



# BM-164 (an H<sub>2</sub>S-donor) modulates microRNA expression associated with reperfusion-induced ventricular fibrillation in rat hearts

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## ABSTRACT

Ventricular arrhythmias are a leading cause of sudden cardiac death in myocardial infarction and heart failure. Reperfusion following ischemia can trigger severe arrhythmias, including ventricular fibrillation (VF), and microRNAs (miRNAs) which are increasingly recognized as critical regulators of these processes.

This study aimed to identify miRNAs associated with ischemia/reperfusion (I/R)-induced ventricular fibrillation by comparing expression profiles between fibrillated (VF) and non-fibrillated (No VF) rat hearts. Nanostring-based screening ( $n = 12$ ; 4 in each group: No I/R control, I/R no VF, and I/R VF) identified several ischemia-associated miRNAs, including rno-miR-30d, rno-miR-99a, rno-miR-130a, rno-miR-133a, rno-miR-181a, and rno-miR-191a. These candidates were subsequently validated in a larger cohort using TaqMan qRT-PCR (qPCR), including BM-164- and ascorbic acid (AA)-pretreated I/R groups administered 10 min before I/R ( $n = 6$  per group). The selection of these miRNAs was guided by prior literature demonstrating their established roles in ischemia-related pathways and cardioprotection, providing a biologically grounded rationale for their inclusion.

qPCR analysis, compared with Nanostring data on a Rat miRNA panel v1.5 (425 miRNAs), revealed significant alterations in a subset of these miRNAs in I/R hearts, particularly in VF-affected samples. BM-164, an H<sub>2</sub>S-donor, and to a lesser extent AA, were associated with partial restoration or upregulation of these miRNAs. These findings suggest that the examined miRNAs, particularly miR-99a, miR-191a, miR-30d, and miR-181a, are likely involved in the development of reperfusion-induced ventricular arrhythmias. BM-164 pretreatment was associated with changes of these miRNAs, which have been previously implicated in autophagy and apoptosis pathways; further studies are warranted to determine whether these alterations directly mediate cardioprotection or anti-arrhythmic effects.

Given the limited sample size, these findings should be considered preliminary. Larger cohorts and additional in vivo studies are required to clarify whether these miRNA alterations causally contribute to arrhythmogenesis or represent protective responses.

## 1. Introduction

Sudden cardiac death is frequently caused by ventricular arrhythmias, particularly ventricular fibrillation (VF), which can occur during

myocardial ischemia and reperfusion. Reperfusion can cause lethal myocardial injury; a major consequence is reperfusion-induced arrhythmia, most commonly ventricular fibrillation (VF), which occurs across species including humans. Despite extensive research, the

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molecular mechanisms underlying reperfusion-induced VF remain incompletely understood (Agiannitopoulos et al., 2021; Austin et al., 2019; Krokhalova and Vaseghi, 2019; Lujan and DiCarlo, 2007; Schirone et al., 2022; Wichter et al., 2002).

MicroRNAs (miRNAs) are small non-coding RNAs that regulate apoptosis, autophagy, and arrhythmogenesis in cardiovascular disease. Among these, miR-133a, miR-30d, miR-99a, miR-130a, and miR-181a have been most strongly associated with arrhythmia susceptibility, cardiomyocyte survival, autophagy, and mitochondrial regulation. Overexpression of miR-133a attenuates I/R-induced apoptosis and endoplasmic reticulum stress, while exosome-mediated delivery reduces post-infarction fibrosis via TGF- $\beta$  signaling. Down-regulation of miR-133a has been reported to potentially contribute to the development of VF in patients with myocardial infarction (Agiannitopoulos, 2021; Boštjančić et al., 2018; Cardin et al., 2012; Han et al., 2016; Kim, 2013; Tang et al., 2015; Zhang et al., 2015). By suppressing ROS production, miR-133a protects cardiomyocytes from apoptosis. miR-30d mimics decrease infarct size, inhibit cardiomyocyte apoptosis, and limit fibroblast proliferation (Boxhammer et al., 2023; Duisters et al., 2009; Ge et al., 2024). In addition, miR-133 and miR-30 directly downregulate the profibrotic protein connective tissue growth factor, thereby playing a key role in remodeling the myocardial extracellular matrix (Duisters et al., 2009). While miR-99a participates in cardioprotective signaling and apoptosis regulation, miR-130a suppresses myocardial I/R injury by downregulating the HMGB2/NF- $\kappa$ B axis and modulates FUNDC1-mediated mitophagy (Li et al., 2021, 2014; Yan et al., 2023). MiR-181a activates Akt/eNOS signaling, reduces apoptosis, and preserves cardiac function in I/R-challenged hearts (Zhang et al., 2019). However, it remains unclear which miRNAs are specifically altered in hearts that develop VF versus those that do not after I/R, and whether pharmacological intervention can prevent these maladaptive changes. A recent study from our group demonstrated that BM-164, a hydrogen sulfide-releasing ascorbic acid derivative, exerts cardioprotective effects in ex vivo rat hearts subjected to I/R by reducing ischemia-induced damage (Tánczos et al., 2024).

In the present study, we aimed to identify miRNAs whose expression is associated with VF development in I/R hearts and to investigate whether BM-164 affects these miRNA-regulated pathways. miRNA profiling was initially performed using the NanoString Rat miRNA Panel and validated by qPCR. This approach enables the identification of miRNAs linked to VF susceptibility and cardioprotection, revealing associations with BM-164 pretreatment and reperfusion-induced arrhythmias.

## 2. Materials and methods

### 2.1. Chemicals

Based on the results of Tánczos et al., 2024, BM-164 (a new H<sub>2</sub>S-releasing ascorbic acid derivative) and ascorbic acid (AA) were used at 30  $\mu$ M concentration for the treatment of hearts. Chemical structure of BM-164 is presented in Fig. 1 (Tánczos et al., 2024).

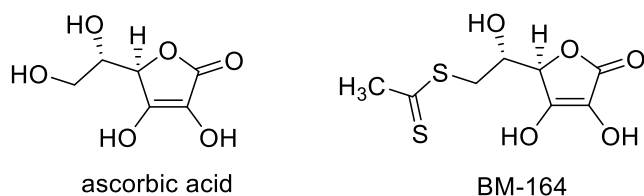


Fig. 1. Chemical structure of ascorbic acid (AA) and BM-164 (Tánczos et al., 2024).

### 2.2. Animals

Male CFY rats with a body weight range of 400–500 g were used for the experiments. All animals received humane care in compliance with the “Principles of Laboratory Animal Care” (EU Directive 63/2010 and The Hungarian Animal Protection Act, Law XVIII/1998). Maintenance and treatment of animals were additionally approved by the Institutional animal care and use committee of the University of Debrecen, (No. 6/2019/DEMAB, Debrecen, Hungary).

### 2.3. Experimental groups and allocation of heart samples

The goal of the experiments was to examine the potential beneficial effects of H<sub>2</sub>S-releasing compound, the modified ascorbic acid-H<sub>2</sub>S donor, BM-164, with regards to I/R injuries in isolated heart model (Skrzypiec-Spring et al., 2007). Beyond the physiological effects of AA and its new H<sub>2</sub>S derivative (data published by Tánczos et al., 2024) our aim was to examine the effects of these biologically active compounds on microRNA expression by rat Nanostring panel and quantitative real-time PCR (qPCR), particularly focusing on the difference between fibrillated (ventricular fibrillation, VF) and non-fibrillated hearts (no ventricular fibrillation, No VF) (Skrzypiec-Spring et al., 2007; Tánczos et al., 2024). Only male CFY rats were used in this study to reduce biological variability in miRNA expression and arrhythmia susceptibility. Due to the limited sample capacity of the NanoString platform (maximum 12 samples per run), inclusion of both sexes was not feasible within the current experimental design. It is important to note that this approach ensured uniform experimental conditions and allowed more reliable comparison between fibrillated and non-fibrillated myocardial samples.

Furthermore, to more accurately evaluate the results, isolated rat hearts were allocated into several experimental groups prior to further analysis. The groups of collected hearts and the overall flow of analyses are shown in Fig. 2.

Isolated rat hearts for further analysis were grouped as follows:

The following groups were used for the Nanostring analysis ( $n = 12$  total, 4 per group):

1. Non - ischemic control hearts (No I/R control,  $n = 4$ ): Hearts excised and placed in Krebs-Henseleit bicarbonate buffer (KHB) without undergoing I/R or any treatment.
2. Ischemic hearts without ventricular fibrillation (I/R no VF,  $n = 4$ ): Hearts subjected to 30 min global ischemia and 120 min reperfusion without pharmacological treatment.
3. Ischemic hearts with ventricular fibrillation (I/R VF,  $n = 4$ ): Hearts subjected to I/R and displaying VF at the onset of reperfusion.

