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# Serum Extracellular Vesicles Attenuate Cardiomyocyte Injury Induced by Hypoxic/Reoxygenation by Regulating miR-1229-5p

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Ischemic heart disease and the resulting heart failure remain the leading causes of death and disability worldwide. This study aimed to investigate the role of miR-1229-5p in serum extracellular vesicles (EVs) mediated myocardial protection by constructing a hypoxia/reoxygenation model (HR) in H9c2 cells. Cardiomyocytes were cultured and divided into different treatment groups: control group, HR group, serum-EVs group, and serum-EVs + miR-1229-5p inhibitor group. The expression levels of miR-1229-5p were detected using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The changes in cell proliferation and apoptosis were detected by MTT assay and flow cytometry. The myocardial injury-related indicators, cardiac troponin I (cTnI), creatinine kinase MB (CK-MB), and lactate dehydrogenase (LDH), were measured by enzyme-linked immunosorbent assay (ELISA). Finally, the luciferase reporter assay was used to verify the miR-1229-5p target. The proliferation of myocardial cells in the HR group was reduced, the number of apoptotic cells was increased, and myocardial injury indicators concentration was decreased. Transfection of miR-1229-5p inhibitor under serum-EVs treatment reduced the protective effect of serum-EVs on myocardial cell injury, decreased cell proliferation, increased the number of apoptotic cells, and increased myocardial injury indicator concentration. Additionally, FOXO4 may be the target of miR-1229-5p. Our data suggest that serum-EVs alleviate HR-induced cardiomyocyte injury by regulating miR-1229-5p/FOXO4.

**Keywords:** hypoxia/reoxygenation; miR-129-5p; myocardial injury; serum extracellular vesicles
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## Introduction

Myocardial ischemia/reperfusion injury (MIRI) refers to the phenomenon in which coronary recanalization after a certain period of myocardial ischemia aggravates the original myocardial injury (Zeng et al. 2018). At present, the most important thing in the treatment of acute myocardial infarction (AMI) is to open infarction-related blood vessels in time to achieve coronary recanalization, and functional damage, which seriously affects the effect of reperfusion therapy (Tang et al. 2020). MIRI has also become an important cause of the hemodynamic disturbance, malignant arrhythmia, pump failure, and even death after coronary recanalization (Wang et al. 2019). The mechanism of MIRI is complex and has not been fully elucidated. Existing studies suggest that free radical injury, calcium overload, endothelial cell damage, and myocardial energy metabolism disorders are all involved in the process of MIRI (Aragón et al. 2011). Besides, the levels of inflammatory factors are related to the damage to cardiac function and the number of myocardial cell necrosis after ischemia, speculating that the inflammatory response also plays a very crucial role in MIRI (Kitazume-Taneike et al. 2019). Thus, it is desperately required to explore novel therapies to protect the myocardium from ischemia-reperfusion injury.

Microvesicles (MVs) are membranous vesicles (diameter: 100 nm-1 $\mu$m) that are released extracellularly when cells are activated or apoptotic, which can carry DNA, RNA, proteins, organelles, and cytokines to target cells and mediate the transfer of information between cells (Sier et al. 2021). In recent years, many studies have been conducted on the role of extracellular vesicles (EVs) in the cardiovascular system, and EVs are involved in the pathophysiological process of many cardiovascular diseases, such as isch-

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emic cardiomyopathy, atherosclerosis, and hypertension (Femminò et al. 2020; Siwaponanan et al. 2022). Circulating EVs can transport various specific proteins, mRNA, and microRNA (miRNA), and can regulate gene expression, proliferation, and differentiation of target cells, participating in the development of cardiovascular diseases (Henning 2021). Circulating miRNAs in plasma are mainly enriched in MVs and exosomes, which, as effective miRNA delivery carriers, can protect the degradation of miRNA, participating in intercellular information transmission (Zhou et al. 2020). For instance, circulating exosomes could repair endothelial cell damage from oxidative stress damage via delivering miR-193-5p (Cao et al. 2021).

