Platelet-Derived Microparticles are Implicated in Remote Ischemia Conditioning in a Rat Model of Cerebral Infarction

Li-Yang Shan,¹ Ji-Zhao Li,² Ling-Yun Zu,³ Chen-Guang Niu,² Albert Ferro,⁴ Ying-Dong Zhang,⁵ Le-min Zheng³ & Yong Ji¹

¹ Key Laboratory of Cardiovascular Disease and Molecular Intervention, State Key Laboratory of Reproductive Medicine, Nanjing Medical University, Nanjing, China

² The Institute of Cardiovascular Sciences and Institute of Systems Biomedicine, School of Basic Medical Sciences, Peking University Health Science Center, Beijing, China

³ Department of Cardiovascular Medicine, Peking University Third Hospital, Beijing, China

⁴ Department of Clinical Pharmacology, Cardiovascular Division, King's College London, London, UK

⁵ Department of Neurology, Affiliated Nanjing First Hospital, Nanjing Medical University, Nanjing, China

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Correspondence

Yong Ji, Key Laboratory of Cardiovascular Disease and Molecular Intervention, State Key Laboratory of Reproductive Medicine, Nanjing Medical University, Nanjing 210029, China.

Tel.: +86-25-86862886;

Fax: +86-25-86508960;

E-mail yongji@njmu.edu.cn

and

Lemin Zheng, The Institute of Cardiovascular Sciences and Institute of Systems Biomedicine, School of Basic Medical Sciences, Peking University Health Science Center, Beijing 100191, China.

Tel.: +86-10-82805452;

Fax: +86-10-82802769;

E-mail zhengl@bjmu.edu.cn

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SUMMARY

Aim: Remote ischemic preconditioning protects against ischemic organ damage by giving short periods of subcritical ischemia to a remote organ. We tested the hypothesis that remote ischemic conditioning can attenuate cerebral stroke in a rat middle cerebral artery occlusion (MCAO) model by microparticles (MPs). **Methods and results:** MPs were extracted from healthy rats that underwent hindlimb ischemia–reperfusion preconditioning (RIPC), and were transfused into rats that had undergone MCAO without RIPC. The transfusion resulted in an increase in platelet-derived MPs in blood and reduction in infarction area, confirmed by both 2-3-5-triphenyltetrazolium chloride staining and magnetic resonance imaging, albeit to a lesser degree than RIPC itself. Behavioral tests (modified Neurological Severity Score [mNSS]) were calculated to judge the behavioral change. However, no significant difference was observed after MP transfusion in 24 h or the following consecutive 9 days. **Conclusions:** RIPC induces an increase in MPs, and platelet-derived MPs may confer at least part of the remote protective effect against cerebral ischemic–reperfusion injury.

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The first two authors contributed equally to this work.

Introduction

Ischemic preconditioning was first reported in myocardial ischemia [1], which was also effective to enhance tolerance to cerebral ischemia reperfusion (I/R) by using cycles of sub-critical cerebral ischemia before occlusion [2]. Such protection against I/R could

be also achieved by delivering preconditioning to distant organs, a phenomenon termed as remote ischemic preconditioning (RIPC) [3–5] and has been widely applied in clinics [6–8].

Microparticles (MPs) are vesicles generated heterogeneously under diverse conditions. Three main categories, namely the endothelial microparticles (EMPs), platelet-derived microparticles

(PMPs), and leukocyte-derived microparticles (LMPs), respond to a variety of pathological conditions, including ischemia, metabolic dysfunction, and inflammation [9–11]. In patients with atherosclerosis, PMPs have been shown to augment recruitment of circulating leukocytes, cellular adhesion, and vascularization potential of circulating angiogenic cells *in vitro* [12]. Similarly, MPs have been found to induce angiogenesis and stimulate post-ischemic revascularization in a stroke model *in vivo* [13]; especially, the PMPs have been demonstrated to augment cell proliferation, neurogenesis, and angiogenesis at the cerebral infarct boundary zone [14,15].

In preliminary experiments, we observed that the levels of circulating MPs in peripheral blood of rats increased in response to RIPC and speculated that the elevation in MPs may be more than merely an epiphenomenon. We tested whether this increase in MPs induced by RIPC may mediate protection against infarction in a rat model of transient middle cerebral artery occlusion (MCAO). By transfusion MPs generated from post-RIPC rats to post-MCAO rats, we observed the protective effect of MPs in a MCAO rat model. The MCAO rats that received RIPC before stroke onset were used as the positive controls.

Materials and Methods

Animal Modeling

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 as shown in Table 1.