Nanostring analysis was carried out for screening purposes. To increase statistical power for qPCR analysis, the number of hearts in groups 1–3 was increased to  $n = 6$ . Furthermore, to investigate the effect of BM-164, a novel H<sub>2</sub>S-releasing compound, two additional groups were included:

4. Ascorbic acid pretreated hearts (AA,  $n = 6$ ): Hearts pretreated with 30  $\mu$ M AA prior to 30 min ischemia and 120 min reperfusion.
5. BM-164 pretreated hearts (BM-164,  $n = 6$ ): Hearts pretreated with 30  $\mu$ M BM-164 prior to 30 min ischemia and 120 min reperfusion.”

It should be emphasized that samples from groups 1–3 were analyzed by Nanostring; a subset of miRNAs identified by Nanostring were then validated by qPCR in groups 1–5, each containing six hearts. In Fig. 2, the upper right panel shows the groups used for the Nanostring analysis, while the lower right panel displays the groups used for the qPCR analysis. All heart samples after collection were immediately preserved in RNA shield reagent (QIAGEN, Venlo, Netherlands) for subsequent RNA analyses.

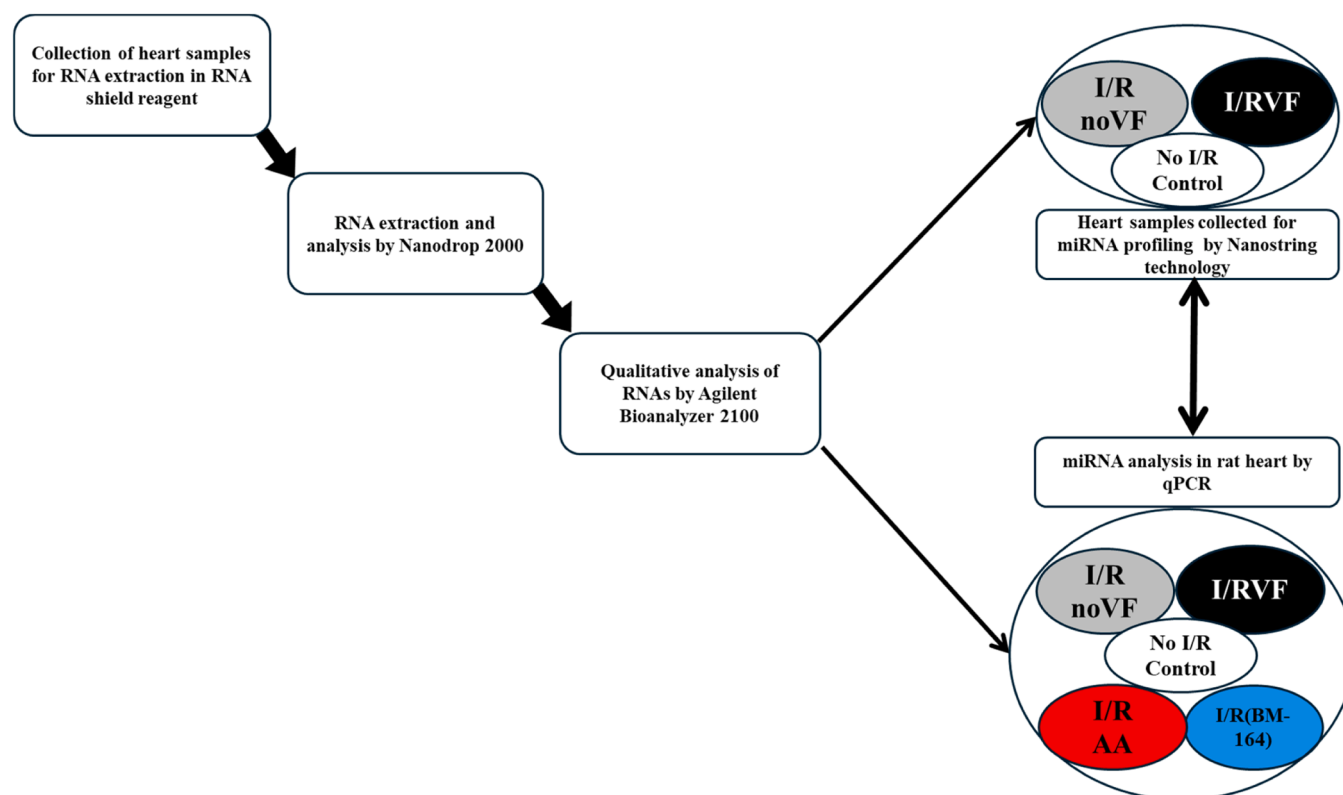


**Fig. 2. Schematic summary of miRNA samples/groups.** The upper-right panel indicates the groups analysed by Nanostring ( $n = 4$  per group, 12 hearts total) using the rat miRNA panel v1.5, which includes 425 miRNAs. The lower-right panel shows the groups analysed by qPCR, with six hearts per group ( $n = 6$  for all five groups). qPCR was used to validate selected miRNAs in each indicated group (see [Section 2.3](#)).

#### 2.4. Isolated heart preparation and cardiac function assessment

The anaesthesia in rats was carried out by ketamine/xylazine (50/10 mg/kg i.p.) and with heparin (400 U) injected intravenously to prevent blood coagulation. After anaesthesia, the chests were opened, and the hearts were excised and placed in modified Krebs–Henseleit bicarbonate buffer (KHB) (118 mM sodium chloride, 4.7 mM potassium chloride, 1.7 mM calcium chloride, 25 mM sodium bicarbonate, 0.36 mM potassium biphosphate, 1.2 mM magnesium sulphate, 10 mM glucose). The hearts were then cannulated via the aorta and perfused by the Langendorff technique at a constant pressure of 71 mmHg (10.0 kPa) ([Skrzypiec-Spring et al., 2007](#)).

Following a brief (5 min) equilibration period, the cannulated hearts were pretreated with 30  $\mu$ M of BM-164 or ascorbic acid in KHB for 10 min and then subjected to 30 min of global ischemia (ISA) followed by 120 min of reperfusion (in total: 30 min ISA + 120 min reperfusion; I/R). At the beginning of the reperfusion period, reperfusion-induced VF or no VF was recorded. In the ischemic control groups, the isolated hearts received only oxygenated KHB buffer prior to I/R. VF was recorded using LabChart Pro 8.1 software (AD Instruments, Sydney, Australia).

Perfusate temperature, gas composition, and baseline coronary flow (CF) were maintained as previously described ([Tánczos et al., 2024](#)). Briefly, the perfusate temperature was kept at 37 °C, the Krebs–Henseleit buffer was continuously oxygenated with a 95 % O<sub>2</sub> / 5 % CO<sub>2</sub> gas mixture, and baseline CF was measured by collecting coronary effluent over a defined time interval under constant perfusion pressure, expressed in mL/min. Baseline CF typically ranged between 8 and 12 mL/min during equilibration, depending on heart size and physiological variability. We note that this work is a direct continuation of [Tánczos et al. \(2024\)](#). All measurement conditions (perfusion pressure, sampling intervals, and calibration procedures) were within the same range as those used in that study. Therefore, CF was not detected in

the present work, as it had already been measured in detail in our previous study, both before ischemia and during reperfusion ([Tánczos et al., 2024](#)).

#### 2.5. RNA extraction

RNA extraction was performed according to the manufacturer's guidelines using the Quick-RNA Miniprep Plus Kit (ThermoFisher Scientific, Waltham, MA, USA). RNA concentration and purity were measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Only samples with acceptable purity values were used for further analyses. The optical density ratios were within the appropriate ranges (OD260/280: 2.05–2.10; OD260/230: 1.9–2.3, Supplementary Table 1). To prevent RNA degradation, all samples were aliquoted and stored at –80 °C.

#### 2.6. Quality control analysis of RNA samples by Agilent Bioanalyzer

RNA integrity was assessed using the Agilent Bioanalyzer a microfluidic chip and providing an objective measurement of RNA quality with RNA Integrity Number (RIN) (Agilent Technologies, Santa Clara, CA, USA). One microliter of total RNA from all extracted samples, with appropriate OD260/280 and OD260/230 values, was used for further analysis. following the instruction of the manufacturer (Agilent RNA 6000 Nano Kit, Santa Clara, CA 95051, USA).

