Platelet Membrane-Camouflaged Nanoparticles Carry microRNA inhibitor Against Myocardial Ischemia-Reperfusion Injury

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Research

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Abstract

Background

The incidence of myocardial ischemia reperfusion injury (MIRI) is increasing year by year, and there is an urgent need to develop new treatment methods. Nrf2 is believed to play a protective role during MIRI and is regulated by microRNAs (miRNAs). Platelets are physiologically targeted to MIRI injury sites. The focus of this study is on platelet membranes, also called platelet membrane vesicles (PMVs), camouflaged PLGA nanoparticles, carrying microRNA inhibitors to regulate Nrf2, and play a therapeutic role during MIRI.

Results

First, we characterized the high transfection efficiency and low toxicity of PLGA in vitro, as well as the good targeting of PMVs in vivo; then, we found a microRNA (microRNA-155-5p) that effectively regulates Nrf2 in vitro, and confirmed its effect on cardiomyocyte apoptosis during MIRI; finally, PMVs camouflaged PLGA-miRNA complexes (PMVs@PLGA-miRNA complexes) were prepared and applied to MIRI treatment, and it was found that it can significantly alleviate the myocardial damage caused by MIRI in vivo and exert effective therapeutic effects.

Conclusions

This research has developed a PMVs camouflaged PLGA nanoparticles, which can carry microRNA inhibitors (PMVs@PLGA-miRNA complexes), which can be targeted and efficiently transfected into cardiomyocytes, and play an important role in the treatment of MIRI. Our results suggest a novel biological delivery system that targets MIRI.

Introduction

Myocardial infarction is a kind of irreversible injury caused by severe and continuous myocardial ischemia, usually caused by thrombosis and vascular occlusion caused by coronary atherosclerosis[1, 2]. The only way to rescue ischemic myocardium from myocardial infarction is timely reperfusion. However, reperfusion can not only save the ischemic myocardium in infarction, but also induce another irreversible injury, which is myocardial ischemia-reperfusion injury (MIRI)[3–5]. MIRI refers to the recovery of the blood supply after myocardial ischemia leading to metabolic dysfunction and structural damage. It involves complex underlying mechanisms, including excessive production of reactive oxygen species (ROS) and oxidative stress, inflammation, calcium overload, and mitochondrial dysfunction[6–9]. More and more evidence indicates that oxidative stress is the major pathological process leading to myocardial injury after reperfusion[10].

Nuclear factor (erythroid-derived 2) related factor 2, otherwise known as NFE2L2 or Nrf2, is indispensable in inducing endogenous antioxidant enzymes in response to oxidative stress[11]. Nrf2 is widely expressed in various oxygen-consuming organs, such as muscle, heart, blood vessel, liver, kidney and brain[12]. Existing studies have shown that Nrf2 plays a variety of important roles in the pathological process of MIRI. Nrf2 is widely involved in reducing oxidative stress, improving endoplasmic reticulum stress, repairing damaged mitochondria, regulating inflammation and autophagy, etc., and protects cardiomyocytes when MIRI occurs[13–17]. In addition, some studies have demonstrated that microRNA (miRNA) can regulate Nrf2 at the post-transcriptional level[18–20].

There have been a lot of studies on applying modified miRNA inhibitor (antagomir) directly to the body for treatment[21–23]. Intravenous injection or in situ injection of antagomir directly antagonizes miRNA in the body, thereby enhancing the expression of a certain gene, which can play a therapeutic role. However, the short half-life and poor targeting of antagomir itself are also difficult to ignore. The development of targeted and effective vectors is the current focus and difficulty of miRNA therapy. Although viral vectors have high transfection efficiency, their cytotoxicity, carcinogenicity, and cellular immune response are terrible[24, 25]. Non-viral vectors have been taken into account the first choice in recent years due to their high safety and biocompatibility[26, 27]. Nanoparticles have a size of about 100nm, so they can easily penetrate cell membranes after being loaded with nucleic acids[28]. Moreover, this delivery system has extremely low cytotoxicity, good cell internalization ability and extremely strong transfection efficiency. Depending on reports, this helps to improve the treatment and diagnosis of cardiovascular diseases and cancers. We have previously found that biocompatible polylactic-glycolic acid (PLGA) nanoparticles have higher transfection efficiency for cells[29–31].

The incidence of MIRI has been increasing in recent years, and Nrf2 is one of the key therapeutic targets. In this study, nanoparticles disguised by platelets, carrying miRNA's inhibitor, through competition to bind miR-155-5p, indirectly increase the expression of Nrf2, thereby playing a role in myocardial protection during MIRI, providing new targeted therapy for MIRI potential method.

