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Original Article

Platelet-derived microvesicles are involved in cardio-protective effects of remote preconditioning

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Abstract: The ischemia-protective mechanism of remote precondition has been a mystery for a long time. Little was known about details of the inter-organ cardio-protective. Microvesicles, also known as microparticles (MPs), are small membrane-vesicles budding from the plasma membrane of cell. Recent studies have indicated MPs to be an important messenger in various biological processes. Our research mainly examined the hypothesis that remote ischemic conditioning can attenuate heart infarction in a rat after they were subjected to 30 min ischemia and 180 min reperfusion (I/R) by MPs. MPs were extracted from three groups of rat: 1) healthy rats, 2) healthy rats that underwent hindlimb ischemia-reperfusion preconditioning (RIPC) immediately, 3) healthy rats that underwent RIPC in 6 hours. Isolated MPs were transfused into rats that had undergone I/R without RIPC. The transfusion of MPs from rats that underwent RIPC immediately resulted in an increase in platelet-derived MPs in blood and reduction in infarction size, confirmed by 2-3-5-triphenyltetrazolium chloride staining. We further observed the contractile function in hearts after they were subjected to different treatments. However, no significant difference was observed in transfusion of MPs from rats that underwent RIPC in 6 hours. RIPC induces an increase in MPs, and platelet-derived MPs may confer at least part of the remote protective effect against cardiac ischemic-reperfusion injury.

Keywords: Heart Ischemia/reperfusion, microparticles, remote ischemia-reperfusion preconditioning

Introduction

Brief episodes of ischemia-reperfusion cycles applied in distant organs initiate a cardioprotective phenotype and render the myocardium resistant to a subsequent sustained ischemic injury [1]. Multiple potential mechanisms have been proposed to explain the unique "transfer" feature of remote ischemia preconditioning (RIPC). Recent studies indicated that microvesicles, also known as microparticles (MPs) might be involved in remote ischemia preconditioning [2, 3].

MPs are small membrane vesicles budding from cell surface. Firstly regarded as cell debris, MPs have garnered a huge amount of interest in recent years as their critical functions in maintaining homeostasis through intercellular communication as well as in different types of disease [4]. Nowadays, MPs were gradually recognized as an important intercellular messenger.

Recently, Giricz and colleagues briefly demonstrated that MPs from RIPC rats' plasma may confer protection to an ex-vivo rat heart suffering I/R injury [3]. However, more work need to be done to accomplish Giricz's seminal work. Firstly, in-vivo experiments were necessary to fully mimic the pathological environments. In addition to that, due to the extremely small size of MPs (< 1 μm), it's generally impossible to efficiently quantify MPs through traditional flow cytometer [5]. Giricz's study failed to identified and quantify of MPs convictively. Finally, it is important to evaluate how long does the protective effects of MPs lasts, given that the clearance of MPs may be very fast, and its half life time may only be 5.8 hours in-vivo.

Therefore, we aimed to investigate whether RIPC-induced cardioprotection can be con-

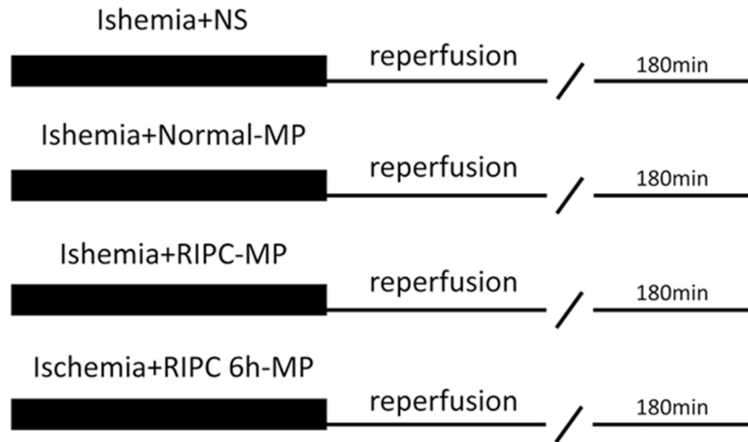

Figure 1. Experimental protocol. Open chest operated Sprague-Dawley rats (240-250 g) were subjected to 30 min of coronary occlusion followed by 180 min of reperfusion and assigned into four groups. Group 1: negative control group (NC, n = 12). Group 2: group injected with MPs from healthy rats not undergoing hindlimb ischemia (normal-MP, n = 12). Group 3: group injected with MPs from rats undergoing hindlimb ischemia immediately (RIPC-MP, n = 10). Group 4: group injected with MPs from rats undergoing hindlimb ischemia in 6 h (RIPC 6 h-MP, n = 9).

ferred by MPs in-vivo, and whether this possible effect remains 6 hours after RIPC.