In the cardiovascular system, miRNAs are involved in regulating the occurrence and development of various diseases, including the cardiovascular system. Circulating miR-1229-5p was one of the differentially expressed and coronary artery disease-related miRNAs that were examined in serum samples from myocardial infarction patients and coronary artery disease (Dégano et al. 2020). The expression of miR-1229-5p can also be measured in serum exosomes (Jin et al. 2019). Whereas, the role of miR-1229-5p in serum EVs-mediated myocardial protection by constructing the hypoxia/reoxygenation (HR) model remains elusive. In the study, we investigated the changes in EVs concentrations and the expression of miR-1229-5p. Moreover, we explored the hypothesis that EVs-mediated transfer of miR-1229-5p can attenuate myocardial ischemia/reperfusion injury.

## Materials and Methods

*Cell culture*

H9c2 cardiomyocytes (Cell Bank of Chinese Academy of Sciences, Shanghai, China) were normally cultured in a DMEM/F-12 medium (Gibco, Eggenstein, Germany) containing 10% exosome-free serum in a 37°C incubator with 95% air and 5% $CO_2$. The cultured cells can be used for experiments when they are in the logarithmic growth phase.

*Establishment of hypoxia/reoxygenation (HR) cell model*

The H9c2 cell suspension (density of $1 \times 10^5$ cells/ml) was seeded in 96-well plates. The complete medium of normal culture of H9c2 cells was removed and an appropriate amount of serum-free and sugar-free hypoxic solution (139 mmol/l NaCl, 4.7 mmol/l KCl, 0.5 mmol/l $MgCl_2$, 1.0 mmol/l $CaCl_2$, 5 mmol/L HEPES, PH 7.4). For preparation of the hypoxia chamber, the chamber was saturated with 95% $N_2$ and 5% $CO_2$ for 10 min to drive off the internal oxygen, and then the cells were placed in the hypoxia chamber and cultured in a 37°C incubator for 1, 6, or 24 h. Subsequently, the cells were treated with a complete medium containing 10% exosome-free serum and cultured for 3 h for re-oxygenation.

*Extraction and transfer of EVs*

The animal studies were approved by the Animal Ethical Committee of The First Affiliated Hospital of Qiqihar Medical College. Blood was collected from the abdominal aorta of the HR model rat [Medical Discovery Leader (MDL), Beijing, China] and serum was separated from blood and stored at −80°C until use. The serum was filtered through a pore size of 0.22 $\mu$m and then added into a Model 8050 rotary ultrafiltration system with a 100,000 NWCO ultrafiltration membrane that was filtered with nitrogen and maximum inlet pressure below 517.122 kPa. The filtrate was ultrafiltered 3 times with phosphate-buffered saline (PBS) to obtain EVs on the filter membrane. The EVs were suspended in PBS and stored for subsequent experiments.

The PKH67 (Sigma-Aldrich, St. Louis, MO, USA) was used to label the isolated EVs for 20 min. H9c2 cells were treated with labeled EVs (20 mg/ml) for 12 h.

*Cell miRNA transfection*

The miR-1229-5p mimic (5'-GUGGGUAGGGUUUG GGGGAGAGCG-3'), mimic negative control (mimic NC; 5'-UUUGUACUACACAAAAGUACUG-3'), miR-1229-5p inhibitor (5'-CGCUCUCCCCCAAACCCUACCCAC-3') and inhibitor NC (5'-UUCUCCGAACGUGUCACGUTT-3') were obtained from RiboBio (Guangzhou, China) and transfected into H9c2 cells (100 nM) using the transfection reagent Lipofectamine 2000 (Life Technologies, Grand Island, NY, USA) for 24 h.

*Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)*

The expression of miR-1229-5p was measured using RT-qPCR. Firstly, total RNA was isolated from H9c2 cells and EVs using miRNeasy Mini Kit (Qiagen, Germantown, MD, USA) and miRCURY RNA Isolation Kit (Exiqon, Vedbaek, Denmark), respectively. Then, reverse transcription was carried out to make total RNA to cDNA using PrimeScript RT Reagent Kit (TaKaRa, Kusatsu, Japan). The RT-PCR was performed using SYBR Premix Ex Taq TM II (TaKaRa) and U6 was employed as an endogenous control. The primers sequences of miR-1229-5p and U6 were as follows: miR-1229-5p, 5'-GCCGAGGUGGGUAG GGUUUGGG-3' (forward) and 5'-CTCAACTGGTGTCGT GGA-3' (reverse), U6, 5'-GCTTCGGCAGCACATATACTA AAAT-3' (forward) and 5'-CGCTTCACGAATTTGCGTGT CAT-3' (reverse). The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of miR-1229-5p.