Treatment procedure for respective group (Figure 1) is as follows:

Donor group: Rats underwent three cycles of hindlimb I/R. Then, the peripheral blood was collected for extracting MPs via ultracentrifugation.

Operation groups:

1 NC: Rats underwent MCAO and were injected with sterile saline 30 min prior to reperfusion;

- 2 PC: Rats underwent MCAO and were subjected to pre-conditioning starting at 30 min prior to reperfusion as previously reported [16];
- 3 R-MP: Rats underwent MCAO and were injected with MPs collected from the donor group 30 min prior to reperfusion;
- 4 N-MP: Rats underwent MCAO and were injected with MPs collected from normal untreated rats 30 min prior to reperfusion.

All injections were administrated via the left femoral vein, and injection volumes were equal in all rats of group NC, R-MP and N-MP. The reperfusion period for all groups is the same as 24 h.

Hindlimb Ischemia Reperfusion

Rats were anaesthetized with chloral hydrate (400 mg/kg body) intraperitoneally. Body core temperature was maintained with a recirculation pad at $37.0 \pm 0.5^\circ\text{C}$, measured with a rectal thermometer. Cycles of hindlimb I/R were performed in rats in the donor group as described [16]. 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

Donor group:

Operation groups:

MCAO+Saline (NC)

Saline injection

MCAO+RPerC (PC)

MCAO+RIPC-MP (R-MP)

MP injection

MCAO+Normal-MP (N-MP)

MP injection

Figure 1 Surgical procedures for donor and temporary MCAO groups. Male Sprague-Dawley rats (240–250 g) were randomly allocated to five groups. Group 1: donor rats subjected to three cycles of hindlimb ischemia–reperfusion. Group 2: negative control group (NC, $n = 12$). Group 3: positive control group (PC, $n = 10$). Group 4: group injected with MPs from donor rats (R-MP, $n = 10$). Group 5: group injected with MPs from healthy rats not undergoing hindlimb ischemia (N-MP, $n = 9$).

Table 1 Allocation of rats for MCAO operation and blood donation

Operation groups	Donor group
Negative control (NC), $n = 12$	$12 \times 3 = 36$
Positive control (PC), $n = 10$	$10 \times 3 = 30$
Post-RIPC MP-treated (R-MP) group, $n = 10$	$10 \times 3 = 30$
N-MP group, $n = 9$	$9 \times 3 = 27$
Total = 41	Total = 123

one rat. Limb ischemia was confirmed by the presence of limb pallor.

Temporary Middle Cerebral Artery Occlusion

Middle cerebral artery occlusion model was established as previously described [16] by an operator who was blinded to the allocation (the same operator was also responsible for neurological scale determination and brain slice staining). Rats were anesthetized by intraperitoneal injection of chloral hydrate (400 mg/kg body weight). A polylysine-coated filament (Shadong Company, Peking) was inserted into the internal carotid artery through the right external carotid artery until it reached the junction of the middle cerebral artery. After 90-min occlusion, reperfusion was initiated by retracting the filament completely, confirmed by recovery to more than 70% of baseline blood flow. Following recovery from anesthesia, rats were scored on a 18-point neurological scale as previously reported [17] (data not shown). Rats without significant behavior changes were excluded.

Infarct volume measurement

After 24-h reperfusion, rats were killed and brains harvested. 2-mm brain slices were achieved with brain mold and were stained as described [16] 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 brain slices were analyzed with ImageJ software for calculating infarct volume as a percentage of whole brain as reported [16].

T2-weighted magnetic resonance imaging (MRI) was also used to evaluate cerebral infarction [18]. Images were collected 24 h after onset of reperfusion. MRI was performed on a high-field micro-MR research scanner (7.0T Bruker PharmaScans, Bruker Biospin, Ettlingen, Germany) in Zhongda Hospital, Medical School of Southeast University. Rats were subjected to isoflurane anesthesia. Respiratory rate and electrocardiogram were monitored using a physiology monitoring unit (Model 1025, SA Instruments Inc, Stony Brook, NY, USA). T2-weighted images were generated using a two-dimensional turbo spin-echo sequence. Each axial slice was 2 mm thick.

Microparticles Collection, Separation, and Transfusion

After ischemia–reperfusion 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 [19], 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

Cryo-EM FEI Tecnai G2 F30 TWIN transmission electron microscope, FEI, Hillsboro, OR, USA) was used to identify the existence of MP [20]. 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 was placed onto carbon-coated holey film supported by a copper grid. The general membrane structure of MPs is presented in Figure 2A.