Materials And Methods

Cells and animals

H9C2 and HEK-293T cells were bought from the National Collection of Authenticated Cell Cultures, Chinese Academy of Sciences (Shanghai, China); they were cultured in DMEM (high glucose) medium complemented with 10% fetal bovine serum. The cells were kept at 37°C in 5% CO₂ at atmospheric pressure. Male Sprague Dawley rats (250±10g, 8 weeks old) were purchased from the Model Animal Research Center of Nantong University (Nantong, China). The rats are kept in a specific pathogen-free (SPF) environment at 22±1°C, a relative humidity of 50±1%, and a normal 12-hour day-night cycle. All animal experiments were approved by the Medical Ethics Committee of Nantong University and conducted under the guidance of the Laboratory of Nantong University. This study was approved by the Ethics Committee of the Affiliated Hospital of Nantong University (No. S20200314-012).

In vitro MIRI cell model and in vivo MIRI rat model

For in vitro experiments, H9C2 cells were cultured in normal medium (DMEM with 10% FBS) to 80% confluences. The cells were then cultured in a serum and sugar-free medium at 37°C in a three-atmosphere incubator under hypoxic conditions for 4 hours, which consisted of 94% N₂, 5% CO₂, and 1% O₂. Finally, the cells are cultured in normal medium under regular oxygen conditions to establish reoxidation. H9C2 cells that not expose to hypoxia conditions were considered the control group.

For in vivo experiments, 24 eight-week-old male Sprague Dawley rats were used for in vivo experiments. The rats were randomly assigned to the different groups. Animal care was in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines. Preparation of myocardial ischemia reperfusion model in rats: Rats were carried out under the Isoflurane throughout the surgery. Rats were artificially ventilated through a tracheal intubation tube connected to a small animal ventilator (VentStar Small Animal Ventilator). The skin of the rat's chest was debrided, disinfected with iodine, the skin was cut along the longitudinal sternum, the subcutaneous tissues and muscles were freed layer by layer with hemostatic forceps, the left 3rd to 4th intercostal space was exposed, and the dark shadow of the pulsating heart could be clearly seen, the rib cage was separated by inserting an elbow hemostatic forceps into the thoracic cavity along the left edge of the sternum at 3-4 rib space, the pericardium was torn open after opening the thoracic cavity, the heart was fully exposed with a chest opener, the left anterior descending branch of the coronary artery was ligated with 6/0 medical sutures for 30 minutes to restore blood perfusion, and the thoracic cavity was sutured layer by layer.

Cell apoptosis analysis

Aspirate the H9C2 cell culture medium into a centrifuge tube, wash the cells once with PBS, add an appropriate amount of trypsin cell digestion solution without EDTA to digest the cells; incubate at room temperature until the adherent cells can be blown down by gentle pipettes, add the collected in the above steps Mix the cell culture medium slightly, transfer it to a centrifuge tube, centrifuge at 1000 rpm for 5 min, carefully aspirate the supernatant; wash the cells twice with 1ml of 4°C pre-cooled PBS, then resuspend the cells with 250µl of diluted Binding Buffer and adjust the concentration 1×10⁶/ml; take 100µl of cell suspension into a 5 ml flow tube, add 5µl Annexin V-FITC, and mix gently; incubate at room temperature for 10 min in the dark; add 5 min before flow cytometry (FACS) detection 10µl propidium iodide solution, mix gently; add 400µl PBS to the reaction tube to resuspend the cells before the FACS detection, store in the dark, and then proceed to the FACS detection.

Preparation of PLGA-miRNA complexes

The miR-140-5p, miR-144-3p, miR-155-5p, miR-340-5p mimic, inhibitor, NC mimic, NC inhibitor, and miR-155-5p antagomir were purchased from GenePharma (Suzhou, China). Nanoparticles were obtained by the double emulsion method as reported in the previous study[30, 31]. The main component of nanoparticles is poly (D, L-lactide-co-glycolide) [PLGA, lactide: glycolide (65: 35), Mw=40–75 kDa] obtained from Sigma-Aldrich (St Louis, MO, USA). In order to load the miRNAs onto the nanoparticles, the

nanoparticles were modified with branched polyethyleneimine (PEI, Mw=25 kDa, Sigma-Aldrich) to make them positively charged for attracting negatively charged miRNAs. Briefly, the nanoparticle solution was mixed with PEI in deionized water, and then the mixed solution was added to the miRNA solutions and vortexed gently and incubated for 20 min at room temperature to formulate PLGA-miRNA complexes (**Fig. 1**).