*MTT assay for cell viability*

Cell viabilities were assessed by MTT assay. After transfection, H9c2 cells ($5 \times 10^4$ cells/well) with different treatments were inoculated in 96-well plates for culture. The 10 $\mu$l MTT (5 mg/ml; Sigma-Aldrich) was added to the 96-well plate and then the cells were incubated for 4 h. The absorbance value (at 490 nm) was detected and the cell activities were normalized to control.

*Cell apoptosis assay*

Apoptosis was measured by flow cytometry using Annexin V-FITC/PI Kit (Biotech, Nanjing, China). Cells from different groups were collected, centrifuged at 1,000 r/min for 3 min, the supernatant was removed, pre-cooled PBS buffer was added, and cells were cleaned. The cells were suspended with 200 $\mu$L binding buffer, mixed with 10 $\mu$l Annexin V-FITC, and reacted at darkroom temperature for 10 min. After adding 300 $\mu$l binding buffer and 5 $\mu$l PI reagent, the cell suspension was filtered on the screen. The change in apoptosis ability was detected by flow cytometry.

*Enzyme-linked immunosorbent assay (ELISA)*

The changes in related biochemical indicators were detected by ELISA. Cell supernatants of different treatment groups were collected according to the requirements of ELISA Kit (Abcam, Cambridge, UK) instruction, and the levels of cardiac troponin I (cTnI), creatinine kinase MB (CK-MB), and lactate dehydrogenase (LDH) were detected.

*Dual-luciferase reporter assay*

The potential target gene of miR-1229-5p was predicted to be FOXO4 by the online database TargetScan. The wild type (FOXO4-WT) and mutant (FOXO4-MUT) recombinant plasmid vectors were constructed. MiR-1229-5p inhibitor, miR-1229-5p mimic, mimic NC, or inhibitor NC and FOXO4-WT or FOXO4-MUT were co-transfected into H9c2 cells, and the cells were lysed after continued culture for 24 h in the incubator. The relative fluorescence intensity (luciferase activities) was detected according to the luciferase activity detection kit instruction.

*Statistical analysis*

GraphPad 7.0 software was used for analysis. Measurement data were expressed as mean ± SD. Repeated measurement data were compared using one-way ANOVA, followed by a post hoc test where appropriate.

## Results

*Effects of hypoxia and re-oxygenation on cell viability*

The effects of hypoxia and re-oxygenation on cell activities in H9c2 cells were measured. As displayed in Fig. 1, we observed that cell activities were decreased after the treatment of hypoxia and reoxygenation ($P < 0.05$). Importantly, among the three-time treatment (1 h, 6 h, and 24 h), the 6 h and 24 h of treatment have shown a significant difference. Therefore, we selected hypoxia for 6 h for the following experiments.

*Effects of serum-EVs on hypoxia/reoxygenation (HR)-H9c2 cell viability and miR-1229-5p expression*

The cell activities were detected after serum-EVs treatment using an MTT assay. Compared to control, HR-treatment decreased the cell viabilities ($P < 0.001$), while the addition of serum-EVs reversed the decreased cell activities ($P < 0.001$, Fig. 2A). Moreover, the levels of miR-1229-5p were detected, and the results showed that miR-1229-5p was decreased in HR-treated cells, while it was increased in serum-EVs ($P < 0.001$, Fig. 2B). These data revealed that miR-1229-5p expression may affect the HR injury in H9c2 cells.

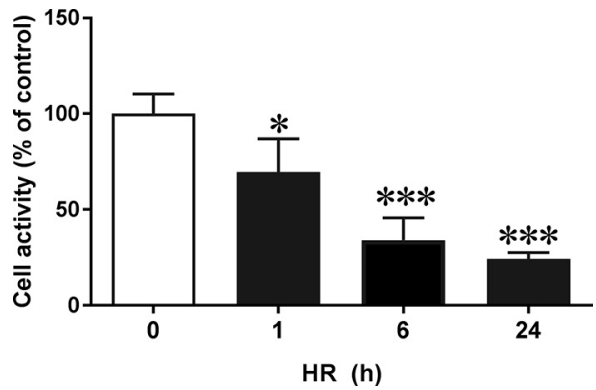


Fig. 1. The time course effect of hypoxia on myocardial cell viability (n = 5).
*$P < 0.05$, ***$P < 0.001$.