Meterials and methods

Experiment protocol

The procedures of animal experiments were approved by the Ethics Committee of Animal Research of Peking University Health Science Center, China, and the provisions of the Declaration of Helsinki (as revised in Edinburgh 2000). Male Sprague-Dawley (SD) rats weighing 240-250 g were purchased from Vital River Laboratory Animal Technology Co. Ltd and kept in SPF environment, fed with normal chow in a temperature and in a light-controlled room (23°C, 12-h light/12-h dark cycle). Animals were fasted for 12 h prior to the experiment. Rats were randomized into five groups, including donor group and four operation groups. Donor group: Rats underwent three cycles of hindlimb ischemia-reperfusion (I/R). Then, the peripheral blood was collected for extracting MPs via ultracentrifugation. Treatment procedure for respective operation group (**Figure 1**) is as follows: 1. I/R+NC: Rats underwent cardiac I/R and were injected with sterile saline; 2. I/R+normal-MP: Rats underwent cardiac I/R and were subjected with MPs collected from normal untreated rats; 3. I/R+RIPC-MP: Rats underwent cardiac I/R and were injected with MPs collected from the donor group; 4. I/R+RIPC 6 h-MP: Rats underwent cardiac I/R and were injected with MPs collected from the donor group after 6 h of RIPC. All injections were administrated via the left femoral vein at the beginning of reperfusion within 5 min, and injection volumes were equal in all rats of group NC, normal-MP, RIPC-MP and RIPC 6 h-MP. The reperfusion period for all groups is the same as 180 min.

Hindlimb ischemia reperfusion

Rats were anaesthetized with chloral hydrate (400 mg/kg body) intraperitoneally. Body core temperature was maintained with a recirculation pad at 37.0±0.5°C, measured with a rectal thermometer. Cycles of hindlimb I/R were performed in rats in the donor group as described [6]. The left femoral vein was exposed through a small cut, and blood supply was occluded by clamping, followed by reperfusion for 5 min by unclamping (forming a 5-5 min I/R cycle). Three such cycles were performed in total for one rat. Limb ischemia was confirmed by the presence of limb pallor.

Animal myocardial ischemia/reperfusion injury model

The I/R model was produced as described previously [7]. Briefly, rats were anesthetized with sodium pentobarbital (40 mg/kg intraperitoneally). Myocardial hypoxia was produced by exteriorizing the heart with a left thoracic incision followed by making a slipknot (6-0 silk) around left anterior descending coronary artery. NC and MPs were administered via intravenously injection within 5 min at the beginning of reperfusion.

Infarct volume measurement

After 180 min reperfusion, rats were killed and hearts harvested. The frozen hearts were cut from apex to base into four transverse slices of