In accordance with Nanostring recommendations, samples with RIN  $\geq 8$  were considered acceptable. It should be noted that two samples with RIN  $< 8$  were excluded from downstream analysis. However, one sample with a RIN value of 7.6, although slightly below the recommended threshold, was deemed to be of high quality based on the technical evaluation of the Nanostring operator and was therefore included in the analyses. Additionally, another sample did not yield an

automatic RIN value due to a measurement artifact; however, visual inspection of the electropherogram indicated good RNA integrity, and this sample was also included. Actual RIN values for all samples, along with notes on inclusion and exclusion, are provided in Supplementary Table 1, additional information related to Agilent analysis is provided in Supplementary Figure 1.

## 2.7. Analysis of selected RNA samples using nanostring technology

Twelve RNA samples from rat hearts, as indicated in Section 2.3 ( $n = 12$  total, 4 per group) (Fig. 2), were used for miRNA screening using Nanostring technology. The assay aimed to detect potential differences in miRNA profiles between non-ischemic control hearts (No I/R control) and ischemic/reperfused hearts that either developed ventricular fibrillation (I/R VF) or did not (I/R no VF). Here, the Nanostring analyses primarily aimed to identify miRNAs associated with rat heart ischemia, with a focus on ventricular fibrillation and non-fibrillated conditions.

For miRNA profiling, samples were processed using the Rat miRNA Panel v1.5 (425 miRNAs) on the NanoString SPRINT Profiler instrument (RT-Europe Research Center Ltd, Mosonmagyaróvár, Hungary; <http://rt-europe.org/>). One hundred nanograms of each sample were processed according to the manufacturer's instructions (user manual MAN—C0009–07). Data quality control (QC) was performed using nSolver Analysis Software 4.0. All samples passed QC without any flags (imaging, binding, positive control, or codeset normalization).

Total RNA was diluted to 33 ng/μl and cooled on ice. Three microliters of RNA were added to 3.5 μl of Annealing Master Mix containing miRNA assay controls and tag reagents in annealing buffer. Following the annealing reaction, ligation was performed by adding 2.5 μl Ligation Master Mix and 1 μl Ligase, incubated according to the manufacturer's instructions. After the clean-up reaction, 40 μl of molecular biology grade water was added. Hybridization was then performed by adding 20 μl of Master Mix containing the Reporter CodeSet hybridization buffer into new tubes. Denatured samples (5 μl) and 5 μl Capture ProbeSet were combined with the 20 μl Master Mix and incubated for 18 h at 65 °C in a thermal cycler. Resulting sample mixes were loaded into the microfluidic sample chambers of the nCounter SPRINT Cartridge (SPRINT-CAR-1.0) prior to running on the nCounter SPRINT Profiler.

Primary data analysis was conducted in nSolver. Raw count data were background-corrected using the mean of negative control values plus two standard deviations. After QC, data were exported and imported into BRB-ArrayTools (<https://brb.nci.nih.gov/BRB-ArrayTools>) for secondary processing. Data were log<sub>2</sub>-transformed prior to downstream statistical analysis. Quantile normalization was applied across samples to reduce technical variability. The final normalization pipeline therefore consisted of: (i) background correction using negative controls, (ii) log<sub>2</sub> transformation, and (iii) quantile normalization in BRB-ArrayTools. No additional scaling, positive control, or codeset normalization was applied beyond the default nSolver QC procedures.

## 2.8. TaqMan® miRNA Quantitative Real-time PCR

Based on Nanostring analysis, among the differentially expressed miRNAs only six (6) were selected for further analysis based on their reported significance in heart ischemia and ventricular fibrillation. Specifically, rno-miR-30d (Assay ID: 000420, catalog number: 4427975), rno-miR-99a (Assay ID: 000435, catalog number: 4427975), rno-miR-130a (Assay ID: 000454, catalog number: 4427975), rno-miR-133a (Assay ID: 002246, catalog number: 4427975), rno-miR-181a (Assay ID: 000480, catalog number: 4427975), and rno-miR-191a (Assay ID: 002299, catalog number: 4427975) were chosen for further validation using TaqMan qRT-PCR assays.

Total RNA (5 ng per reaction) was reverse transcribed using TaqMan™ MicroRNA Assays (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. qPCR reactions were

performed in triplicate using TaqMan® microRNA assays. For normalization, U6 snRNA (Assay ID: 001973, catalog number: 4427975), 5S rRNA (Assay ID: 001717, catalog number: 4427975), and rno-miR-20a were evaluated as endogenous controls. The stability of three candidate small RNA reference genes (U6, 4.5S RNA, rno-miR-20a) was evaluated across all experimental groups using NormFinder and geNorm. NormFinder analysis showed that U6 was the most stable (stability value = 0.147), followed by miR-20a (0.541) and 4.5S RNA (1.111). geNorm analysis yielded similar results (M-values: U6 = 0.40, miR-20a = 0.57, 4.5S RNA = 1.02) with pairwise variation V2/3 = 0.13, indicating that two reference genes (U6 + miR-20a) were sufficient for normalization. Accordingly, qPCR data were normalized using the geometric mean of U6 and miR-20a, resulting in consistent expression patterns. To ensure robust normalization and account for potential treatment-induced variation, the geometric mean of U6 snRNA and rno-miR-20a was used (Supplementary Table 2) (Andersen et al., 2004; Huang et al., 2006; Vandesompele et al., 2002).

Real-time PCR was performed on a CFX96 Real-Time PCR System (Bio-Rad Laboratories, Hercules, CA, USA) under the following cycling conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 1 min. All assays were run in triplicate. Triplicate Ct values were averaged and normalized to the geometric mean Ct values of U6 snRNA and rno-miR-20a. Relative expression levels were calculated using the  $2^{-\Delta\Delta Ct}$  method. No-RT and no-template controls (NTC) were included to ensure specificity and to exclude contamination, and no amplification was detected in these controls.

## 2.9. Bioinformatical analyses

### 2.9.1. In silico miRNA analyses

In silico study was carried out for rno-miR-30d-5p, rno-miR-99a-3p, rno-miR-130a-3p, rno-miR-133a-3p, rno-miR-181a-5p and rno-miR-191a-5p with the purpose to find and compare miRNAs specific targets. Each relevant miRNA was analysed by TargetScan and miRDB, using a computational algorithm to predict biological gene targets of miRNAs. We attempted to find link between the targets found in the databases and the most typical miRNA signaling pathway and cellular processes. Only predicted targets were included; no additional filtering for conserved sites or experimentally validated targets (e.g., miRTarBase, TarBase) was applied. Pathway enrichment analysis was conducted using ShinyGO (<https://bioinformatics.sdstate.edu/go/>) with *Rattus norvegicus* as the species. The complete list of predicted targets from both databases was used. Enrichment for KEGG pathways was assessed, and statistical significance was determined using Benjamini–Hochberg FDR correction, with pathways considered significant at FDR < 0.05.

### 2.10. Statistical analysis

Nanostring data ( $n = 4$ ) were quantile-normalized and background-corrected using negative control values. After background correction, 60 miRNAs were retained for downstream analysis. Differential expression between groups was assessed using a moderated *t*-test in the limma package (R). Multiple testing correction was performed using the Benjamini–Hochberg method, and targets with an adjusted *p*-value ≤ 0.05 were considered significant. qPCR data ( $n = 6$  per group) were analysed using two-tailed Welch's *t*-tests to account for unequal variances. Exact *p*-values and effect sizes (Cohen's *d*) are reported in Figures and Supplementary Table 3.

## 3. Results

### 3.1. miRNA Nanostring profiling of heart samples; heat map analysis

We aimed to characterize miRNA expression patterns in RNA isolated from rat hearts subjected to I/R, assigned to three experimental groups: hearts that developed ventricular fibrillation during I/R (I/R

VF), hearts subjected to I/R without fibrillation (I/R no VF), and non-ischemic control (No I/R control) (Fig. 2).

Twelve samples ( $n = 4$  per group) were analysed using the Rat miRNA Panel v1.5 on the NanoString SPRINT Profiler, which quantifies 425 individual miRNAs. Global miRNA expression patterns were visualized using a heat map (Fig. 3).

To ensure analytical robustness and reduce the likelihood of false positives, we applied a global pre-filtering step. Specifically, we defined the limit of detection (LOD) per sample using the mean signal of the negative control probes plus two standard deviations (mean + 2 SD), resulting in a threshold of 67.34. miRNAs with raw counts below this threshold were considered indistinguishable from background noise and were excluded from further analyses. This strategy adheres to the recommendations in the nCounter Gene Expression Data Analysis Guidelines for background thresholding (Malkov et al., 2009; Wang et al., 2016). This step allowed us to focus on reliably detected, above-background miRNAs.