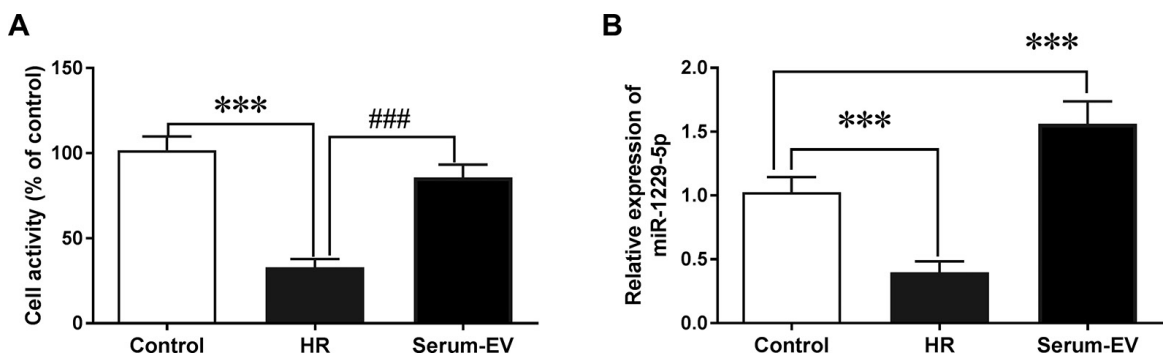


Fig. 2. Hypoxia/reoxygenation (HR)-treatment and serum extracellular microvesicle (EV)-treatment affected cell activities and miR-1229-5p expression levels.
(A) The changes of cell activities (n = 5). (B) The expression of miR-1229-5p in different treatment groups (n = 5).
***$P < 0.001$ vs. control. $^{###}P < 0.001$.

*Knockdown of miR-1229-5p decreased the protective role of serum-EVs to HR injury in H9c2 cells*

Firstly, the serum-EVs and miR-1229-5p transfer efficiency were confirmed. The expression of miR-1229-5p levels was high in serum-EVs, and transfection of miR-1229-5p effectively decreased the miR-1229-5p expression level in HR-treated H9c2 cells ($P < 0.001$, Fig. 3A). By using the MTT assay, the cell viabilities were increased by serum-EVs compared to the untreated-serum-EVs HR cells, but knockdown of miR-1229-5p weakened the increased cell proliferation abilities induced by serum-EVs ($P < 0.001$, Fig. 3B). Moreover, the cell apoptosis rate was significantly decreased after serum-EVs treatment, while the decreased apoptosis rate was diminished by a miR-1229-5p inhibitor ($P < 0.001$, Fig. 3C). These data suggested that serum-EV increased cell proliferation and inhibited apoptosis, while miR-1229-5p downregulation weakened the protective effects caused by serum-EVs, that is, serum-EVs may protect the myocardial injury from HR by regulating miR-1229-5p.

*Effect of serum-EVs on HR caused myocardial cell injury indicators after inhibiting miR-1229-5p*

Cell supernatant of different treatments was collected to examine the changes in the concentrations of cTnI, CK-MB, and LDH, which are indicators of myocardial cell injury. After serum-EVs treatment, the concentrations of cTnI ($P < 0.001$, Fig. 4A), CK-MB ($P < 0.05$, Fig. 4B), and LDH ($P < 0.001$, Fig. 4C) were significantly inhibited. However, after the miR-1229-5p inhibitor was transferred to serum-EVs treatment, the indicators of cardiac cell injury increased ($P < 0.05$). The results showed that serum EVs had a protective effect on HR-induced myocardial injury, but the protective effect of serum EVs decreased when miR-1229-5p was reduced.