Microparticles analysis by flow cytometry

MPs were measured using a Beckman Coulter Gallios™ 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 μ m, 0.9 μ m, and 3 μ m fluorescent beads [21]. Standardization of MP count requires mastering this limit located at 0.5 μ m 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 μ m 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 μ m, and the upper side is the end of the 0.9 μ m bead cloud. On the SS Log x FS Log cytogram, an MP autogate with maximum sensitivity around 0.9 μ m was created (Figure 2B, C).

Characterization of MP subsets was performed by Annexin V and fluorescence label staining of characteristic ligands [22]. EMPs, PMPs, and LMPs were identified using phycoerythrin (PE)-CD144, fluorescein isothiocyanate (FITC)-CD41 and PerCP-CY5.5-CD45 (KeyGen Biotech Company, China), respectively. Background reactivities to isotypic irrelevant IgG were subtracted. 50 μ L of serum sample was added to a mixture of four fluorescence stains (4 μ L each) and 4 μ L heparin. The mixture was incubated at room temperature for 15 min, and 200 μ L loading buffer (KeyGen Biotech Company, China) was added to get the final analytes for flow cytometry detection.

Behavioral tests (Neurological Severity Score, mNSS)

All animals received behavioral tests according to mNSS before and after the MCAO operation. The first examination was carried out 24 h after the MCAO operation. For the following 9 consecutive days, mNSS was performed once per day. The tests were performed by an investigator who was blinded to the experimental groups [23,24]. Table 2 shows a set of the mNSS. Neurological function was graded on a scale of 0–18 (normal score = 0; maximal deficit score = 18).

Statistical Analysis

Data were expressed as mean $\pm$ 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

Figure 2 MPs originating from endothelial cells (EMP), platelets (PMP), and leukocytes (LMP) were identified by phycoerythrin (PE)-CD144, fluorescein isothiocyanate (FITC)-CD41, and PercpCY5.5-CD45, respectively. Enumeration of subsets of MP were confirmed by dual-positive Annexin V with the three fluorescence label staining, respectively. During MPs quantization, the background in isotype-nonspecific IgG was subtracted. 50 μ L of each sample was added to a mixture of four fluorescence stains (4 μ L each) and 4 μ L heparin, incubated at room temperature for 15 min, and then mixed with 200 μ L loading buffer (KeyGen Biotech) to get the final sample for flow cytometry detection. 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. Particles were of various sizes, among which the ones with smaller diameters were found in neighbor of the larger one. Figure 2B, 2C 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. Figure 2D, 2E, 2F represent isotype controls for PMP (Figure 2G), LMP (Figure 2H), and EMP (Figure 2I), respectively.

performed by one-way ANOVA with Student–Newman–Keuls post-test. Differences with $P < 0.05$ were considered statistically significant.

The animal experiment procedures in this study were in accordance with the standards set forth in the eighth edition of Guide for the Care and Use of Laboratory Animals.

Table 2 Behavioral tests (Neurological Severity Score, mNSS)

Modified Neurological Severity Score Points

One point for inability to perform the tasks or for lack of a tested reflex.

Motor tests	
Raising rat by tail	3
Flexion of forelimb	1
Flexion of hindlimb	1
Head moved >10° to vertical axis within 30 seconds	1
Placing rat on the floor (0 = normal; 3 = maximum)	3
Normal walk	0
Inability to walk straight	1
Circling toward paretic side	2
Falls down to paretic side, unable to move	3
Sensory tests	
Placing test (visual and tactile test)	1
Proprioceptive test (deep sensation, pushing paw against table edge to stimulate limb muscles)	1
Beam balance tests (0 = normal; 6 = maximum)	
Balances with steady posture	0
Grasps side of beam	1
Hugs beam and 1 limb falls down from beam	2
Hugs beam and 2 limb falls down from beam, or spins on beam (> 60 s)	3
Attempts to balance on beam but falls off (>40 seconds)	4
Attempts to balance on beam but falls off (>20 seconds)	5
Attempts to balance on beam or hang on to beam (<20 seconds)	6
Reflex absence and abnormal movements	
Pinna reflex (head shake when auditory meatus is touched)	1
Corneal reflex (eye blink when cornea is lightly touched with cotton)	1
Startle reflex (motor response to a brief noise from snapping a clipboard paper)	1
Seizures, myoclonus, myodystonia	1

Generally, scores 13–18 mean severe injury; 7–12, moderate injury and 1–6, mild injury. All rats (except those died within 24 h) had scores over 10 after MCAO operation.