Z-scores in the heat map were truncated to the range of  $-2$  to  $+2$  for visualization clarity. Based on above-background counts, red regions indicate highly expressed miRNAs, whereas green regions represent miRNAs with lower expression levels (Fig. 3). Using this threshold, approximately 60 miRNAs were identified as differentially expressed across the experimental groups (Supplementary Figure 2 and Supplementary Table 4). From these candidates, a subset of seven miRNAs—rno-miR-191a, rno-miR-181a, rno-miR-133a, rno-miR-130a, rno-miR-100, rno-miR-99a, and rno-miR-30d—was selected for further analysis, based on their reported relevance in the literature to ischemia/reperfusion-induced cardiac injury or potential roles in cardioprotection, as discussed in the Introduction. These miRNAs were highlighted separately in Fig. 4.

### 3.2. Differential miRNA expression across groups

#### a) No I/R Control vs. I/R no VF

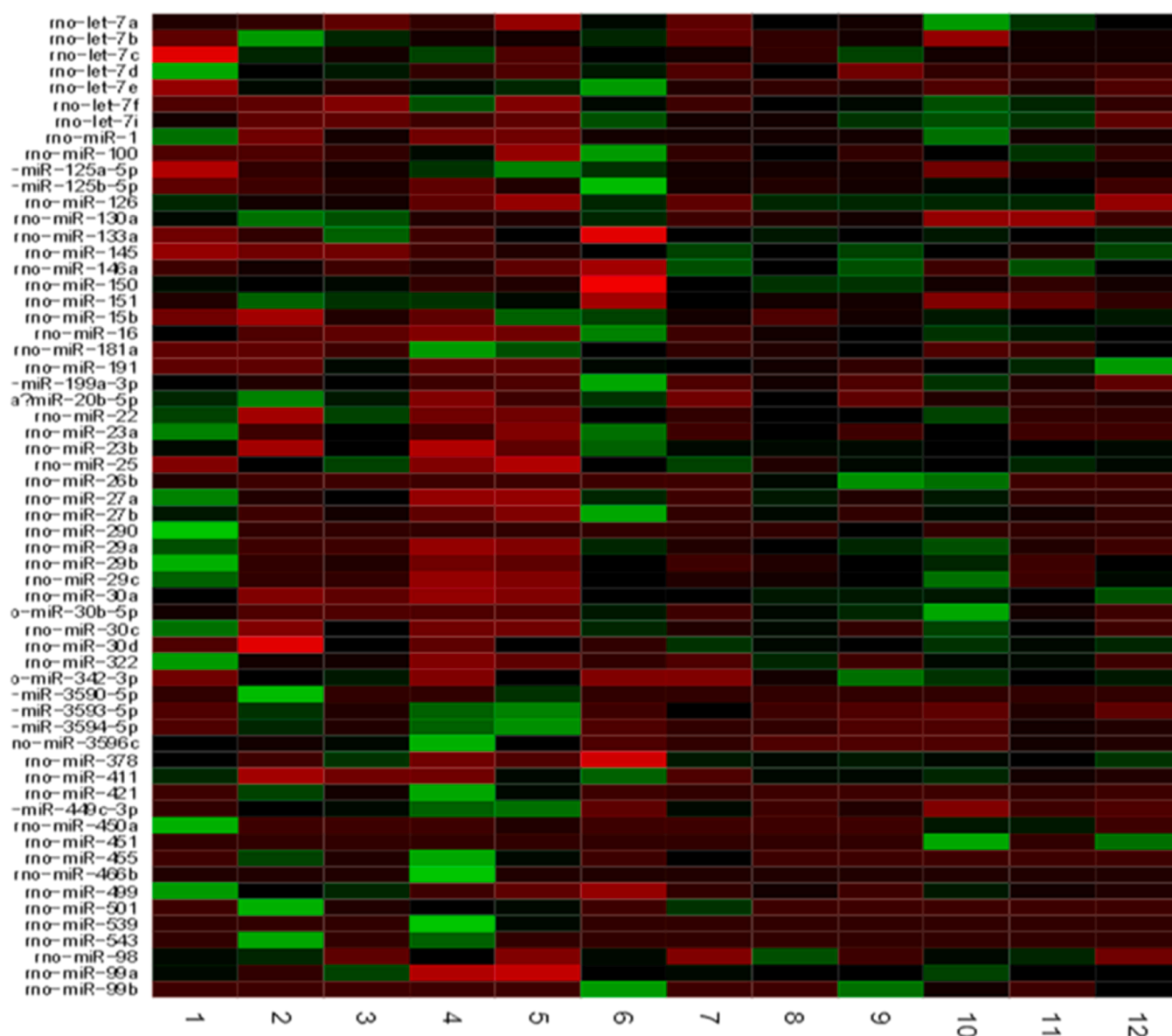


**Fig. 3.** Heat map analysis of miRNA profiles using the NanoString Rat miRNA panel. A total of 12 hearts (non-ischemic controls, hearts that developed VF upon reperfusion (I/R VF), and hearts that did not develop VF upon reperfusion (I/R no VF)) were analysed using the Rat miRNA Panel v1.5 (425 miRNAs) on the NanoString SPRINT Profiler instrument. miRNAs with raw counts below the threshold of 67.34 (calculated as the means of the negative control values plus two standard deviations) were excluded from analysis. Red areas indicate highly expressed miRNAs in the samples, and green areas indicate lowly expressed miRNAs.

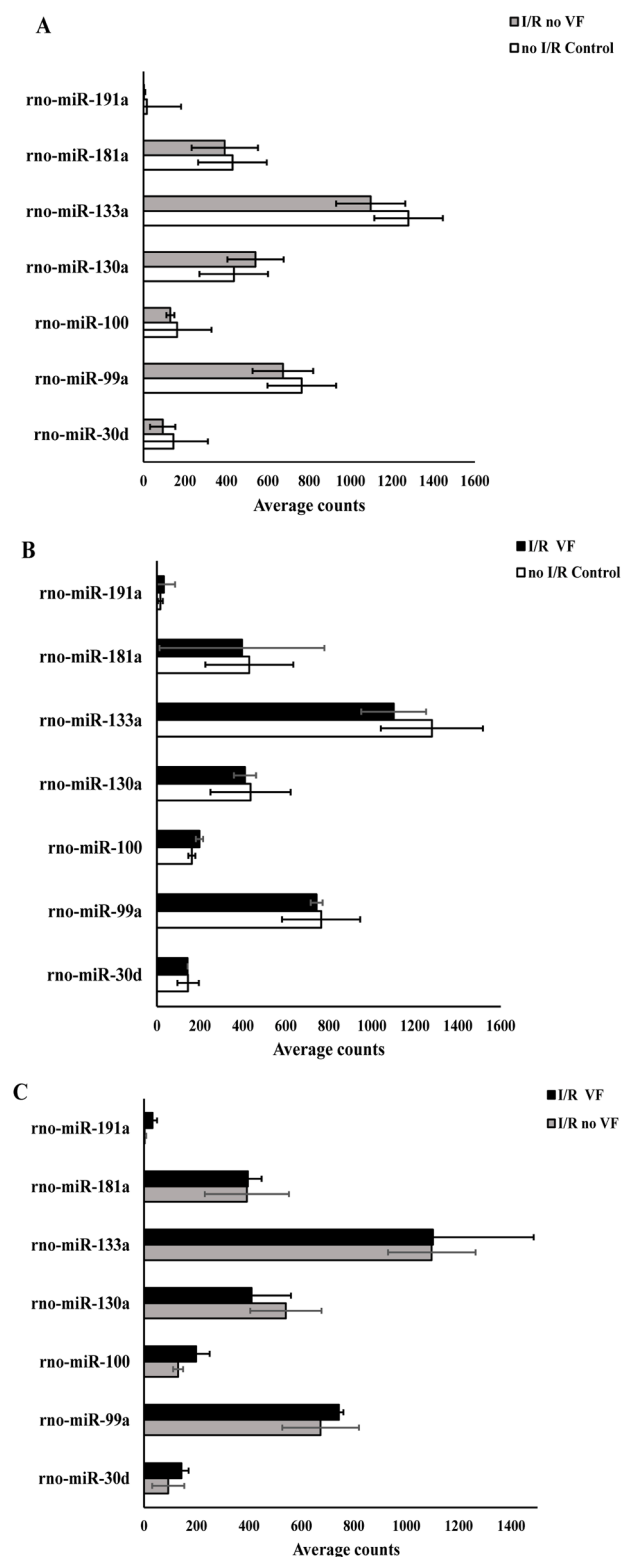


**Fig. 4.** Expression of selected miRNAs across experimental groups. Bar graphs show the detected counts of seven miRNAs (rno-miR-191a, rno-miR-181a, rno-miR-133a, rno-miR-130a, rno-miR-100, rno-miR-99a, and rno-miR-30d) in rat hearts from three experimental groups: non-ischemic controls (No I/R control), ischemic hearts without ventricular fibrillation (I/R no VF), and ischemic hearts with ventricular fibrillation (I/R VF). Statistical comparisons were performed using two-tailed *t*-test; however, false discovery rate (FDR) correction was applied to account for multiple testing. After FDR adjustment, none of the differences reached statistical significance. Error bars indicate standard deviation (SD), *n* = 4.