*FOXO4 is a direct target of miR-1229-5p*

The online TargetScan database showed the potential

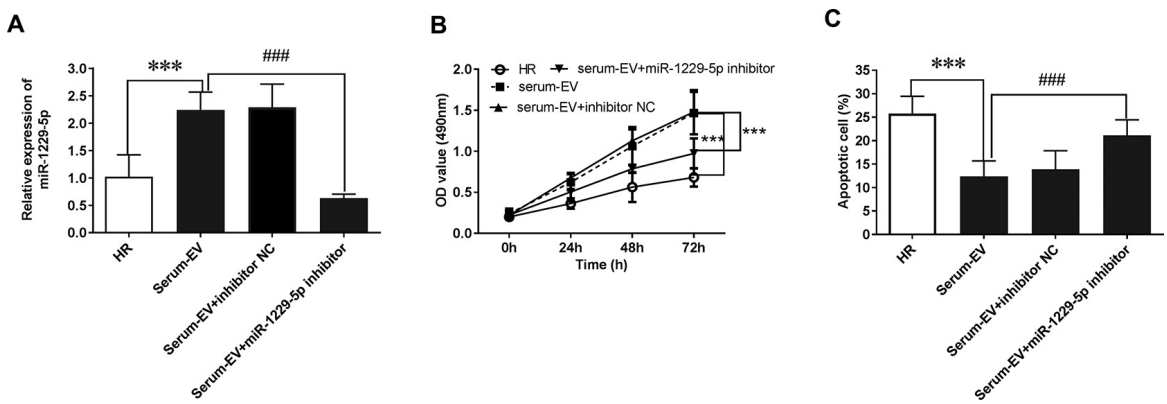


Fig. 3. Effects of miR-1229-5p inhibitor on proliferation and apoptosis of H9c2 cells.
(A) Transfer and incubation efficiency was detected by RT-qPCR (n = 5). MiR-1229-5p inhibitor decreased the increased expression of miR-1229-5p in serum-EV. $^{***}P < 0.001$ vs. HR group. $^{###}P < 0.001$ vs. serum-EV group. (B) The proliferation activities were measured using MTT assay (n = 5). $^{***}P < 0.001$. (C) The cell apoptosis capacities were measured by flow cytometry assay (n = 5). $^{***}P < 0.001$ vs. HR group. $^{###}P < 0.001$ vs. serum-EV group.
Inhibitor NC, inhibitor negative control.

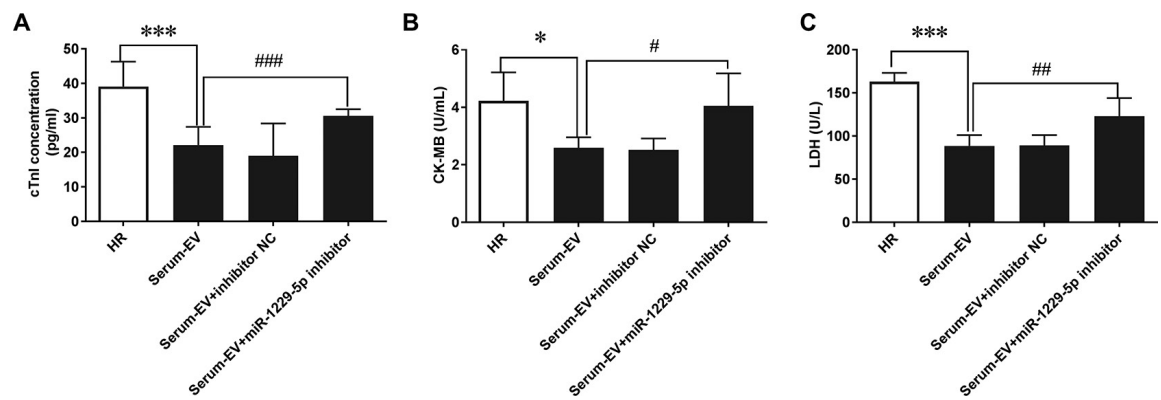


Fig. 4. Effects of serum extracellular microvesicles (serum-EVs) on HR myocardial cell injury indicators after miR-1229-5p inhibition.
(A-C) The expression levels of cTnl (A), CK-MB (B), and LDH (C) were measured using ELISA assay (n = 5). $^{*}P < 0.05$, $^{***}P < 0.001$ vs. HR group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. serum-EVs group.
Inhibitor NC, inhibitor negative control.

target of miR-1229-5p and FOXO4 had binding sites with miR-1229-5p (Fig. 5A). The increased expression of miR-1229-5p decreased the luciferase activities of the FOXO4-WT group, while no effect on luciferase activity was observed in the FOXO4-MUT group ($P < 0.001$, Fig. 5B). The data replied that miR-1229-5p may participate in the protective effect of serum-EVs on HR-induced myocardial injury through targeted regulation of FOXO4.