Results

Cryo-Electron Microscopy Image of Microparticle and the Methodologies of Flow Cytometry for Detecting Microparticles

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. Particles were of various sizes, among which the ones with smaller diameters were found in neighbor of the larger one. 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 2B and 2C. As isotype controls

may generate falsely negative or positive microparticles, the background was evaluated using a control in which all stains were used: PMPs were identified as CD41+/annexin V+. LMPs and EMPs were characterized by CD 144+ and CD45+, respectively. Figure 2D, 2E, and 2F represent for isotype controls of PMP (Figure 2G), LMP (Figure 2H) and EMP (Figure 2I), respectively.

Microparticle Transfusion from Donor rats to Rats Undergoing Middle Cerebral Artery Occlusion Induces an Increase in Platelet-Derived Microparticle Levels Only

The representative images for EMPs and PMPs were for the control group, post-RIPC group (without MCAO), post-MCAO group and MP-treated post-MCAO group, which are shown in Figure 3A and 3B. Procoagulant PMPs were identified as CD41 + and annexin V +, and EMPs were characterized as CD144 + and annexin V+. EMP increased in response to RIPC from $316 \pm 265/\mu\text{L}$ to $3602 \pm 4108/\mu\text{L}$ (Figure 3C, $*P < 0.05$). However, there was no significant difference between EMP level in MCAO group ($828 \pm 1162/\mu\text{L}$) and MP-treated MCAO group ($263 \pm 147/\mu\text{L}$). The RIPC also induced an PMP increase from ($1356 \pm 2260/\mu\text{L}$) to ($4292 \pm 4401/\mu\text{L}$), with significant difference between the two (Figure 3D, $*P < 0.05$). Besides, PMPs showed further increase after MP treatment to ($9270 \pm 8568/\mu\text{L}$) compared with MCAO group ($1886 \pm 2538/\mu\text{L}$) (Figure 3D, $P < 0.05$). The LMPs were characterized as CD45+ and annexin V+, but showed no significant change in response to RIPC. The representative image of flow cytometry for LMP of the said four groups are shown in Figure 3E, and the corresponding LMP count was ($715 \pm 183/\mu\text{L}$), ($908 \pm 453/\mu\text{L}$), ($957 \pm 436/\mu\text{L}$) and ($757 \pm 553/\mu\text{L}$), respectively. The standardization columns for LMP are shown in Figure 3F.

Protection by Microparticle Transfusion to Rats Undergoing MCAO Led to a Lesser Degree than RIPC

Degree of infarction was assessed by TTC staining (Figure 4A) or MRI (Figure 4B). For TTC staining, infarcted area percentages with edema correction for groups (1) to (4) were 22.74 ± 4.13 ($n = 12$), 11.00 ± 3.81 ($n = 10$), 16.47 ± 3.19 ($n = 10$), and 21.66 ± 2.84 ($n = 9$), respectively. By MRI measurement, infarcted area percentages with edema correction for groups (1) to (3) were 33.20 ± 2.20 ($n = 8$), 20.03 ± 2.51 ($n = 8$), and 23.77 ± 3.59 ($n = 5$), respectively. The standardization columns analysis for TTC staining and MRI result are presented in Figure 4C and 4D accordingly. Both TTC staining and MRI analysis revealed that MP treatment exerted some protective effect compared with the negative control group (1).

The Behavioral Tests of Neurological Severity Score (mNSS) Showed no Significant Change after MP Transfusion Compared with post-MCAO Rats Without MP Transfusion

All animals received behavioral tests according to mNSS first at 24 h after MCAO operation and for the following 9 days (Fig-

Figure 3 MP content changes were detected by flow cytometry. Circulating microparticles increased in response to cycles of hindlimb RIPC and to MP transfusion. EMPs and PMPs were characterized respectively by phycoerythrin (PE)-CD144 and fluorescein isothiocyanate (FITC)-CD41 positivity. Figure 3A, 3B indicated the representatives of flow cytometry for post-RIPC (blood samples taken immediately after RIPC from donor group) and post-transfusion rat serum. EMP increased in response to RIPC from $316 \pm 265 /\mu\text{L}$ to $3602 \pm 4108 /\mu\text{L}$ (Figure 3C, $*P < 0.05$). RIPC induced a PMP increase from $1356 \pm 2260 /\mu\text{L}$ to $4292 \pm 4401 /\mu\text{L}$, with significant difference between the two (Figure 3D, $*P < 0.05$). Besides, PMPs were shown to further rise after MP treatment from $1886 \pm 2538 /\mu\text{L}$ to $9270 \pm 8568 /\mu\text{L}$. LMP was characterized by CD45-PerCP-Cy5.5 and Annexin V positivity. Figure 3E presented the representative image of LMP of the said four treatment groups. The standardization columns for LMP is shown in Figure 3F. No significant difference was detected between the four groups.