Characterization of PLGA-miRNA complexes in vitro

The size and morphology of the PLGA-miRNA complexes were characterized by scanning electron microscopy (SEM, Carl Zeiss, Oberkochen, Germany). Briefly, the complexes were coated with platinum after freeze drying, and then photographed by SEM. The average diameter and size distribution of the complexes were determined by dynamic light scattering (DLS) using a Mastersizer 3000 laser particle size analyzer (Malvern Instruments Ltd., Malvern, UK). The in vitro cumulative release of miRNA from complexes was also measured in triplicate after they were incubated in phosphate-buffered saline (PBS) at 37°C under gentle agitation. The released RNA was detected using the Quant-iT™ RNA Assay Kit (Invitrogen; Grand Island, NY, USA). Gel retardation assay was used to evaluate the interaction between PLGA nanoparticles and miRNAs at different N/M ratios (the ratio of moles of the amine groups of polyethyleneimine to the moles of the phosphate groups of RNA). The potential of PLGA-miRNA complexes was also determined using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK).

The biocompatibility of PLGA-miRNA complexes (2.5 µg/mL, i.e., 1 mL medium contained 2.5 µg miRNAs loaded on 50 µg nanoparticles) against tenocytes was evaluated by MTT assay in vitro. The other concentrations of the complexes were also analysed in this study. The culture method of cells was similar to the previous study[29-31]. Transfection efficiency of PLGA-miRNA complexes was also evaluated by flow cytometry analysis in vitro.

Platelet isolation and platelet membrane derivation

Blood from SD male rats was collected into an EDTA tube and then centrifuged at 200g for 20 mins at room temperature to separate red blood cells and white blood cells. The supernatant was collected containing platelets (PRP). PBS with 5 mM of prostaglandin E1 (PGE1, GlpBio, USA) was added to the purified PRP to keep platelets inactivated. Isolated platelets were then pelleted by centrifugation at 1800g for 20 min at RT. After removing the supernatant, the platelets were re-suspended in PBS containing 5 mM of prostaglandin E1. The prepared suspended platelets are counted in the flow cytometer and control the number of platelets per tube $(2.5-3.0) \times 10^5$. To fabricate platelet membrane vesicles (PMVs), pelleting platelet from SD male rats plasma was repeatedly freeze-thawed, centrifuged at 8000g for 15 min, then sonicated for 2 min in a FS30D bath sonicator (Fisher Scientific, Waltham, MA, dUSA)[7]. The platelet membrane after the bath sonicator is filtered by sterile filters of 0.2µm, which is PMVs. PMVs were aliquoted into 1ml samples and placed in -80°C for storage before use. The size distribution and morphology of PMVs were examined using transmission electron microscopy respectively.

The preparation of PMVs@PLGA-miRNA complexes

The PMVs vesicles were fused with an equal volume of PLGA-miRNA complexes by ultrasound (5min, 42khz, 100w). The sample was filtered 20 times using a porous syringe filter with a membrane pore size of 200nm, and centrifuged (2500 rpm, 10 minutes) to remove excess PMVs to divide PMVs@PLGA-miRNA complexes.

Platelet Target Validation

In order to verify the platelet targeting in MIRI in rats, we injected platelets dyed red with infrared fluorescent dye into the tail vein during 30 min of reperfusion after ligation of the LAD branch of the rat heart coronary artery. In the control group, the LAD branch was threaded without ligation. We use IVISLumina XRMS Series III to detect the results of the ligation group and the control group after 24 hours to verify whether the PMVs@PLGA-miRNA complexes have the effect of targeting the MIRI injury site.

qPCR

Total RNA was isolated from cells using the Invitrogen TRIzol reagent (Thermo Fisher Scientific, USA). For mRNA analysis, reverse transcription reaction was performed via the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. qPCR was performed using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) on the QuantStudio 5 Real-Time PCR Systems (Thermo Fisher Scientific). All target genes were standardized to GAPDH. The primer sequences used are listed as follows:

rno-Nrf2 forward, 5'-TCCAAGTCCAGAAGCCAAACTGAC-3';

rno-Nrf2 reverse, 5'-GGAGAGGATGCTGAAGGAATC-3';

rno-GAPDH forward, 5'-GACATGCCGCCTGGAGAAAC-3';

rno-GAPDH reverse, 5'-AGCCCAGGATGCCCTTTAGT-3'.