Average counts suggested modest differences between non-ischemic controls and ischemic hearts without fibrillation. A two-tailed *t*-test indicated that rno-miR-191a was significantly upregulated in controls compared with I/R no VF ( $p = 0.0126$ ). After false discovery rate (FDR) correction to account for multiple comparisons, no miRNA remained statistically significant (adjusted  $p = 1$ ) (Fig. 4A). b) No I/R Control vs. I/R VF

In fibrillated hearts, rno-miR-191a and rno-miR-181a were slightly elevated, whereas the remaining miRNAs were lower than in controls (Fig. 4B). A two-tailed *t*-test suggested down-regulation of rno-miR-99a in the I/R VF group ( $p = 0.0163$ ). However, this difference was not significant after FDR adjustment (adjusted  $p = 0.980$ ). c) I/R no VF vs. I/R VF

Among ischemic hearts, rno-miR-133a (~1200 counts) and rno-miR-99a (~1300 counts) showed high expression in the VF group. A *t*-test indicated slightly higher rno-miR-133a levels in I/R VF hearts ( $p = 0.0394$ ), while rno-miR-130a was lower, though not significant. After FDR correction, rno-miR-133a and all other miRNAs were not statistically significant (adjusted  $p = 1$ ) (Fig. 4C).

In summary, none of the seven selected miRNAs reached statistical significance after FDR-based multiple-testing correction, despite some nominal differences. These results highlight the limited statistical power due to the small sample size and underscore the need for validation in larger cohorts using qPCR.

Only FDR-corrected results are shown in Fig. 4, corrected *p*-values are not shown because they were not significant.

### 3.3. Individual miRNA expression detected by Taqman qRT-PCR

Nanostring analysis was used to screen miRNA expression in rat hearts subjected to ischemia/reperfusion, comparing fibrillated and non-fibrillated hearts with non-ischemic controls. Due to the high cost of the arrays, only a limited number of samples could be analysed (a maximum of 12 per panel), which made statistical comparisons and definitive conclusions challenging. For the qPCR analysis, the number of samples per group was increased ( $n = 6$ ) to achieve more robust and statistically reliable results. Additional experimental groups were also included (see Materials and methods), such as hearts treated with ascorbic acid (AA) or with a novel H<sub>2</sub>S-releasing ascorbic acid derivative, BM-164. The groups included in the study are shown in Fig. 2.

A subset of miRNAs was selected based on their high counts in the Nanostring analysis and on literature linking them to ischemia and ventricular fibrillation (VF) (see Introduction).

Results of qPCR analysis are shown in Figs. 5 and 6. Figure 5 (A–F) presents the expression of rno-miR-191a, rno-miR-181a, rno-miR-133a, rno-miR-30d, rno-miR-99a, and rno-miR-130a in I/R no VF and I/R VF groups compared to non-ischemic controls (No I/R control). Almost all examined miRNAs showed decreased expression in ischemic hearts, both fibrillated (VF) and non-fibrillated, compared to non-ischemic controls.

In the I/R no VF group, all miRNAs were moderately but not significantly downregulated, indicating I/R without VF did not significantly alter miRNA expression (exact *p*-values are shown in Figure 5). In contrast, significant decreases were observed for most miRNAs in I/R VF hearts compared to both I/R no VF and No I/R controls, with the exception of rno-miR-181a and rno-miR-99a, whose expression did not change significantly in either I/R no VF or I/R VF hearts compared to No I/R controls.

The most pronounced downregulation was observed for rno-miR-191a in I/R VF hearts versus I/R no VF ( $p = 0.0007$ ), importantly indicating the potential effect of ventricular fibrillation on its expression. This VF effect is further supported by the lower abundance of rno-miR-191a in I/R VF hearts compared to No I/R controls ( $p = 0.0202$ ) (Figure 5B). Similarly, rno-miR-130a showed significantly lower expression in I/R VF than in I/R no VF ( $p = 0.0099$ ) (Figure 5F) and was also decreased compared to No I/R controls ( $p = 0.0210$ ) (Figure 5F),

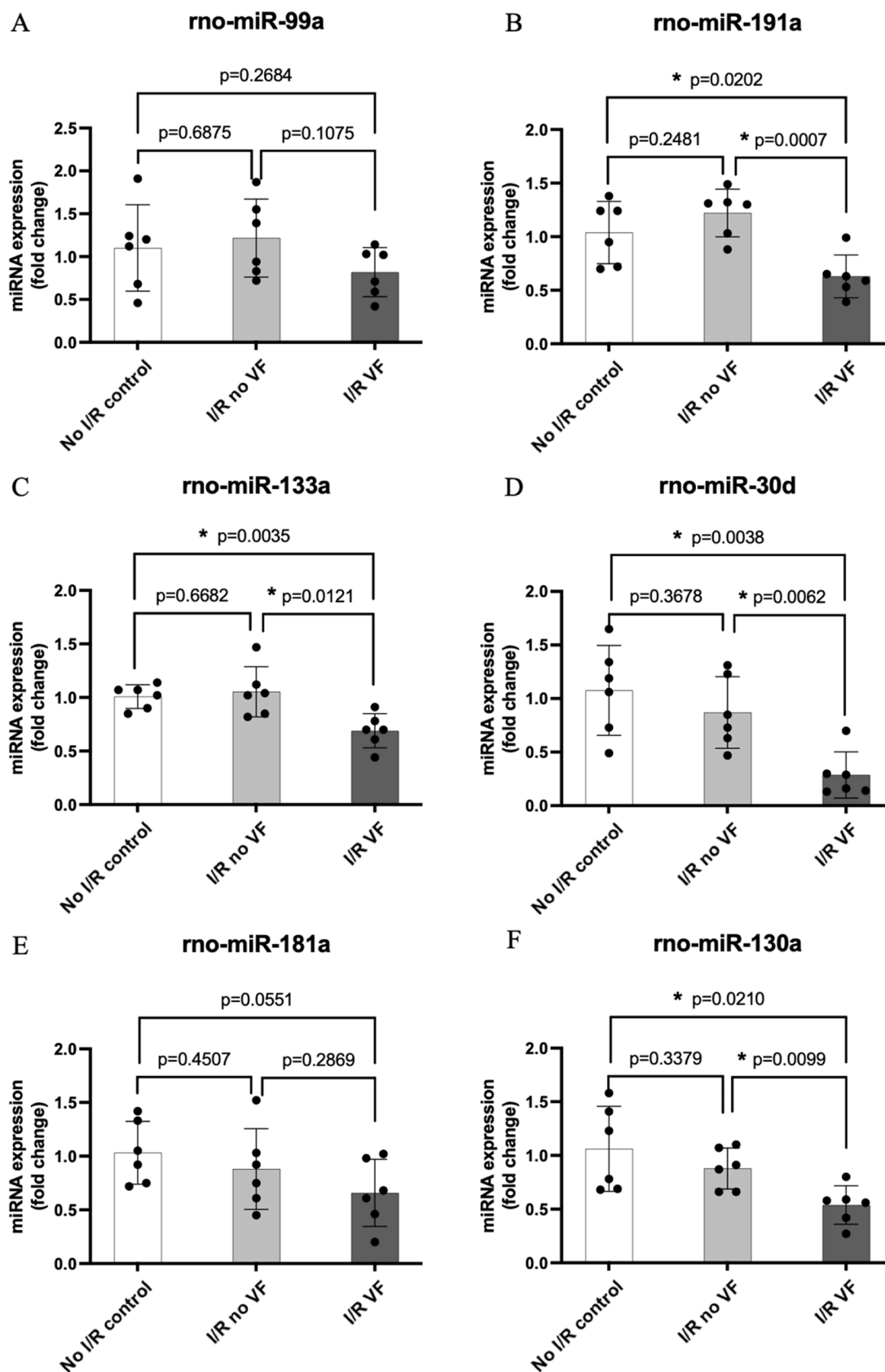


**Fig. 5.** qPCR analysis of cardiac miRNA expressions after ischemia/reperfusion (I/R). Panels A–F show the expression of rno-miR-191a, rno-miR-181a, rno-miR-133a, rno-miR-30d, rno-miR-99a, and rno-miR-130a in I/R no VF and I/R VF hearts compared to non-ischemic controls (No I/R Control). Bars represent group means with individual data points overlaid to illustrate sample variability. Data are presented as mean  $\pm$  SD (fold change), normalized to geometric mean of U6 and rno-miR-20a; p-values were calculated using two-tailed Welch's *t*-tests. \*  $p < 0.05$ , ( $n = 6$ ). Cohen's *d* values for all comparisons are provided in Supplementary Table 3.