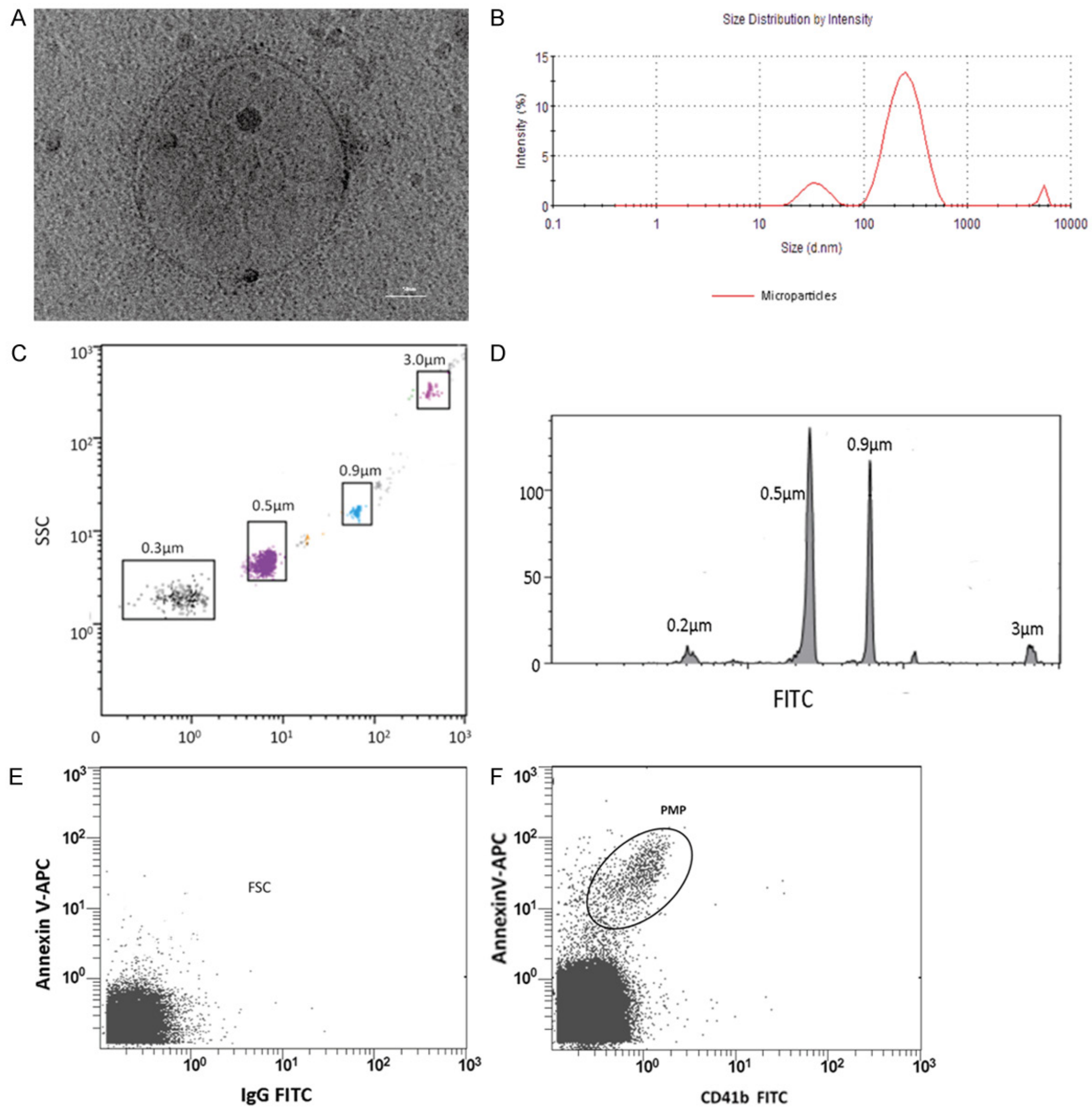

Figure 2. MPs originating from platelets (PMP) were identified by fluorescein isothiocyanate (FITC)-CD41. Enumeration of subsets of MPs was confirmed by dual-positive Annexin V with fluorescence label staining. During MPs quantization, the background in isotype-nonspecific IgG was subtracted. General structure of microparticles by Cryo-EM is shown in (A). Cryo-images of the microparticles showed dense particles with overall spherical shape and homogeneous electron-scattering density. (B) Shows the diameter distribution of MPs performed by dynamic light scattering analysis. (C, D) Show MP separation with Megamix beads and setup of MP gate. As isotype controls may generate falsely negative or positive microparticles, the background was evaluated by using a control in which all stains were used. (D and G) Represent isotype controls for PMP.

approximately equal thickness and were stained as described [6] with 0.5% w/v 2-3-5-triphenyltetrazolium chloride (TTC) (Sigma, USA) dissolved in phosphate-buffered saline (PBS), followed by fixation in 4% formalin overnight. Digital images of heart slices were analyzed with ImageJ software for calculating infarct volume as a percentage of whole heart as reported [6].

