocytes were chosen based on their circularity and whether they had centrally located nuclei. Circularity was calculated using the shape factor feature in NIS-Elements AR 4.0. Cardiomyocytes were chosen based on a shape factor between 1.0 and 0.895 (radius ratio of 1:1 to 1:1.4).

Capillaries density

Cardiac sections were stained with isolectin B4 (fluorescein-labeled Griffonia simplicifolia Lectin I; Vector Labs) as we have described previously [41, 42]. Capillary density was determined by dividing the total number of isolectin B4-positive vessels by the area of the image (number of capillaries per mm²).

Picrosirius red stain

Myocardial collagen content was quantitated on picrosirius red-stained heart images acquired under polarized light microscopy by determining collagen density (arbitrary unit) per mm² of risk region or noninfarcted region with the NIS-Elements software.
TUNEL assay

The TUNEL assay was performed according to the manufacturer’s instructions on LV sections using In-Situ Cell Death Detection Kit, TMR red (Roche). Sections were also stained with DAPI to identify nuclei. Sections were imaged using a Nikon Eclipse Ti using a 20x objective. TUNEL positivity was calculated by dividing the total number of TUNEL-positive cells by the number of nuclei.

Statistical analysis

Data are presented as mean ± SEM. All data were analyzed with Student’s t tests or one-way ANOVA for normally distributed data followed by unpaired Student’s t tests with the Bonferroni correction, or Kruskal–Wallis one-way analysis of variance on ranks for data that are not normally distributed, as appropriate. A P value < 0.05 was considered statistically significant. All statistical analyses were performed using the Sigma Stat software system [43–45].

Sample size calculation for multiple group means was conducted via G*Power software [46]. A fixed effects omnibus one-way ANOVA F-test was used [46]. Type I error rate was set at (α) = 0.05, type II error rate (β) = 0.2, and power (1-β) = 0.8. Based on previous literature [8], the major endpoint (EF) in the control group and miR-199a-3p group was 24 ± 8.1% and 37.5 ± 4.5%, respectively. Assuming that the effects of miR-149-3p and miR-149-5p are not less than those of miR-199a-3p, the calculated minimum total sample size is 28. Assuming a 30% mortality rate, the minimum sample size required is 28 × 130% = 37.

Results

A total of 42 mice were originally enrolled for coronary artery occlusion/reperfusion. One mouse died during occlusion; another was excluded because the ischemic ECG changes were not persistent throughout ischemic period. The remaining 40 mice were randomly assigned to four groups, originally labeled as Group A-D. Three mice died right after the miR injections (one in Group B and 2 in Group D) and before closing the chest. Another 3 mice died overnight (one each in Group A, C, and D). One mouse in Group D died at 35 days after treatment. After all results were submitted to the principal investigator, the group code was broken, revealing that Group A was hsa-miR-199a-3p, Group B hsa-miR-149-3p, Group C hsa-miR-149-5p, and Group D control miR (Fig. 1B and Supplemental Table 2). The body weight (Supplemental Fig. A and Supplemental Table 3) and lung weight/body weight ratio (Supplemental Fig. B) demonstrated no differences among the four groups.

Echocardiographically assessed cardiac function is not improved in miR mimics-treated hearts

Echocardiographic studies were performed at 3 d (Post-MI), 5 weeks, and 8 weeks after treatment. All mice that completed the protocol were included in the dataset (6 mice in the control group and 9 in each miR mimic-treated group). Comprehensive quantitative analyses of echocardiographic parameters are presented in Fig. 2A–D (the full set of detailed echocardiographic results can be found in Supplemental Table 3) and representative echocardiographic recordings are illustrated in Fig. 2E. As shown in Fig. 2, at 3 d after MI EDV, ESV and EF were not significantly different among mice assigned to control, hsa-miR-199a-3p, hsa-miR-149-3p, or hsa-miR-149-5p, indicating that the severity of post-MI LV dysfunction was comparable in the four groups. In all groups of mice, EDV and ESV increased significantly from Post-MI to end of protocol (Fig. 2A, B). Accordingly, a deterioration in LV function was observed in all groups during the 8 weeks of follow-up. There were no significant differences in LV EF among groups at 5 weeks or 8 weeks after treatment, with final EF of 27.1 ± 0.4% in the control group and 25.9 ± 0.5%, 26.0 ± 0.8%, and 26.6 ± 0.6% in the hsa-miR-199a-3p, hsa-miR-149-3p, and hsa-miR-149-5p groups, respectively (Fig. 2C and Supplemental Table 3). The net change (delta) in EF between Post-MI and 8 weeks also showed no significant difference: average, − 4.1% in control and − 3.2%, − 2.4%, and − 0.4% in the other groups (Fig. 2D).