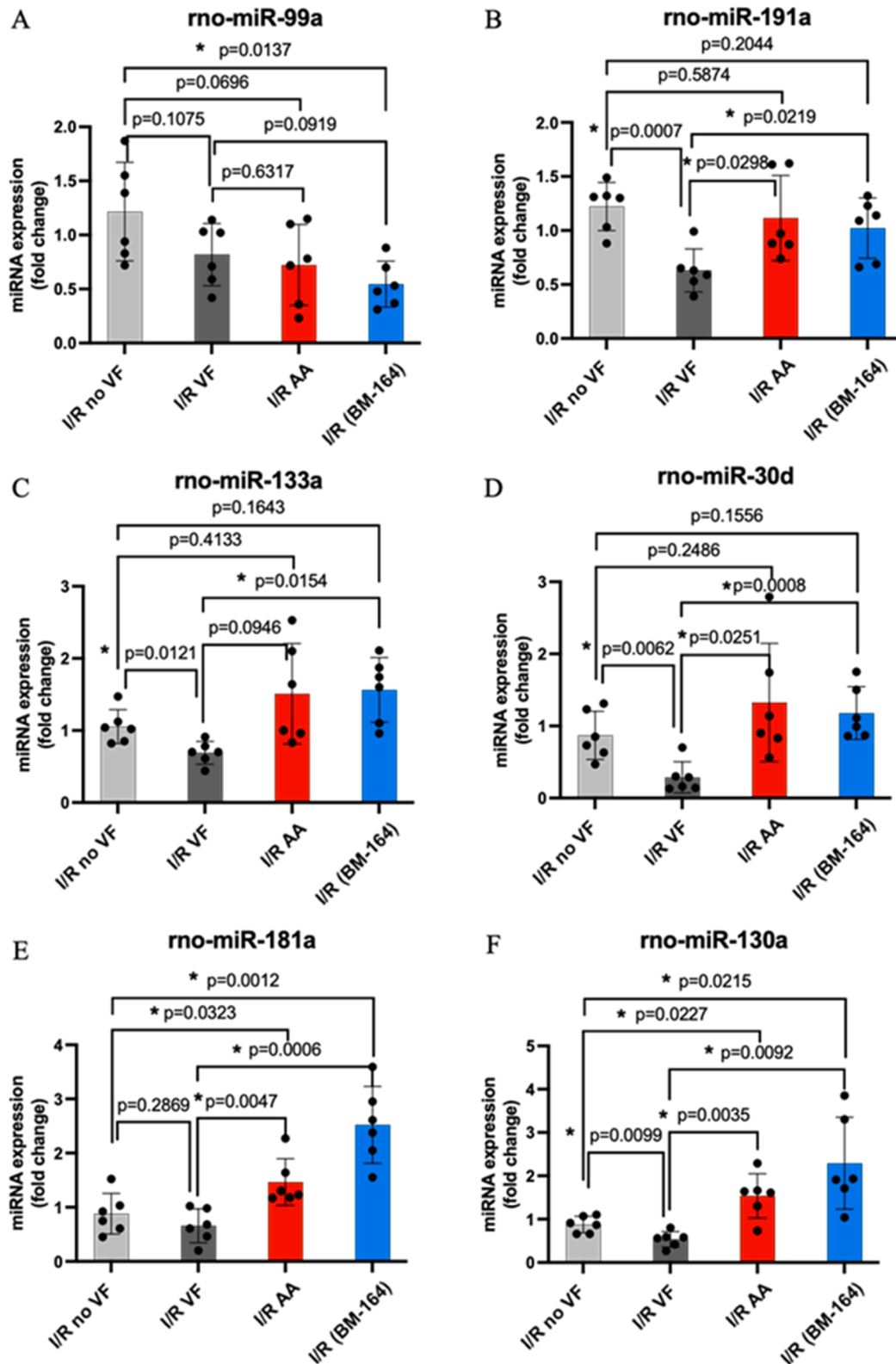


**Fig. 6.** Effects of AA and BM-164 pre-treatment on cardiac miRNA expression after I/R. Expression of (A) rno-miR-99a, (B) rno-miR-191a, (C) rno-miR-133a, (D) rno-miR-30d, (E) rno-miR-181a, and (F) rno-miR-130a in hearts subjected to 30 min ischemia and 120 min reperfusion, with or without VF (I/R VF or I/R no VF), and pretreated with AA or BM-164. BM-164 markedly upregulated several miRNAs downregulated by I/R and VF (miR-30d, miR-181a, miR-130a), while AA partially restored selected miRNAs. Bars represent group means with individual data points overlaid to illustrate sample variability. Data are presented as mean  $\pm$  SD (fold change), normalized to geometric mean of U6 and rno-miR-20a; p-values were calculated using two-tailed Welch's *t*-tests. \* $p < 0.05$ ,  $n = 6$ . Effect sizes (Cohen's *d*) further support the stronger modulatory effect of BM-164 (Supplementary Table 3).

suggesting a strong VF-dependent modulation. A comparable effect of VF was observed for rno-miR-30d ( $p = 0.0062$  for I/R VF vs I/R no VF;  $p = 0.0038$  for I/R VF vs No I/R control) (Figure 5D). Overall, these miRNAs displayed the lowest expression in VF-affected hearts.

Cohen's  $d$  values were calculated to assess the effect size between experimental groups. Beyond the  $p$ -values, the calculated Cohen's  $d$  effect sizes corroborate the significance of these findings. The statistically significant  $p$ -values demonstrate that the observed differences are unlikely to be due to a chance, while Cohen's  $d$  quantifies the magnitude of the effect and its potential biological relevance. Cohen's  $d$  analysis revealed that ventricular fibrillation (I/R VF) strongly modulates specific miRNAs. The most pronounced effects were observed for rno-miR-30d, rno-miR-130a, and rno-miR-191a, all showing large effect sizes ( $d = 1.72$ – $2.84$ ) when comparing I/R VF with either I/R no VF or non-ischemic controls. In contrast, ischemia alone (I/R no VF) produced small to medium effects ( $d = 0.22$ – $0.58$ ) for these miRNAs, indicating more modest changes. These results suggest that while ischemia alone moderately alters miRNA expression, ventricular fibrillation exerts a substantial impact on the cardiac miRNA profile, highlighting potential targets involved in VF-associated molecular pathways. Detailed Cohen's  $d$  values for all miRNAs and group comparisons are provided in Supplementary Table 3.

Fig. 6 shows the expression levels of selected miRNAs in hearts pre-treated with AA or BM-164, compared with the I/R VF and I/R no VF groups. miR-99a expression was highest in the I/R no VF group and decreased progressively in I/R VF, I/R AA, and I/R (BM-164) hearts. A significant reduction was observed in the BM-164-treated group compared with I/R no VF ( $p = 0.0137$ ), whereas AA had no significant effect (Fig. 6A). Comparison of I/R VF with I/R (BM-164) revealed that BM-164 did not significantly alter miR-99a expression in this setting ( $p = 0.0919$ ) (Fig. 6A).

As previously noted in Figure 5, miR-191a was markedly down-regulated in I/R VF compared with I/R no VF ( $p = 0.0007$ ). In Fig. 6 is presented that both AA and BM-164 partially restored miR-191a expression to levels approaching those observed in I/R no VF hearts (Fig. 6B). When comparing BM-164 and AA with I/R VF, miR-191a expression was significantly increased ( $p = 0.0219$  for I/R (BM-164) vs. I/R VF;  $p = 0.0298$  for I/R AA vs. I/R VF) (Fig. 6B).

Rno-miR-133a levels showed an upward trend in both pre-treated groups. AA increased rno-miR-133a expression relative to I/R no VF and I/R VF, though not significantly, while BM-164 induced a significant increase compared with I/R VF ( $p = 0.0154$ ). BM-164 also produced a non-significant elevation versus I/R no VF ( $p = 0.1643$ ) (Fig. 6C).

Rno-miR-30d was significantly reduced in I/R VF compared with I/R no VF ( $p = 0.0062$ ). Both AA and BM-164 significantly increased its expression relative to I/R VF ( $p = 0.0251$  and  $p = 0.0008$ , respectively). However, compared with I/R no VF, neither treatment significantly altered miR-30d abundance ( $p = 0.1556$  for AA;  $p = 0.2486$  for BM-164) (Fig. 6D).