Cardiac function assay

Cardiac function was continuously monitored during the entire reperfusion procedure. A fluid filled-latex balloon connected to a pressure transducer was inserted into the left ventricle via a small incision in the left atrium and inflated to set a left ventricular end-diastolic pressure (LVEDP) (5-10 mmHg) using a hemody-

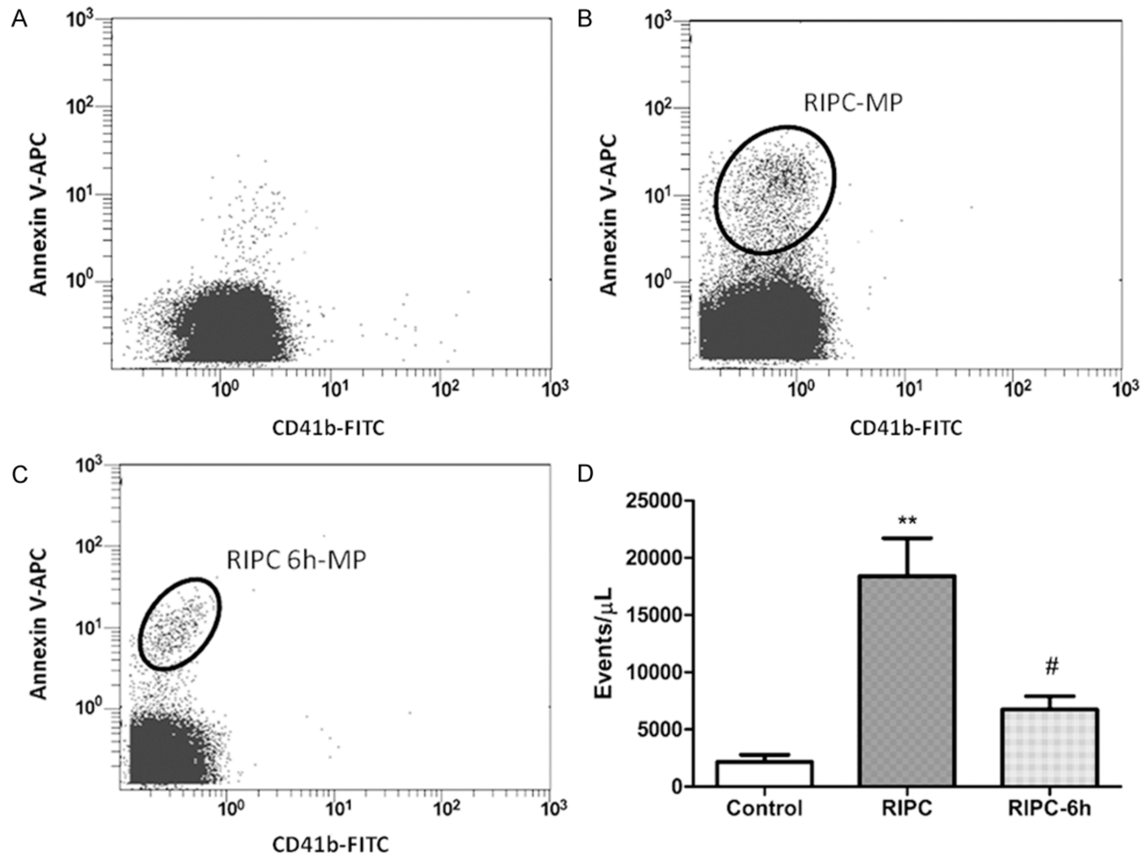

Figure 3. MP content changes were detected by flow cytometry. Circulating microparticles increased in response to cycles of hindlimb RIPC. PMPs were characterized by fluorescein isothiocyanate (FITC)-CD41 positivity. A-C. Indicated the representatives of flow cytometry for healthy, post-RIPC (blood samples taken immediately after RIPC) and RIPC-6 h (blood samples after 6 h of RIPC) rat serum. RIPC induced a PMP increase from (2356±660 μL) to (17292±6401/μL), with significant difference between the two (D, $^{**}P < 0.01$). PMP decreased from (17292±6401/μL) to (7568±1573/μL) after 6 h of RIPC, with significant difference between the two (D, $^{\#}P < 0.05$). No significant difference was detected between the healthy and RIPC-6 h groups.

namic analysis system (Taimeng Co., Chengdu, China) for the recording of functional parameters, such as heart rate (HR), left ventricular systolic pressure (LVSP), and the rate in rise and fall of ventricular pressure (±dp/dtmax).