Hemodynamically assessed cardiac function is not improved in miR mimics-treated hearts

Mice were subjected to hemodynamic studies via pressure–volume loop assessment after the final echocardiographic study. The results demonstrated no significant improvement in cardiac function in the hsa-miR-199a-3p, hsa-miR-149-3p, or hsa-miR-149-5p group compared with the control miR mimic group (Fig. 3). First, we examined systolic functional parameters including LV EF, LV dP/dt maximum (+dP/dt), and LV end-systolic elastance (Ees). The EF measured via a conductance catheter was 28.6 ± 0.7% in the control group and 26.4 ± 1.7%, 26.6 ± 1.1%, and 26.1 ± 1.0% in the hsa-miR-199a-3p, hsa-miR-149-3p, and hsa-miR-149-5p groups, respectively (Fig. 3A). Another load-dependent parameter (LV +dP/dt) also showed no difference (Fig. 3C). Moreover, Ees, a load-independent parameter, further confirmed the similarity of systolic function among all groups (Fig. 3B). Regarding diastolic function, LV dP/dt minimum (−dP/dt) and
Fig. 2 Echocardiographically assessed cardiac function is not improved in miR mimics-treated hearts. Mice underwent coronary occlusion/reperfusion and were monitored for 8 weeks. Serial echocardiographic studies were performed at 3 days (Post-MI), 5 weeks, and 8 weeks (n=6 in the control group; n=9 in hsa-miR-199a-3p, hsa-miR-149-3p, and hsa-miR-149-5p groups). A LV end-diastolic volume (EDV); B LV end-systolic volume (ESV); C ejection fraction (EF); D ΔEF (compared with Post-MI). E Representative end-diastolic long-axis B-mode images in each group. Data are mean ± SEM.
Fig. 3  Hemodynamically assessed cardiac function is not improved in miR mimics-treated hearts. Hemodynamic assessment was performed before euthanasia. A EF; B end-systolic elastance (Ees); C LV dp/dt maximum (+dp/dt) and dp/dt minimum (−dp/dt); D relaxation time constant of LV pressure (Tau). E Representative PV loops of each group. Data are mean±SEM.
relaxation time constant of LV pressure (Tau) both demonstrated that there was no difference among the four groups (Fig. 3C, D). The full set of detailed hemodynamic results can be found in Supplemental Table 4.

LV morphometric analysis

Representative trichrome-stained heart sections are shown in Fig. 4A. All four groups exhibited similar levels of LV dilatation and thinned infarcted wall with a confluent scar. In each heart, a detailed quantitative analysis was performed on two sections (one from each of two mid-ventricular slices); the results are summarized in Figs. 4B–D. The LV mass (Supplemental Fig. C) and risk region mass/LV mass ratio (Fig. 4D) were similar in all four groups, indicating that the size of the risk region was comparable. We observed no difference in scar size, measured either as scar/LV mass ratio or scar/risk region ratio, between miR-treated groups and the control group (Fig. 4B, C).

Cardiomyocyte hypertrophy

To further evaluate whether miR mimic treatments affected cardiomyocyte hypertrophy, WGA staining was performed. As shown in Fig. 4E, WGA demarcates the membrane of individual cells. We did not observe any significant difference in cardiomyocyte cross-sectional area in the infarct zone, border zone, or remote (noninfarcted) zone of hearts treated with hsa-miR-199a-3p, hsa-miR-149-3p, or hsa-miR-149-5p compared with control miR mimic-treated hearts (Fig. 4F–H).

Capillary density

We next assessed the impact of miR mimics on angiogenesis by isolectin B4 staining (Fig. 4E). Similar to cardiomyocyte size analysis, we did not observe any significant difference in capillary density in the infarct zone, border zone, or remote (noninfarcted) zone of hearts treated with miR mimics compared to control miR mimic-treated hearts (Fig. 4I–K).

Collagen content

Quantitative analysis of collagen content was performed using picrosirius red staining and polarized light microscopy of LV sections (Fig. 5A). Collagen content was evaluated with pixel density analysis and expressed as a percentage of total myocardial area. There was no statistically significant difference in collagen content among groups in either the risk region or the noninfarcted region (Fig. 5B, C).

TUNEL analysis

To examine whether miR mimic treatments affected myocardial apoptosis, TUNEL analysis was performed on LV sections (Fig. 5D). The ratio of TUNEL + cells to total cells in the infarct zone, border zone, and remote (noninfarcted) zone is presented in Fig. 5E–G. We did not observe any significant difference in TUNEL positivity among the 4 groups.