Figure 4 Infarcted areas detected by TTC staining. A: TTC-stained brain sections for representative subjects of NC, PC, R-MP, and N-MP. B: Infarcted areas detected by T2-weighted MRI for representative subjects of NC, PC, R-MP, and N-MP. The infarcted area percentage stained with TTC with edema correction for groups was qualified as shown in Figure 4C. The MRI-detected infarcted area percentages with edema correction for groups (1) to (3) were standardized as shown in Figure 4D. Both TTC staining and MRI analysis revealed that 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). * $P < 0.05$.

Figure 5 The behavioral tests of Neurological Severity Score (mNSS) showed no significant change after MP transfusion compared with post-MCAO rats without MP transfusion. Animals received behavioral tests according to mNSS (Table 2), and the test results during day 0 to day 9 post-MCAO are shown in Table 2. Slight differences with regard to MCAO abnormality were observed between MP treatment and other groups; however, the differences are not significant ($P > 0.05$).

ure 5). Slight differences with regard to MCAO abnormality were observed between MP treatment and other groups; however, the differences are not significant ($P > 0.05$).

Discussion

A few studies have related MP to cerebral ischemia. Increase in PMPs has been shown to occur with ischemic stroke [25–27], and changes in MPs of different origins have been considered as biomarkers of pathological development [28,29]. More recently, MPs

have been considered to be more than biomarkers that may exert functional and biochemical effects, not only in the brain [30], but also in I/R damage in other organs such as the kidney [31]. PMPs have been demonstrated to aid ischemic brain tissue rehabilitation [15] by promoting neurogenesis via stimulating endogenous neural stem cell proliferation and migration, angiogenesis, and the release of neurogenic signals [32,33]. In our study, we reported for the first time that PMPs can mediate remote RIPC in a rat MCAO model.

MPs released from activated peripheral mast cells contain tumor necrosis factor and other proteins, facilitating communication between peripheral sites of inflammation and remote secondary lymphoid tissue [34]. A previous report examined changes in circulating MPs in a heart ischemia rat model, and the effect of treatment with post-RIPC MPs. It was found that only EMPs increase significantly after RIPC and no protective effect observed by transfusion MPs to myocardial ischemia rats [35]. Such results differed with this present study. In our study, we similarly examined the effect of transfusing MPs from the donor animals to animals with post-I/R injury. To isolate MPs by ultracentrifugation, we used a higher rotating speed compared with previous reports, for the following reasons.

It is known that particles shed under stimulation varied in size. Higher ultracentrifugation speed is required to isolate particles with smaller diameters [36]. However, the separation standard for these particles remains vague. Suggestions for separating so-called “microparticles” have ranged from 10,000 g [37] to 100,000 g [38], let alone variations in suggested centrifugation time. MPs sediment at $< 100,000$ g has been reported, while exosome ($< 0.1 \mu\text{m}$) isolation requires ultracentrifugation at $> 100,000$ g [9], yet other papers have used 100,000 g to isolate exosomes [39].

The possibility has recently been raised that particles of different size (diameters from 40 to 1000 nm) can exert some different, even opposite, biochemical effects [9,40,41]. We chose 100,000 g to ensure that MPs be pelleted as completely as possible; however, this leaves open the question about whether the observed biological effect can be ascribed to microparticles, microvesicles, exosomes or the combined effect of two or more of these. On the other hand, since we could distinguish particle size of MPs by flow cytometry and were able specifically to detect an increase in PMP in response to hindlimb I/R, our conclusion concerning PMPs in RIPC protection still held water.

The neurological scores showed no significant alleviation compared with other groups without MP treatment. Although it has previously been reported that treatment with microparticles can alleviate Neurological Severity Score, the significance did not show up until 90 days after the treatment [14].

Conclusion

MPs generated in response to hindlimb I/R cycles exert protective effect on cerebral ischemic damage when transfused into rats undergoing MCAO, replicating the effect – albeit to a lesser degree

– of RperC. Platelet-derived MPs may provide protection against I/R damage, suggesting the promise of developing new therapies for stroke.

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Disclosures

The manuscript has not been published or submitted elsewhere. No relationship of authors with companies may have a financial interest in the information contained in the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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