Microparticles collection, separation, and transfusion

After I/R treatment as above, blood from rats in the donor group was collected via the abdominal aorta and immediately centrifuged at 1000 g for 15 min, at 4°C, and subsequently at 15,000 g for 3 min to obtain platelet-free plasma (PFP). PFP was ultracentrifuged (Hitachi CP-WX, Japan) at 100,000 g (4°C, 1 h) to pellet MP. Approximately 10 mL blood could be collected from each donor rat and, since the estimated whole blood volume of individual rats is around 30 mL [8], blood collected from 3 rats was amalgamated to extract enough MP to transfuse into one recipient rat. MP pellets were resuspended in sterile saline. The volume injected into each rat was < 500, in order to minimize possible blood volume (and hence pressure) changes.

Observation of microparticles by cryo-electron microscopy and dynamic light scattering

Cryo-EM FEI Tecnai G2 F30 TWIN transmission electron microscope, FEI, Hillsboro, OR, USA) was used to identify the existence of MP [9]. PFP achieved from centrifugation (1000 g, 15 min, 15,000 g, 3 min) was diluted with PBS buffer (1:10), followed by ultracentrifugation at 100,000 g for 1 h. MPs were resuspended in PBS, and a droplet of resuspension mixture

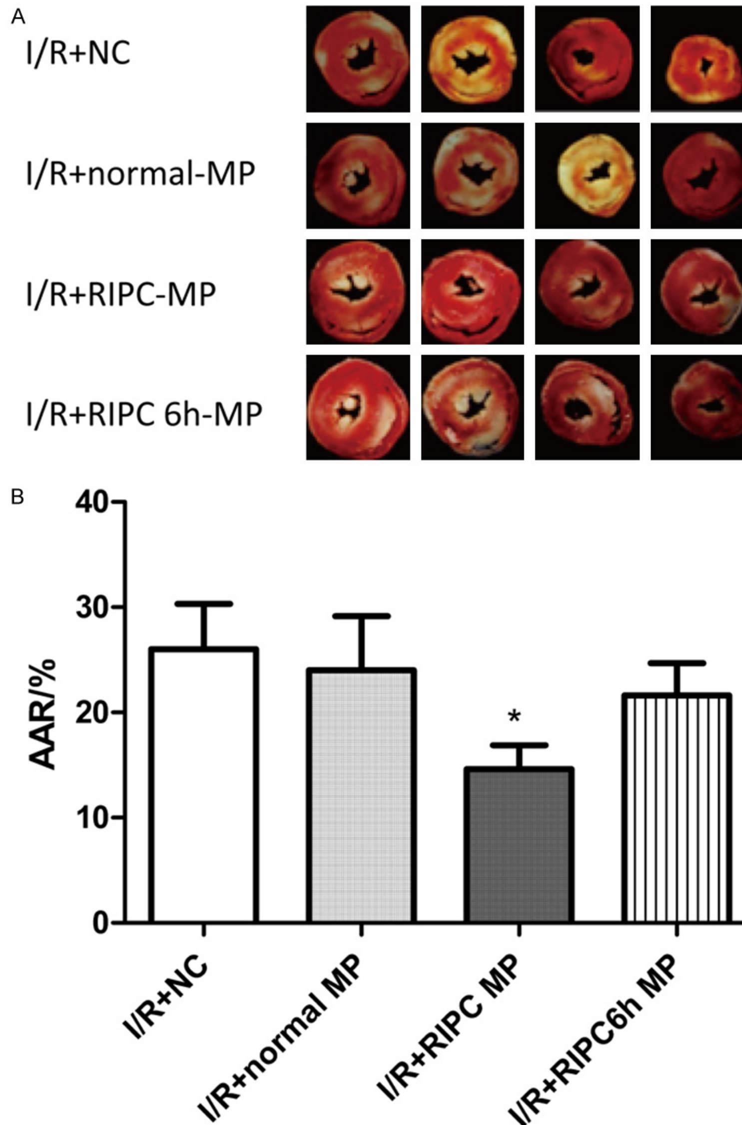

Figure 4. Infarcted areas detected by TTC staining. (A) TTC-stained heart transverse sections for representative subjects of I/R+NC, I/R+normal-MP, I/R+RIPC-MP and I/R+RIPC 6 h-MP. The infarcted area percentage stained with TTC with edema correction for groups was qualified as shown in (B). TTC staining revealed that RIPC-MP treatment exerted some protective effect compared with the negative control group (1). The edema condition has been considered by following the equation: edema degree (percentage) = [(area of lesioned hemisphere-area of contralateral hemisphere)]/(area of contralateral hemisphere). $^{*}P < 0.05$.