For miRNA analysis, reverse transcription reaction was performed via the EZ-press microRNA Reverse Transcription Kit (EZBioscience, USA) according to the manufacturer's instructions. qPCR was performed using EZ-press microRNA qPCR Kit (ROX2 plus) (EZBioscience) on the QuantStudio 5 Real-Time PCR Systems (Thermo Fisher Scientific). All target genes were standardized to U6. The primer sequences used are listed as follows:

rno-miR-144-3p forward, 5'-GCGCGCGTACAGTATAGATGA-3';

rno-miR-155-5p forward, 5'-GCGCGTTAATGCTAATTGTGAT-3';

rno-miR-140-5p forward, 5'-CGCGCAGTGGTTTTACCCTA-3';

rno-miR-153-3p forward, 5'-CGCGTTGCATAGTCACAAAA-3';

rno-miR-410-3p forward, 5'-CGCGGCAATTTAGTGTGTGT-3';

rno-miR-27a-3p forward, 5'-TTCACAGTGGCTAAGTTCCGC-3';

rno-miR-27b-3p forward, 5'-TTCACAGTGGCTAAGTTCTGC-3';

rno-miR-340-5p forward, 5'-GCGCGTTATAAAGCAATGAGA-3';

rno-miR-106b-5p forward, 5'-TAAAGTGCTGACAGTGCAGAT-3';

rno-miR-495 forward, 5'-AAACAAACATGGTGCACTTCTT-3';

rno-miR-142-5p forward, 5'-GCGCGCATAAAGTAGAAAGC-3';

rno-miR-17-5p forward, 5'-CAAAGTGCTTACAGTGCAGGTAG-3';

rno-miR-93-5p forward, 5'-CAAAGTGCTGTTCGTGCAGGTAG-3';

rno-miR-128-3p forward, 5'-CGCGTCACAGTGAACCGGT-3';

U6 forward, 5'-CCTGCTTCGGCAGCACA-3'.

The reverse primers for miRNAs and U6 were provided in EZ-press microRNA qPCR Kit (ROX2 plus). Quantification of qPCR results was performed by the $2^{-\Delta\Delta C_t}$ method.

Transfection of miRNA mimic, inhibitor and antagomir

For in vitro transfection, take one well of a 6-well plate as an example. After the cells grow to 60-80% confluence, add 10ul PLGA and 10ul miRNA inhibitor/mimic to 125ul basal medium and mix thoroughly and gently. After standing for 15 minutes, add it to the complete medium of the 6-well plate to complete the transfection.

For in vivo transfection, each rat was prepared with antagomir at 10 mg/kg for transfection. The corresponding weight of antagomir was made into PMVs@PLGA-miRNA antagomir in vitro. About 10 minutes after the completion of myocardial ischemia-reperfusion model, it was injected into the rat from the tail vein to complete the transfection.

Dual-Luciferase Reporter Gene Assays.

HEK-293T cells were seeded into a 24-well plate per well, incubated overnight, and then transfected with the dual luciferase plasmid vectors and miR-140-5p, miR-144-3p, miR-155-5p, miR-340-5p mimics. According to the manufacturer's protocol, the luciferase activity was checked by FLUOROSKAN FL SYSTEM (Thermo) using the Dual-Luciferase Reporter Assay System (Promega, WI, USA). With Renilla activity as a control, the firefly luciferase activity was normalized to the Renilla activity in each well.

Western Blot Analysis and Immunofluorescence Analysis

Cell pellet or tissue homogenate was lysed with RIPA lysis buffer (NCM biotech, China, WB3100) and PMSF (Solarbio, China, P0100) for 1 hour; after centrifugation at 12000 rpm for 15 minutes, aspirate the supernatant, mix with loading buffer, and incubate at 100°C for 10 minutes to obtain protein samples; Western Blot experiments use SDS-PAGE Gel electrophoresis was performed. The primary antibodies used were: The secondary antibodies were: Proteins were detected by Odyssey Infrared Imaging System (Licor, USA), and the gray scale analysis was performed using imageJ software. The primary antibody is: CD41 (Proteintech, China, 24552-1-AP; 1:1000), CD62P (Proteintech, 60322-1-Ig; 1:1000), GAPDH (Proteintech, 60004-1-Ig; 1:1000).

Frozen sections were used for immunofluorescence. The slide was first sealed with 5% BSA for 30 mins, and then incubated overnight with Bax (Proteintech, 50599-2-Ig; 1:100), BCL-2 (Proteintech, 12789-1-AP; 1:100) at 4°C. The sections were incubated with the second antibody (1:500) combined with appropriate Alexa Fluor 488 at room temperature for 1 h, and mounted with DAPI (P36935, Life Technologies).