Rno-miR-181a was strongly upregulated by both treatments. Its expression was significantly higher than in I/R no VF ( $p = 0.0006$  for AA;  $p = 0.0012$  for BM-164) and significantly elevated compared with I/R VF ( $p = 0.0323$  and  $p = 0.0047$ , respectively) (Fig. 6E).

A significant increase in miR-130a was also observed in treated samples. The most pronounced elevation occurred in AA- and BM-164-treated hearts compared with I/R VF ( $p = 0.0035$  and  $p = 0.0032$ , respectively) (Fig. 6F).

Overall, BM-164 pre-treatment exerted more pronounced and consistent modulatory effects on cardiac miRNA expression than AA. While AA partially restored some miRNAs, BM-164 significantly upregulated several miRNAs (miR-30d, miR-181a, miR-130a) that were downregulated by ischemia/reperfusion and VF, suggesting a stronger cardioprotective potential in modulating miRNA responses to ischemic injury.

Cohen's  $d$  analysis was performed to quantify the effect size of pre-treatment with AA or BM-164 relative to I/R no VF and I/R VF

groups. Overall, BM-164 demonstrated stronger modulatory effects on miRNA expression than AA. For rno-miR-99a, large effect sizes were observed in comparisons involving BM-164 (Cohen's  $d = 1.93$  for I/R no VF vs. I/R BM-164), whereas AA had smaller effects ( $d = 1.19$ ). rno-miR-191a exhibited large negative effect sizes when comparing I/R VF with BM-164 ( $d = -1.65$ ), indicating partial restoration toward control levels.

BM-164 significantly increased rno-miR-30d, rno-miR-181a, and rno-miR-130a expression compared with I/R VF, with large effect sizes in all relevant comparisons (e.g.,  $d = 3.54$  for I/R VF vs. I/R BM-164 for rno-miR-181a). In contrast, AA generally produced moderate or small effects. All Cohen's  $d$  values for the comparisons between experimental groups are provided in Supplementary Table 3. These results suggest that BM-164 pretreatment may be associated with a potential cardioprotective role of cardiac miRNA expression following ischemia-reperfusion and VF, while AA provides only partial restoration for select miRNAs.

However, discrepancies between qPCR and Nanostring datasets highlight the impact of sample size and biological variability. qPCR results ( $n = 6$ ) were not always consistent with Nanostring ( $n = 4$ ), likely due to smaller sample size and individual differences in the response to 30 min of ischemia followed by 120 min of reperfusion, which reduced RNA sample homogeneity.

In conclusion, I/R induced distinct changes in cardiac miRNA expression, with the most pronounced downregulation occurring in hearts that developed VF. While some miRNAs were moderately decreased in I/R no VF hearts, substantial reductions were primarily associated with VF, suggesting that these miRNAs may contribute to arrhythmogenesis.

#### 3.4. In Silico miRNA target database analysis

In Silico analysis of two different miRNA databases TargetScan ([http://www.targetscan.org/cgi-bin/targetscan/vert\\_80/targetscan.cg](http://www.targetscan.org/cgi-bin/targetscan/vert_80/targetscan.cg)), and miRDB (<http://mirdb.org>), was performed for search of the potential targets of studied miRNAs (rno-miR-30d-5p, rno-miR-99a-3p, rno-miR-130a-3p, rno-miR-133a-3p, rno-miR-181a-5p, rno-miR-191a-5p). These databases were selected based on literature evidence indicating their frequent use in the analysis of rat samples. The aim of the screening was to identify the primary specific targets of miRNAs associated with myocardial ischemia. By analysing the studied miRNAs using these two databases, we identified their most common targets (Fig. 7A). The total number of predicted targets per miRNA is reported in the Supplementary Table 5.

Pathway enrichment analysis was performed for all predicted targets obtained from miRDB and TargetScan using ShinyGO (<https://bioinformatics.sdstate.edu/go/>), with *R. norvegicus* species. Enrichment for KEGG pathways was evaluated, and statistical significance was determined using Benjamini–Hochberg FDR correction, considering pathways significant at  $FDR < 0.05$ . The analysis identified apoptosis and autophagy among the top enriched pathways (Supplementary Table 6). Full FDR values and fold enrichment metrics for all pathways are provided in the Supplementary Table 6.

The enriched pathways also indicate that the studied miRNAs are likely involved in several biological processes, including apoptosis, autophagy, inflammatory signaling, and other mechanisms potentially relevant to the development of cardiac ischemia. Notably, comparative assessment of pathways and targets revealed that the shared targets of the examined miRNAs predominantly regulate the apoptosis and autophagy pathways (Fig. 7B).

For rno-miR-30d-5p, a total of 11 targets were identified in miRDB and 14 in TargetScan, with nine common targets (BCL2L11, PPARGC1B, MAPK8, RAB10, RAB22A, USP44, PTBP3, GJA1, and BECN1) mainly associated with both autophagy and apoptosis. For rno-miR-99a-3p, two targets (TMEM41A and MAPK9) were identified in two databases were used and both are related to the regulation of apoptosis or autophagy. Additionally, four other targets were identified by the TargetScan

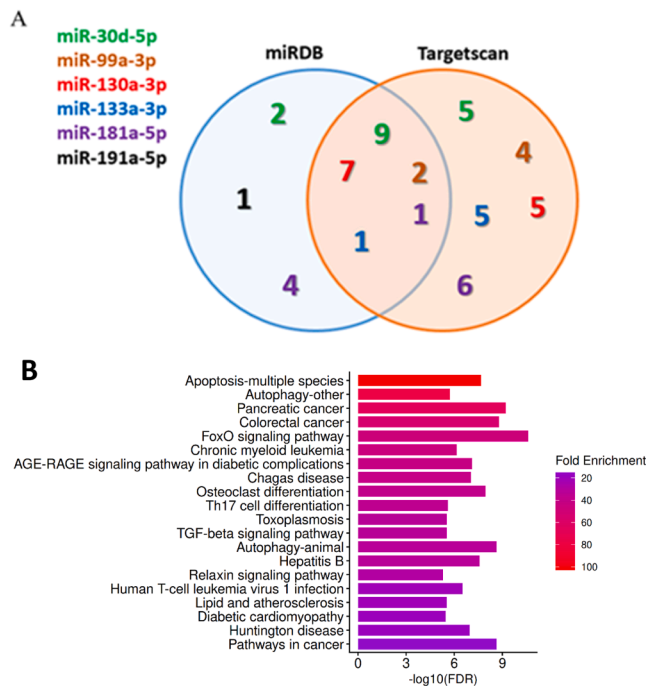


**Fig. 7.** Numbers of common targets identified in two different databases (A) and the classification of the predicted targets based on their associated signaling pathways (B). Potential targets for each studied miRNA were identified through screening in two different databases. The Venn diagram (A) visualizes the targets detected in each individual database, highlighting the overlapping targets found in both databases. Panel (B) Pathways enrichment analyses. The horizontal bar plot shows significantly enriched pathways, with the x-axis representing  $-\log_{10}(\text{FDR})$ . Bar colors correspond to the degree of Fold Enrichment, ranging from lower (purple) to higher (red) enrichment levels. The analysis highlights strong enrichment in pathways related to apoptosis, autophagy, cancer progression, and immune regulation.

database (Supplementary Table 5).

For *rno-miR-130a-3p*, seven targets were identified, all of which were also present in both miRDB and TargetScan (ULK2, PPARG, TGF $\beta$ 2, ATG16L1, BMPR2, ROCK2, and GJA1). Regarding *rno-miR-133a-3p*, BCL2L2, a key apoptosis-related gene, was the only target has been identified in both databases. Similarly, for *rno-miR-181a-5p*, BCL2L11 was the only common target in both miRDB and TargetScan databases. Interestingly, for *rno-miR-191a-5p*, RAB4A was identified as a target only in miRDB, whereas no targets were detected for this miRNA in TargetScan (Supplementary Table 5).

#### 4. Discussion

Ischemia/reperfusion (I/R)-induced ventricular arrhythmias, particularly VF, remain a leading cause of sudden cardiac death. Although several mechanisms have been identified, no effective targeted therapy or preventive strategy is yet available, making further elucidation of VF mechanisms and the identification of predictive molecules a key research priority (Austin et al., 2019; Demidova et al., 2015; Engstrom et al., 2022; Krokhalava and Vaseghi, 2019; Li et al., 2024; Narins et al., 2022; Schirone et al., 2022; Wichter et al., 2002).