was placed onto carbon-coated holey film supported by a copper grid. The general membrane structure of MPs is presented in **Figure 2A**. Dynamic light scattering analysis for MPs was performed by using a Delsa Nano C particle analyzer (Beckman Coulter, USA). Briefly, a volume of 1 mL free-debris MPs containing PBS was transferred into a cuvette, placed in the analyzer and detected for three times.

Microparticles analysis by flow cytometry

MPs were measured using a Beckman Coulter GalliosTM flow cytometer (Beckman Coulter, Inc. Brea, CA, USA). The MP-gate determination was based on the use of fluorescent calibrated beads (Megamix beads; BioCytex, Marseille, France), comprising 0.5 μm, 0.9 μm, and 3 μm fluorescent beads [10]. Standardization of MP count requires mastering this limit located at 0.5 μm for an optimal compromise between MP analysis and background exclusion. Set FL1 PMT and SS PMT to locate beads cloud separately. Adjust FS PMT to let 0.5 μm bead percentage in FS Log x Count histogram close to 50%. The MP analysis region is defined as follows: The lower side is defined by the threshold allowing acquisition of events of at least 0.5 μm, and the upper side is the end of the 0.9 μm bead cloud. On the SS Log x FS Log cytogram, an MP autogate with maximum sensitivity around 0.9 μm was created (**Figure 2B, 2C**). Characterization of MP subsets was performed by Annexin V and fluorescence label staining of characteristic ligands [11]. PMPs were identified using fluorescein isothiocyanate (FITC)-CD41. Background reactivities to isotypic irrelevant IgG was subtracted. 50 μL of serum sample was added to a mixture of two fluorescence stains (4 μL each) and 4 μL heparin. The mixture was incubated at room temperature for 15 min, and 200 μL loading buffer (KeyGen Biotech Company, China) was added to get the final analytes for flow cytometry detection.

Table 1. Effect of RIPC-MP on parameters of myocardial function in I/R rats

Groups	LVSP (mmHg)	LVEDP (mmHg)	+dp/dtmax (mmHg/s)	-dp/dtmax (mmHg/s)	HR (beats/s)
I/R+NC	91±14	15±4	2875±905	2013±751	209±32
I/R+normal-MP	94±13	14±5	3028±871	2289±734	213±37
I/R+RIPC-MP	120±15**	11±5*	3759±614*	2916±684*	304±36**
I/R+RIPC 6h-MP	105±10	13±6	3145±804	2278±910	231±44

The data are expressed as mean ± SD from six independent experiments. *P<0.05, **P<0.01 versus I/R+NC.

Statistical analysis

Data were expressed as mean ± SD and analyzed by GraphPad Prism5 software. The two groups were compared using Student's t test, and comparison between more than two groups was performed by one-way ANOVA with Student-Newman-Keuls post-test. Differences with $P < 0.05$ were considered statistically significant.

Results

Cryo-electron microscopy image and dynamic light scattering of microparticle

The visualization of microparticles prepared from blood was performed by cryo-electron microscopy (cryo-EM). General structure of microparticles by Cryo-EM is shown in **Figure 2A**. Cryo-images of the microparticles showed dense particles with overall spherical shape and homogeneous electron-scattering density. Then we performed a dynamic light scattering analysis to detect the size distribution of MPs population. As shown in **Figure 2B**, the size of FC-EVs ranged from tens to hundreds of nanometers in diameter, and the peak value of diameter distribution is 367.6 nm.

Methodologies of flow cytometry for detecting microparticles

Beckman Coulter Gallios, a high-sensitivity cytometry that has high reproducibility in MPs measurement containing 0.5 μm and 0.9 μm fluorescent beads, was applied to ensure the accurate definition of MPs in the flow cytometry and MP separation with Megamix beads, and the setup of MP gate is shown in **Figure 2C** and **2D**. As isotype controls may generate falsely negative or positive microparticles, the background was evaluated using a control in which CD41+/annexin V-APC stains were used for PMPs. **Figure 2E** represent for isotype controls of PMP (**Figure 2F**).