Statistical Analysis

The data were evaluated by GraphPad Prism software and expressed as mean±SD. Differences between groups were assessed by one-way analysis of variance and subsequent Tukey post-tests. P<0.05 proves the significance.

Results

Characterization of PLGA-miRNA complexes in vitro

We observed the PLGA nanoparticles with SEM and found that the size of the PLGA-miRNA inhibitor/mimic/antagomir complexes (PLGA-miRNA complexes) was almost uniform, showing a spherical profile (**Fig 2A**), with an average diameter of 155.3±32.21nm, as measured by DLS (**Fig 2B**). We matched nanoparticles and miRNA inhibitor/mimic/antagomir in different mass ratios, and performed gel retardation experiments. The results showed that the best N/M ratio between nanoparticles and miRNA inhibitor/mimic/antagomir was 4:1. Nanoparticles containing plasmids showed excellent retardation at this ratio (**Fig 2C**). The zeta potential of the complex (N/M ratio is 4:1) is about 32.6 millivolts. The slow-release curve of the plasmid in the PLGA-miRNA complexes was detected, and about 80% of the loaded plasmid was released within 21 days (**Fig 2D**). In vitro MTT experiments showed that after 48 hours of exposure, compared with phosphate-buffered saline (PBS), both pure nanoparticles and PLGA-miRNA complexes had no significant cytotoxicity (**Fig 2E**). For the evaluation of transfection efficiency in vitro, microscopic observation and FACS showed that there was no miRNA expression in the control group (**Fig 2F-G**). Otherwise, it is obvious that the expression of miRNA in H9C2 cells treated with the PLGA-miRNA complexes is extremely high, and the transfection efficiency reaches more than 95% (**Fig 2H-I**).

Characterization of PMVs in vitro

We used SEM to observe the platelets extracted from the blood of adult SD rats and the PMVs prepared therefrom, and found that the platelets extracted had a complete structure (**Fig 3A**), while the PMVs had a complete vesicle-like structure (**Fig 3B**). In order to further verify whether PMVs have the biological characteristics of PLTs, we tested the specific marker of platelet membrane: CD41, and the specific marker of platelet activation: CD62P. CD41 detection results showed that there was no significant difference in CD41 expression between PLTs and PMVs made with the same amount of PLTs, that is, the PMVs made by us kept the membrane protein of PLTs relatively intact (**Fig 3C**). The CD62P test results show that the PLTs we extracted have not already been activated, and the PMVs made therefrom are also activated, which shows that the PMVs we made can be activated after being injected into the body and play a biological role. At the same time, the GAPDH test results show that the PMVs we made have removed most of the platelet cytoplasm, which proves the purity of PMVs, implying that they have a targeting effect and are not easy to accumulate and embolize in the body (**Fig 3D**). Finally, we labeled PMVs with infrared fluorescence, injected them into SD rats through the tail vein, and performed in vivo imaging 24 hours later. The results showed that, compared with the control group, the MIRI group showed obvious infrared fluorescence signals and concentrated on the MIRI injury site, which indicated that the PMVs we made had good MIRI targeting in vivo (**Fig 3E**).

MiRNA regulation of Nrf2 in MIRI

Many studies have shown that Nrf2 plays a protective role in MIRI[32]. We have also confirmed in previous studies that the activation of Nrf2 can reduce oxidative stress during MIRI by inducing the secretion of anti-inflammatory factors. Here, we once again use the RT-qPCR method to confirm that after myocardial ischemia-reperfusion injury occurs, the expression of Nrf2 in myocardial tissue decreases significantly (**Fig 4A**). Therefore, how to increase the expression of Nrf2 after the occurrence of MIRI is the focus of this study. We searched two databases, miRDB and Targetscan, and found 32 and 47 potential miRNAs that can bind to Nrf2 mRNA, respectively. Among them, a total of 14 miRNAs appeared in the intersection of the prediction results of the two databases (**Fig 4B**). Subsequently, we used RT-qPCR to detect the changes in the expression levels of these 14 miRNAs in the rat heart tissue after MIRI, compared with the sham operation group, and found that only 4 miRNA expression levels increased, namely miR-144-3p, miR-155-5p, miR-140-5p, miR-340-5p (**Fig 4C**).