Discussion

The salient results of the current study can be summarized as follows: (1) a rigorous, randomized, blinded preclinical study was successfully conducted; (2) assessment of cardiac function via echocardiography showed no differences in LV EF among the hsa-miR-199a-3p, hsa-miR-149-3p, hsa-miR-149-5p, and control miR mimic groups; (3) assessment of cardiac function by hemodynamic analysis via a PV loop conductance catheter, a method independent of echocardiography, also confirmed that there was no difference in either systolic or diastolic function among groups; (4) consistent with the functional results, histological analyses including measurements of scar size, cardiomyocyte area, capillary density, collagen content, and apoptosis also showed no difference among groups. To our knowledge, this is the first randomized, blinded study to evaluate the efficacy of synthetic miR mimics in a murine model of MI induced by coronary occlusion and reperfusion.

We designed this study in an effort to provide solid supportive data for the strategy of using synthetic miR mimics as a therapy for MI. Our results, obtained with a rigorous preclinical experiment, are basically negative and contrast with previous reports from other researchers [8, 9]. Lesizza et al. [8] reported that in a murine model of MI induced by permanent LCA ligation, a single injection of 1.4 μg of hsa-miR-199a-3p and hsa-miR-590-3p mimic right after ligation was sufficient to stimulate cardiac repair, reduction of infarct size, and restoration of cardiac function (with a striking 13.5-point and 24.5-point increase in mean EF in the hsa-miR-199a-3p and hsa-miR-590-3p groups, respectively, compared with control). We were unable to reproduce this study. Another group reported that injection of a single dose of 10 μg of miR-19a/19b right after LCA ligation restored cardiac function at 5 days after treatment and this effect lasted for 12 months [9]. Conflicting results are not uncommon in a new and rapidly evolving field. Such discrepancies underscore the necessity of evaluating a therapeutic strategy in multiple centers with rigorous experimental design before that strategy can be advanced to clinical translation [11–17].

The reason(s) for the discrepancy between our results and previous results [8, 9] is unclear. There are several differences between our study and that by Lesizza et al. [8],
Fig. 4  Histological analysis of LV scar, cardiomyocyte size, and capillary density. A Representative trichrome staining images in the control, hsa-miR-199a-3p, hsa-miR-149-3p, and hsa-miR-149-5p groups. Sections from the apical, middle, and basal slice of the heart are shown. B scar mass to LV mass ratio. C scar mass to risk region mass ratio. D risk region mass to LV mass ratio. E Representative confocal microscopic images of WGA and isolectin B4-stained heart sections acquired from the infarct zone (IZ), border zone (BZ), and remote zone (RZ). (Blue: DAPI, green: WGA, red: isoletin). F–H Cardiomyocyte cross-section area in IZ, BZ, and RZ. I–K Capillary density per mm² in IZ, BZ, and RZ. Data are mean ± SEM.
whose results we were unable to reproduce. The first notable difference is the methodological design. Our study was designed and conducted in a randomized and blinded manner. Details regarding hiding the randomized assignment allocation, exclusion criteria, blinding, and group code breaking methods are reported in our Methods and Results. Lesizza et al. [8] did not mention randomization or blinding in their report, while Gao et al. [9] only mentioned randomized allocation but did not indicate how they hid the randomizing scheme or blinding. Neither study reported reasons for exclusion or inclusion. As suggested first by our group [11–17] and later by the Guidelines for experimental models of myocardial ischemia and infarction [18], to improve scientific rigor, investigators should be blinded, animals should be randomized to control or treated groups whenever possible, exclusion and inclusion criteria should be declared in advance, and details of such exclusions should be specified in any publications. Unfortunately, the methodological shortcomings of preclinical cardiovascular studies have not improved significantly over the past 10 years [10]. Increased awareness of these issues, leading to more rigorous design and conduct of experiments, are essential to move forward [11–17].

Regarding the assessment of cardiac function, we used the same echocardiographic system, Vevo 2100 (Visual Sonics), as the others groups [8, 9]. However, we used long-axis B-mode image tracings to measure LV volumes and calculate EF, while the other two groups used only M-mode tracings to calculate fractional shortening (FS) or EF [8, 9]. Both sets of guidelines on measurement of cardiac function via echocardiography [19, 20] recommend B-mode instead of M-mode evaluation to calculate LV systolic function in animal models of MI since M-mode is not accurate and leads to greater errors in post-MI remodeling because of the irregular geometric shape of the left ventricle after infarction. Especially when the M-mode

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**Fig. 5** Left ventricular collagen content and apoptosis. A Representative picrosirius red staining and polarized light microscopy images from middle sections of the hearts in the four groups. B and C Collagen content in the risk region and noninfarcted (non-risk) region. D Representative confocal microscopic images of TUNEL-stained heart section from IZ, BZ, and RZ (Blue: DAPI, pink: TUNEL). E–G TUNEL-positive cells (% of total cells) in IZ, BZ, and RZ. Data are mean ± SEM. (Color figure online)
measurement is performed by a person not blinded to group assignment, the potential bias from image acquisition and analysis undermines the validity of the results. To provide additional support for the functional results, in the current study, we also utilized hemodynamic analysis via PV loop measurements, a method independent of echocardiography. As summarized above, the results from both of these methods are consistently negative. The use of two independent methods to assess cardiac function is rare in the literature but important, because it greatly corroborates conclusions.