MPs transfusion from donor rats to rats undergoing I/R induces an increase in PMP levels

The image for PMPs for the control group was shown in **Figure 3A**. Procoagulant PMPs were identified as CD41b and annexin V-APC. The RIPC induced an PMP increase from (2356±660 μL) to (17292±6401/μL), with significant difference between the two (**Figure 3D**, $^{**}P < 0.01$). Besides, if we collected the blood from rats undergoing RIPC in 6 hours, the counts of PMP decreased from (17292±6401/μL) to (7568±1573/μL), with significant difference between the two groups (**Figure 3D**, $^{\#}P < 0.05$). However, no significant difference was detected between the normal untreated and RIPC-6 h groups. The standardization columns for PMP are shown in **Figure 3D**.

Protection by microparticle transfusion to rats undergoing I/R

Degree of infarction was assessed by TTC staining (**Figure 4A**). For TTC staining, infarcted area percentages with edema correction for four groups were 25.74±4.13 (n = 12), 22.00±3.81 (n = 10), 14.47±3.19 (n = 10), and 20.6±2.84 (n = 9), respectively. The standardization columns analysis for TTC staining is presented in **Figure 4B**. The results from TTC staining revealed that injection of RIPC-MP exerted some protective effect compared with the NC injection group. To be mentioned, injection of RIPC 6 h-MP did not decrease the infarction size compared to the NC injection group. The possible explanation is the half life of MP is 5.8 hour [12]. This result indicated the protective effects of RIPC-MP indirectly.

RIPC-MP improved cardiac function of I/R injured rat heart

We also detected the cardiac function of rat during the entire reperfusion procedure.

Compared with the NC group, the RIPC-MP treatment led to an increase in left ventricular function characterized by a significant increase in HR, LVSP, and ±dp/dtmax and decrease in LVEDP. The parameters were not significantly changed in RIPC 6 h-MP injection group compared to the NC group, which is consistent with the TTC staining results (**Table 1**).

Discussion

Microparticles were highly cardioprotective when introduced either into isolated perfused rat hearts or intravenously into anesthetized mice immediately before reperfusion. The mechanism by which exosomes confer cardioprotection seems to involve the activation of the same cardioprotective kinase pathways as preconditioning [13-15].

When it comes to the studies of microparticles, methods, more specifically, the quantification of microparticles is always a problem [5]. Microparticles are extremely small membrane vesicles with diameters less than 1 μm [5]. Electronic microscope (EM) is the ideal tool for its identification, but not suitable for quantification. Flow cytometer is the primary tool for microparticles measurement. However, the resolution limitation of traditional flow cytometer is around 0.5 μm [10]. Most flow cytometer cannot distinguish microparticles and background noisy very well. It was not until very recently that high-resolution flow cytometer and reproducible microparticles measurement protocols became available [11]. In Giricz's study, it seems that they didn't take advantage of these powerful tools, which makes their results less convictive. In our study, microparticles were confirmed by EM and dynamic light scanning. With the help of Gallios, a powerful high-resolution flow cytometer, the quantification of microparticles in our study is more reliable.

In our studies, we estimated the in-vivo interorgan myocardium protective effects of microparticles for the first time. Besides, we collected the circulating microparticles from rats undergoing RIPC in 6 hours, and indicated the protective effects of microparticles reduced sharply after 6 hours. One possible mechanism underlining this phenomenon may be that microparticles could be rapidly cleared in circulation, with half-life time less than 6 hours in human circulation.

Besides, another earlier study showed that microparticles from RIPC rats failed to evoke the resultant infarct-sparing effect [2]. The roles of microparticles in RIPC are remaining uncertain. Hence, further investigation is needed.

In summary, this study demonstrated that RIPC induces MPs release in both rats, especially PMPs. We demonstration that MPs are the carrier mechanism of the cardioprotective effect of RIPC of the heart, although, further molecular and pathway experimentation is warranted to decipher the nature of actual effector factors carried by these vesicles.

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Disclosure of conflict of interest

None.

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