In order to further explore the regulatory relationship between these 4 miRNAs and Nrf2, we used inhibitor and mimic to interfere with and over-exposure of these 4 miRNAs, and used RT-qPCR to detect the interference/overexpression efficiency and the change of Nrf2 expression level. The results showed that the 4 miRNA inhibitor all achieved significant interference effects, and after interference, the expression of Nrf2 increased significantly (**Fig 5A-B**). On the other hand, the mimic of the 4 miRNAs all achieved significant over-exposure effects, and after overexpression, the expression of Nrf2 was significantly reduced (**Fig 5C-D**). The above results indicate that miR-144-3p, miR-155-5p, miR-140-5p, miR-340-5p, these four miRNAs may bind to Nrf2 mRNA to reduce the expression of Nrf2 after MIRI occurs, thereby playing a role of injury in the process of MIRI.

MiR-155-5p plays a key role in the miRNA regulatory network of Nrf2

In order to confirm whether the above four miRNAs bind to Nrf2 mRNA and play a key role in the MIRI process, we constructed a wild-type dual-luciferase plasmid vector of Nrf2 mRNA and the mutant vector corresponding to the four miRNAs (**Fig 6A**). The results of dual-luciferase experiments showed that only miR-144-3p, miR-155-5p, miR-140-5p these three miRNAs directly bind to Nrf2 mRNA (**Fig 6B-E**). Subsequently, we used PLGA nanoparticle/inhibitor/mimic complexes to transfect H9C2 cells with three miRNA inhibitor and mimic, and then established a MIRI model in vitro to detect apoptosis by flow cytometry. The results showed that after interfering with miR-155-5p, the apoptosis of H9C2 cells after MIRI was significantly reduced. On the contrary, after overexpression of miR-155-5p, the apoptosis of H9C2 cells after MIRI was significantly increased, while the other two miRNAs were not good. The above results indicate that miR-155-5p plays a key role in the miRNA regulatory network of Nrf2 in the process of MIRI (**Fig 6F-I**). Therefore, in the following in vivo experiments, we chose miR-155-5p as the target of PMVs@PLGA nanoparticle-antagomir complexes interference.

PMVs@PLGA nanoparticle-antagomir complexes have a therapeutic effect on MIRI in vivo

In the in vivo experiment, we chose to inject PMVs@PLGA nanoparticle-antagomir complexes about 10 minutes after MIRI occurred (**Fig 7A**). We performed echocardiography on the rats 24 hours after the injection (**Fig 7B**). Depending on echocardiographic data, we calculated EF (ejection fraction) and FS (fractional shortening). The results showed that EF and FS increased significantly after injection of PMVs@PLGA nanoparticle-antagomir complexes (**Fig 7C-D**). In addition, we also harvested rat heart tissues, made frozen sections, and detected the expression levels of BAX and BCL-2 using immunofluorescence methods. Among them, BAX is a marker for promoting apoptosis, and BCL-2 is a marker for inhibiting apoptosis. The results showed that after the injection of the complex, the expression of BAX decreased significantly, while the expression of BCL-2 increased significantly (**Fig 8A-D**). The above results indicate that injection of the PMVs@PLGA-antagomir complexes into rats has a significant myocardial protective effect in MIRI. The PMVs@PLGA nanoparticle-antagomir complexes are a potential MIRI treatment modality.

Discussion

Nrf2 has traditionally been regarded as a key regulator for maintaining redox balance and is involved in initiating the transcriptional expression of downstream antioxidant enzymes[11]. In addition, Nrf2 can also be adjusted in various ways. Nrf2/Keap1/ARE is also one of the important signal pathways to reduce the area of myocardial infarction and protect heart function after MIRI[33]. A large number of studies have shown that ischemic preconditioning and ischemic postprocessing have obvious protective effects on MIRI, and the potential role of Nrf2 signaling pathway in cardioprotection is worth exploring. However, how to effectively intervene the expression of Nrf2 in the body has always been a difficulty in MIRI-related research. This study uses miRNAs that regulate the expression of Nrf2 as an entry point,

trying to find a key miRNA as a target, and indirectly increase the expression of Nrf2 through in vivo transfection, thereby playing a therapeutic role.