Over the past years, our group has focused on synthesizing and evaluating compounds that mitigate I/R-induced myocardial damage, including EV-34, BM-88, and BM-164 (Gyöngyösi et al., 2021; Tanczos et al., 2024; Vass et al., 2023). Among these, BM-164—a water-soluble H<sub>2</sub>S-releasing ascorbic acid derivative—has shown beneficial effects in ex vivo rat hearts, improving functional parameters such as coronary flow, heart rate, and the incidence of reperfusion-induced VF. Furthermore, infarct size was reduced in hearts treated with either ascorbic acid

or BM-164 compared to untreated controls, supporting the potential cardioprotective effect of BM-164 under ischemic conditions (Tanczos et al., 2024).

The molecular background of VF and ischemia is complex and not fully understood (Prag et al., 2022; Schirone et al., 2022). Regulatory networks, including short non-coding RNAs such as miRNAs, have been increasingly recognized as modulators of cardiac fibrosis, remodeling, apoptosis, autophagy, and arrhythmogenesis. miRNAs can influence cardiac contractility, ion channel function, and the balance between cardiomyocyte survival and death, which is likely critical during ischemic injury and VF. Despite numerous studies, miRNAs directly linked to VF incidence remain largely unidentified, and their precise functional roles are still poorly understood (Elton et al., 2011; Makkos et al., 2021; Ságthy et al., 2020; Varga et al., 2014).

Heart samples were collected post-reperfusion for RNA extraction, and miRNA profiles were analysed using the NanoString Rat miRNA Panel v1.5 (425 miRNAs) on the NanoString SPRINT Profiler, a widely recognized method for quantitative assessment of small RNAs (Goytain and Ng, 2020). Non-ischemic control, ischemic no VF, and ischemic VF hearts were screened for miRNA expression. Importantly, after background subtraction and p-value adjustment, no significant differences were found among the groups for the miRNAs selected for further study. Some discrepancies were observed between qPCR and Nanostring results, likely due to differences in detection chemistry, dynamic range, normalization strategies, and reference gene choice. qPCR may be more sensitive for low-abundance miRNAs, whereas Nanostring can be less reliable near the detection limit; these technical factors were considered when interpreting the overall results (Mestdagh et al., 2014; Pescarmona et al., 2019).

A major limitation of our study is the small sample size of the NanoString discovery cohort ( $n = 4$  per group), resulting in low statistical power. Although the platform provides exploratory data, the small cohort increases the risk of false positives and false negatives, limiting statistical comparisons and definitive conclusions. For qPCR validation, the sample size was increased ( $n = 6$  per group) to achieve more robust and statistically reliable results. The miRNAs selected for independent TaqMan-based qPCR assays were only partially based on NanoString-detected expression. Importantly, the majority of miRNAs included in further analysis (*rno-miR-191a*, *rno-miR-181a*, *rno-miR-133a*, *rno-miR-130a*, *rno-miR-99a*, *rno-miR-30d*) were chosen based on their established roles and relevance in ischemia, ventricular fibrillation, cardioprotection, or other heart-related conditions, as supported by previously published literature, highlighting the critical contribution of prior knowledge to study design and interpretation. For instance, miR-30d is involved in cardiac remodeling, miR-181a mediates Akt-dependent cardioprotection and ischemia postconditioning (Zhang et al., 2019), and miR-130a regulates mitophagy (Yan et al., 2023). Additionally, Boxhammer et al. (2023) confirmed the cardioprotective role of miR-30d-5p in ischemia (Boxhammer et al., 2023).

In recent years, increasing attention has been directed toward the role of miR-133 in cardioprotection. miR-133a is downregulated in rodent models of angiotensin II-dependent hypertension and fibrosis and, together with miR-30, regulates cardiac fibrosis (Duisters et al., 2009). miR-133a/b and miR-208 modulate ion channel function and myocardial contractility, contributing to arrhythmogenesis (Boštjančič et al., 2018; Canale et al., 2022; Chen et al., 2017). Its anti-fibrotic and cardioprotective roles have been further supported in models of diabetes-induced fibrosis, apoptosis, and autophagy regulation, including CASP9 modulation (Ciccacci et al., 2015).

Consistent with these observations, our study revealed downregulation of miR-133a in the I/R VF group by qPCR, while treatment with AA or BM-164 partially restored its expression, BM-164 showing the strongest effect. Other miRNAs analysed (*rno-miR-191a*, *rno-miR-30d*, *rno-miR-130a*, *rno-miR-99a*) displayed similar patterns, generally decreased in I/R VF hearts and partially restored by pretreatment, supporting a potential contribution to VF susceptibility and

cardioprotection. In our study, these miRNAs generally showed similar changes, being decreased in the I/R VF group. An exception was miR-99a, whose expression further decreased significantly after pretreatment with AA or BM-164. Pretreatment with AA or BM-164 partially restored the expression of the other miRNAs, with BM-164 showing the most pronounced effect. These results suggest that, alongside miR-133, miR-191a, miR-130a, miR-30d, and miR-99a contribute to the response to I/R injury, with miR-99a exhibiting a distinct pattern in response to the applied pretreatments.

Pathway enrichment analysis of all predicted targets further indicates that BM-164 and AA may modulate miRNAs influencing apoptosis and autophagy pathways, providing mechanistic support for their observed effects.

Although these data are consistent with previous reports, the current study cannot establish causality between the observed miRNA changes and the cardioprotective effects of BM-164 or AA. Therefore, these results should be interpreted as preliminary associations between treatment and altered miRNA profiles, potentially linked to autophagy and apoptosis regulation. Future *in vivo* studies, including gain- and loss-of-function experiments and protein-level analyses of predicted miRNA targets, are necessary to clarify whether these miRNA alterations mediate the protective effects of BM-164. This study has several limitations that should be considered when interpreting the findings.

Study limitations:

- First, the NanoString discovery cohort had a small sample size ( $n = 4$  per group), limiting statistical power and increasing the risk of false-positive and false-negative results. Although qPCR validation used a larger cohort ( $n = 6$  per group), and thus provides stronger support, but caution is still needed for the interpretation of our results.
- Second, methodological differences between the NanoString platform and qPCR—such as sensitivity, dynamic range, amplification steps, and normalization strategies—may account for discrepancies between the datasets.
- Third, biological variability inherent to the ischemia–reperfusion model may influence injury severity and miRNA expression, contributing to inter-individual differences within groups.
- Fourth, the study was conducted exclusively in male rats. Because sex hormones affect myocardial energetics, ion channel function, arrhythmia susceptibility, and miRNA expression, the results primarily reflect male physiology. Future studies including both sexes are needed to determine sex-specific effects.
- Fifth, although the *ex vivo* Langendorff-perfused heart model offers controlled experimental conditions, it does not fully replicate *in vivo* hemodynamics or neurohumoral regulation; therefore, extrapolation of findings—particularly regarding ventricular fibrillation risk—should be performed cautiously.
- Finally, only a subset of NanoString-identified miRNAs was validated by qPCR, and no downstream protein-level targets or mechanistic pathways were examined, limiting conclusions about the functional impact of the observed miRNA changes.

## 5. Conclusion

Our results show that ischemia/reperfusion (I/R)-induced ventricular fibrillation (VF) is associated with changes in the expression of specific microRNAs. As a novel aspect of our study, we observed significant changes in the expression of certain miRNAs following treatment with ascorbic acid and BM-164. All the miRNAs examined in this study are known to be involved in various cardiovascular processes and influence the expression of autophagy- and apoptosis-related proteins.

Taken together, our findings support the hypothesis that ascorbic acid and the H<sub>2</sub>S-donating compound BM-164 may modulate expression of miRNAs involved in cardioprotection. While our study identified changes in miRNA expression following ischemia/reperfusion, these alterations may reflect indirect effects or shifts in cellular composition

rather than direct transcriptional regulation. Future studies incorporating protein-level measurements and functional assessments of autophagy and apoptosis pathways are needed to clarify the biological relevance of these findings.

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## Institutional Review Board Statement

Animal use for research: 13/2017/DEMÁB

## CRediT authorship contribution statement

**E. Szabó:** Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **J. Király:** Software, Formal analysis. **A.B. Mondal:** Software, Formal analysis. **I. Bereczki:** Resources, Methodology, Funding acquisition. **Z. Szabó:** Writing – original draft, Supervision, Conceptualization. **Á. Tószaki:** Writing – original draft, Supervision, Funding acquisition, Conceptualization.

## Conflicts of interest

All authors declare no conflict of interest.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.ejps.2026.107433](https://doi.org/10.1016/j.ejps.2026.107433).

## Data availability

All data presented in this study are available in the main text.

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