## Discussion

Ischemic heart disease is mainly caused by the reduction of blood supply in the heart, resulting in insufficient oxygen supply to the blocked part of the heart, abnormal myocardial energy metabolism and the disease caused by myocardial cell apoptosis (Liu et al. 2020). The incidence and mortality of ischemic heart disease are increasing year by year in the world, and the incidence trend is getting younger and younger. Numbers of studies have demonstrated that ischemic preconditioning (IPC) can effectively play an anti-myocardial ischemia role, mainly in reducing the area of myocardial infarction and restoring cardiac function (Wang et al. 2018). Studies have shown that IPC-MVs in circulating blood can effectively reduce myocardial I/R injury in rats and reduce myocardial cell apoptosis (Zhang et al. 2020a). Many studies have also demonstrated that miRNAs carried by MVs can participate in the regulation of myocardial I/R injury (Ou et al. 2020). Therefore, we hypothesize that IPC-MVs can carry specific miRNAs, transport them to target cells, and change the expression levels of the corresponding miRNAs in target cells to play anti-myocardial I/R injury effects.

Increasing evidence has indicated that EVs have a protective effect against ischemia-reperfusion injury in many diseases (Bei et al. 2017; Quaglia et al. 2020; D'Ascenzo et al. 2021). For instance, exercise-induced circulating EVs could strengthen the protective effects of endogenous EVs from cardiac ischemia-reperfusion injury (Bei et al. 2017). However, some other studies reported that MVs may adversely affect cardiovascular disease. For instance, diabetic serum EVs injected into the intra-myocardium of non-diabetic hearts can aggravate myocardial ischemia/reperfusion injury (Gan et al. 2020). In this study, we first validated the influence of serum-EVs on cell activities and the results revealed that myocardial hypoxia significantly reduced cell activities and serum-EVs-treatment. Thus, the effects of EVs on cardiovascular disease are "two-sided", and the influence of EVs produced by different stimuli and cell sources are different.

In the cardiovascular system, miRNAs are involved in regulating the occurrence and development of various diseases. Studies have shown that miRNAs mainly control the growth and contraction of cardiomyocytes, maintain heart rate, and affect plaque formation, lipid metabolism, and blood vessel generation. miR-1229-5p was dysregulated in several diseases with high expression levels, such as noise-induced hearing loss (Forouzanfar and Asgharzade 2020)