Antagomir, as a kind of modified small RNA, has anti-degradation properties and has a longer half-life in the body, and has been extensively studied for in vivo transfection[21–23]. However, it is worth noting that if antagomir enters the body directly via intravenous injection, it will still face a variety of enzymes in the circulatory system, so that antagomir will be rapidly degraded. Secondly, its transfection efficiency is worrying, and it does not have the characteristics of slow release at all, so it cannot be used as a long-term effective treatment. Therefore, it is particularly important to study new types of vectors to carry antagomir for in vivo transfection. There have been many studies on the use of viruses as vectors. But the biological safety and compatibility of the virus itself are also worrying[24, 25]. As a degradable functional polymer organic compound, PLGA has good biocompatibility, non-toxicity, good encapsulation and film-forming properties, and is widely used in pharmaceuticals, medical engineering materials and modern industrial fields. At present, many studies have applied PLGA to carry various plasmids or RNA for treatment. In our previous study, we also used PLGA to carry plasmids for effective tendon repair treatment[29–31]. In this study, we also used PLGA as a carrier to carry miRNA inhibitor/mimic/antagomir for transfection experiments in vivo and in vitro. We found that the PLGA-miRNA complexes is spherical with a small size, about 150nm. These vectors easily penetrate the cell membrane and release plasmids within the cell. The complex also has a slow-release function, prolonging the release and action time of miRNA. Transfection efficiency is an important indicator of the quality of in vivo/external nucleic acid delivery systems. In our previous research, it has been proved that PLGA nanoparticles have good transfection efficiency as a vector to deliver plasmids. In this study, we found through experiments that PLGA nanoparticles and miRNA inhibitor/mimic/antagomir have the best carrying efficiency when the mass ratio is 4:1 (N:M=4:1). In the subsequent transfection experiment, we also confirmed that the use of PLGA nanoparticles to transfect miRNA inhibitor/mimic/antagomir has good interference and overexpression efficiency. More importantly, we found through MTT experiments that this compound has good biological safety, which is consistent with our previous research results. This makes us think that PLGA-miRNA complexes is a promising delivery system.

Although we have proved through a series of experiments that PLGA nanoparticles as a carrier for miRNA transfection have good transfection efficiency, biological safety and compatibility, there is no doubt that PLGA nanoparticles have almost no targeting when transfected in vivo characteristic. This makes its therapeutic effect worrying when it is used in treatment. How to make PLGA nanoparticles targetable in MIRI treatment is the focus of this research.

There is no doubt that platelets will accumulate to the site of myocardial ischemia injury during MIRI. This physiological property can be used to add targeting to PLGA nanoparticles. At present, many studies have shown that the use of cell membranes to camouflage nanoparticles can allow them to target treatment tasks in the body[34–39]. In this study, we used an appropriate method to obtain the platelet membrane, namely PMVs. Through SEM observation, we found that PMVs still maintain a relatively complete structure. Furthermore, we have verified through Western blot experiments that the PMVs we

extracted are relatively intact. The purity is high (the cytoplasm contained is relatively reduced), it has the surface markers that platelets should have, and it has not been activated. More importantly, we injected fluorescently labeled PMVs into rats intravenously. The results showed that PMVs have good cardiomyocyte targeting after MIRI in rats, and they can be used for a relatively long period of time. Staying at the injury site provides time for the transfection of the PLGA nanoparticle-antagomir complexes and the slow release of antagomir. What is particularly gratifying is that when we use the combined PMVs@PLGA-miRNA complexes for MIRI in vivo treatment, significant results have been achieved. Whether it is echocardiography or immunofluorescence detection of BAX and BCL-2 of tissue samples, it shows that PMVs@PLGA-miRNA complexes has a relatively ideal therapeutic effect. At present, there have been some studies that apply the fusion membrane to camouflage nanoparticles, which has achieved better targeting and biocompatibility. In the future, we also plan to fuse myocardial cell membranes and platelet membranes to disguise nanoparticles for MIRI treatment.

Conclusions

In this study, we constructed a method to deliver miRNA inhibitor in vivo through PLGA nanoparticle-inhibitor complexes camouflaged by platelet membranes. We found that in vitro, PLGA nanoparticle-inhibitor complexes can effectively transfect miRNA inhibitor into cells, and play a significant protective role in the process of MIRI. In vivo, with the targeting of PLT itself, the PLGA nanoparticle-antagomir complexes disguised by PMVs can also target the heart, protect the myocardium after MIRI occurs, and reduce the apoptosis of myocardial cells. Our research results show that a potential and promising biological treatment method can be applied to the treatment of MIRI.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

Tianyi Wang and Tingting Zhou designed part of the research, conducted most of the experiments including in vitro experiments and in vivo experiments, analyzed most of the experimental data, wrote the first draft of the manuscript, and revised the manuscript. Mingming Xu partially designed and supervised the experiment. Xuechao Yang and Shuai He assisted in in vitro experiments, participated in cell culture, and revised the manuscript. Shuo Wang and Anqi Wu assisted in in vivo experiments and revised the manuscript. Mingyang Zhang and You Lang Zhou partially designed the experiment, supervised the experiment and revised the manuscript. Jiahai Shi provided preliminary ideas for this research, partially designed experiments, and revised the manuscript.