The second notable difference between our study and the previous ones [8, 9] is the animal model. We used 60 min of ischemia followed by reperfusion rather than the permanent ligation model used in the previous studies [8, 9]. The purpose of the animal model in our study is to evaluate a therapeutic strategy that has a clinical translation potential, and to provide mechanistic insights from an experimental study for translation to the clinical situation. Therefore, according to the Guidelines for experimental models of myocardial ischemia and infarction, experimental models used for this purpose must mimic the clinical setting as closely as possible [18]. Nowadays, most of patients with acute MI undergo reperfusion therapy. Thus, a model of coronary occlusion/reperfusion will have more clinical relevance. The difference in animal model and the time of miR mimic injections may also explain the difference between our results and the previous studies [8, 9]. In our study, the heart underwent 60 min of ischemia before miR injection, while in the previous studies, the myocardium was treated with miR mimics at the same time when the ischemia started (right after LCA ligation). That time frame of treatment, however, has no clinical relevance because, with very rare exceptions, patients cannot be treated at the very onset of MI.

There are also differences in the time course of cardiac function. Our results show that at 3 days after MI and miR treatment, there was no improvement in EF measured by echocardiography, while in the two previous studies, the EF of miR mimics-treated hearts already improved significantly as early as 5 days (miR-19a/19b treatment) [9] and 7 days (miR-590-3p) [8] after treatment. Lesizza et al. [8] reported that miR-199a-3p administration right after coronary occlusion did not affect EF during the first 2 weeks but prevented EF from declining over the subsequent 6 weeks, while we did not observe such a late-phase protection effect with the same dose of miR-199a-3p given after 60 min of ischemia. Perhaps the time of miR administration is important, but if that is so, there is no clinical applicability for miR administration at the very onset of coronary occlusion in patients, as mentioned above. Another critical question is whether the beneficial effects of miR mimics observed in the two above-mentioned studies in a model of acute MI can be reproduced in more chronic MI models, after LV remodeling and dysfunction have been established [47]. Further experiments should be considered to address this question.

Regarding the dose of miR mimics, we used the same dose as Lesizza et al. [8] (1.4 μg of miR mimics per heart), while Gao et al. [9] used 10 μg of miR mimics per heart. In another study claiming that synthetic miR-302b/c mimics improved cardiac function after MI, a dose of 10 μg miR-302b/c mimics per heart per day was delivered via intravenous tail-vein injection for 7 days [48]. As for any therapy, it is theoretically possible that higher doses or repeated doses of miR mimics could have been more effective in our study. The optimal dose of miR is unknown. However, the purpose of our study was to reproduce the data of Lesizza et al. [8], which we were unable to do.

All the studies [8, 9, 48] mentioned above with positive results on cardiac function also claimed that the mechanism of beneficial effects was cardiomyocyte proliferation and cardiac regeneration, based upon the results of BrdU-positive [48] or ethynyl-2-deoxyuridine (EdU)-positive [8, 9] cell counting 4–12 days after treatment. In our original design, we also intended to monitor any potential formation of new cells and, thus, treated the animals with BrdU. But since there was no effect of miR mimics on cardiac function, analysis of BrdU positivity became unnecessary.

In conclusion, we present the results of a randomized, blinded preclinical study which demonstrate that a single injection of synthetic hsa-miR-199a-3p, hsa-miR-149-3p, or hsa-miR-149-5p, at doses previously reported to be beneficial by others, does not improve cardiac function or remodeling in a murine model of reperfused MI. Whether the strategy of using a single administration of synthetic miR molecules to repair the infarcted heart is effective in preclinical studies remains an open question. Our data do not undermine the importance of miRs as therapeutic targets in heart disease, but rather underscore the need for rigorous preclinical testing to provide a foundation for safely moving forward with potential clinical translation of this strategy in patients with ischemic heart disease [49, 50].

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Authors’ contributions RB designed experiments, supervised study, and revised manuscript. YN performed experiments, analyzed data, and wrote manuscript. YG supervised findings. AG, QL, AT, and XZ performed experiments and analyzed data.

Data availability All data and material are available from the corresponding author on reasonable request.
Declarations

Conflict of interest The authors have no conflict of interest to declare.

Ethical approval All animal procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Louisville Institutional Animal Care and Use Committee (protocol number: 14034).

Consent to participate All the participants for the study have signed the written-informed consent.

Consent for publication All of the authors have approved the final version of this manuscript and have consented to the submission of this manuscript to the journal.

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