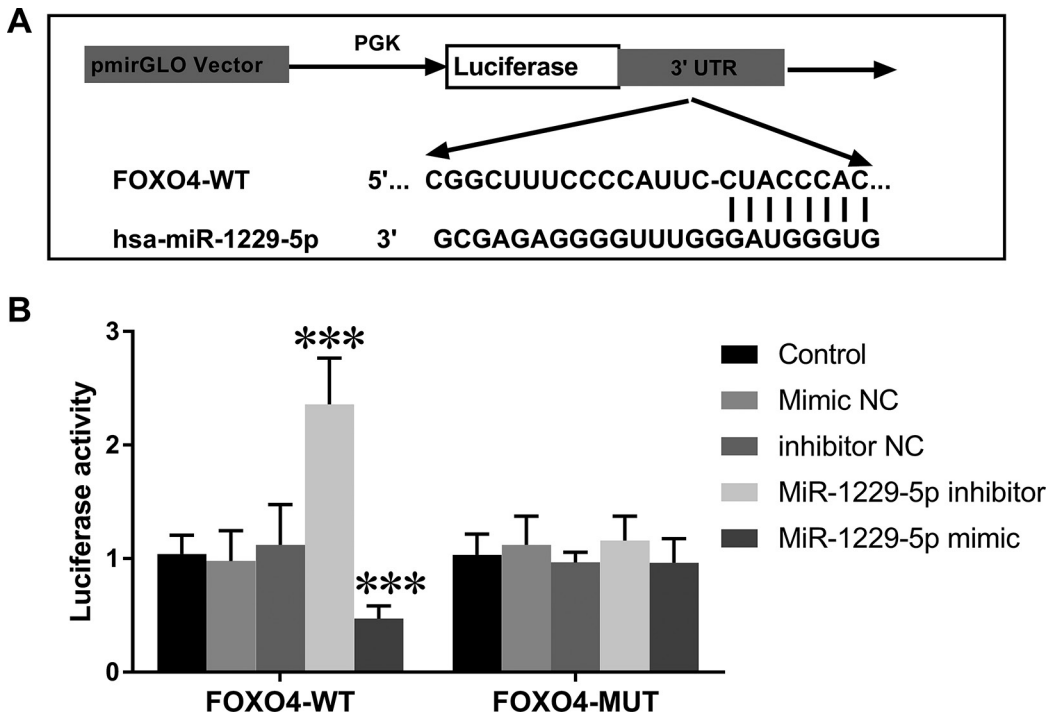


Fig. 5. FOXO4 is a direct target of miR-1229-5p.
(A) The binding sites between miR-1229-5p and FOXO4 were verified using TargetScan. (B) Upregulation of miR-1229-5p decreased the luciferase activities of the WT-FOXO4 group while it did not affect the luciferase activities of the MUT-FOXO4 group (n = 5). ***$P < 0.001$.
NC, negative control.

and coronary artery disease (Liu et al. 2015). The expression of miR-1229-5p was also observed in serum exosomes in colon cancer (Ogata-Kawata et al. 2014; Hu et al. 2019). Herein, the serum of miR-1229-5p was decreased in HR H9c2 cells, while serum EV-miR-1229-5p levels were elevated compared with control. These results replied that miR-1229-5p may be involved in the process of myocardial ischemia/reperfusion injury.

Studies indicated that miRNAs mainly control cardiomyocyte growth and contraction, maintain heart rate, affect plaque formation (Koroleva et al. 2017), lipid metabolism (Singh et al. 2018), and angiogenesis (Bassand et al. 2021). Furthermore, the effect of serum-EVs and miR-1229-5p were explored in HR H9c2 cells after transfection. The miR-1229-5p inhibitor was transfected into HR H9c2 cells and then co-incubated with serum-EVs. The results revealed that the pro-proliferative and anti-apoptotic effect of serum-EVs on HR H9c2 cells is counteracted. Moreover, myocardial cell injury indicators cTnl, CK-MB, and LDH levels were measured. We observed that serum-EVs declined the expression levels of cTnl, CK-MB, and LDH, while decreased expression of miR-1229-5p reversed the expression levels of myocardial cell injury indicators, which suggests that miR-1229-5p in serum-EVs does play a protective role in myocardial ischemia injury. Furthermore, the downstream target of miR-1229-5p was investigated and FOXO4 was a possible target of miR-1229-5p. Previous studies have demonstrated that FOXO4 is involved in myocardial ischemia-reperfusion injury (Yu et al. 2018; Huang et al. 2021) and myocardial infarction (Zhu et al. 2015). We speculated that serum-RVs may act a protective effect on myocardial ischemia-reperfusion injury by modulating miR-1229-5p/FOXO4 expression. In previous studies, EVs could deliver miR-221-3p (Zhang et al. 2020b), miR-10b, miR-223-3p (Parrizas et al. 2020), and miR-155 (Gomez et al. 2020) that significantly associated with progression of diseases. Considering that in addition to miR-1229-5p, serum-EVs may carry a variety of miRNAs or other genes, all of which may act on cardiomyocytes, so we cannot deny whether other substances carried by serum-EVs to cardiomyocytes are also involved in regulation, which will be further explored in our future experimental studies as well as detailed mechanism.

In conclusion, endogenous released serum EVs can reduce myocardial HR cell apoptosis to play the protection effect. The serum-RVs may act as a protective effect on myocardial ischemia-reperfusion injury by modulating miR-1229-5p/FOXO4 expression in H9c2 cells.

## Acknowledgments

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## Conflict of Interest

The authors declare no conflict of interest.

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