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Availability of data and materials

Not applicable.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Schematic diagram of PMVs@PLGA-miRNA complexes treating MIRI.

Figure 2

Characterization of PLGA-miRNA complexes in vitro. (A) SEM image of PLGA-miRNA complexes. (B) Hydrodynamic diameter distribution of PLGA-miRNA complexes. (C) Agarose gel electrophoresis to determine complexes with different N/M ratios (N: nanoparticles; M: miRNAs). (D) In vitro cumulative release profiles of the miRNA from the PLGA-miRNA complexes in PBS at pH 7.2. (E) Cell viability of H9C2 cells treated in vitro with PBS, PLGA nanoparticles and PLGA-miRNA complexes. At different time points, there were no significant differences between PBS, PLGA nanoparticles, and PLGA-miRNA complexes. (F) Microscopic pictures of untransfected H9C2 cells in brightfield and fluorescence mode (10 $\times$). (G)

Untransfected H9C2 cells detected by FACS. (G) Microscopic pictures of H9C2 cells transfected with PLGA-miRNA complexes in brightfield and fluorescence mode (10×). (I) H9C2 cells transfected with PLGA-miRNA complexes detected by FACS.

Figure 3

Characterization of PMVs in vitro. (A) SEM image of PLTs. (B) SEM image of PMVs. (C) CD41 expression levels of PLT and PMVs. (D) CD62P expression levels of PLT, PLT(active) and PMVs (PLTs were activated with 1% CaCl_2 for 2 hours). (E) In the control group (threaded but not ligated) and MIRI group rats were injected with infrared fluorescence-labeled PMVs into the tail vein, and in vivo imaging was performed 24 hours later.

Figure 4

MiRNA regulation of Nrf2 in MIRI (1). (A) PCR was used to detect the expression of Nrf2 in the sham operation group and MIRI 6, 12, 24, and 48 hours later. (B) Two databases, miRDB and TargetScan, were used to predict miRNAs bound to Nrf2 mRNA. (C) PCR was used to detect the expression of 14 miRNAs in the sham operation group and MIRI 6, 12, 24, and 48 hours later.

Figure 5

MiRNA regulation of Nrf2 in MIRI (2). (A) PCR was used to detect the interference efficiency of miR-140-5p, miR-144-3p, miR-155-5p, miR-340-5p. (B) PCR was used to detect the expression of Nrf2 after the interference of miR-140-5p, miR-144-3p, miR-155-5p, miR-340-5p. (C) PCR was used to detect the overexpression efficiency of miR-140-5p, miR-144-3p, miR-155-5p, miR-340-5p. (D) PCR was used to detect the expression of Nrf2 after the overexpression of miR-140-5p, miR-144-3p, miR-155-5p, miR-340-5p. *P<0.05; **P<0.01; *** P<0.001; **** P<0.0001.

Figure 6

MiR-155-5p plays a key role in the miRNA regulatory network of Nrf2. (A) The dual-luciferase plasmid vector structure of miR-140-5p, miR-144-3p, miR-155-5p, miR-340-5p. (B-E) The results of Dual-Luciferase Reporter Gene Assays for miR-140-5p, miR-144-3p, miR-155-5p, miR-340-5p. (F-G) After miR-140-5p, miR-144-3p, miR-155-5p, miR-340-5p inhibitor were transfected into H9C2 cells, the MIRI model was constructed and FACS was used to detect cell apoptosis. (H-I) After miR-140-5p, miR-144-3p, miR-155-5p, miR-340-5p mimic were transfected into H9C2 cells, the MIRI model was constructed and FACS was used to detect cell apoptosis. *P<0.05; **P<0.01; *** P<0.001; **** P<0.0001.

Figure 7

PMVs@PLGA nanoparticle-antagomir complexes have a therapeutic effect on MIRI in vivo (1). (A) Schematic diagram of the use of PMVs@PLGA-miRNA complexes in the treatment of MIRI in vivo. (B-D) The results of echocardiography of rats after treatment with PMVs@PLGA-miRNA complexes (control group: no ligation after threading; PMVs group: only injected PMVs after MIRI; PMVs@PLGA-miRNA complexes group: injected PMVs@PLGA-miRNA complexes after MIRI, of which PLGA carries miR-155-5p antagomir). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Figure 8

PMVs@PLGA nanoparticle-antagomir complexes have a therapeutic effect on MIRI in vivo (2). After using PMVs@PLGA-miRNA complexes to treat MIRI in vivo, the expression of BAX and BCL-2 in myocardial tissue (use immunofluorescence detection). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

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