Original Article

Transfusion of Plasma Collected at Late Phase after Preconditioning Reduces Myocardial Infarct Size Induced by Ischemia-reperfusion in Rats *In vivo*

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Abstract

Background: Plasma transfusion is a common clinical practice. Remote ischemic preconditioning (RIPC) protects organs against ischemia-reperfusion (IR) injury. Whether preconditioned plasma (PP), collected at late phase after RIPC, could protect organs against IR injury *in vivo* is unknown. This study explored whether transfusion of PP could reduce myocardial infarct size (IS) after IR in rat *in vivo*.
Methods: Eighty Lewis rats were randomized to eight groups (n = 10 for each group). Two groups of plasma donor rats donated plasma at 48 h after transient limb ischemia (PP) or control protocol (nonpreconditioned plasma [NPP]). Six groups of recipient rats received normal saline (NS; NS-IR 1, and NS-IR 24 groups), NPP (NPP-IR 1 and NPP-IR 24 groups), or PP (PP-IR 1 and PP-IR 24 groups) at one or 24 h before myocardial IR. Myocardial IR consisted of 30-min left anterior descending (LAD) coronary artery occlusion and 180-min reperfusion. The area at risk (AAR) and infarct area were determined by double-staining with Evans blue and triphenyltetrazolium chloride. IS was calculated by infarct area divided by AAR. This was a 3 × 2 factorial design study, and factorial analysis was used to evaluate the data. If an interaction between the fluid and transfusion time existed, one-way analysis of variance with Bonferroni correction for multiple comparisons was used to analyze the single effects of fluid type when the transfusion time was fixed.
Results: IS in the NPP-IR 1 and PP-IR 1 groups was smaller than in the NS-IR 1 group (F = 6.838, P = 0.005; NPP-IR 1: 57 ± 8% vs. NS-IR1: 68 ± 6%, t = 2.843, P = 0.020; PP-IR 1: 56 ± 8% vs. NS-IR 1: 68 ± 6%, t = 3.102, P = 0.009), but no significant difference was detected between the NPP-IR 1 and PP-IR 1 groups (57 ± 8% vs. 56 ± 8%, t = 0.069, P = 1.000). IS in the NPP-IR 24 and PP-IR 24 groups was smaller than in the NS-IR 24 group (F = 24.796, P < 0.001; NPP-IR 24: 56% ± 7% vs. NS-IR 24: 68 ± 7%, t = 3.102, P = 0.026; PP-IR 24: 40 ± 9% vs. NS-IR 24: 68 ± 7%, t = 7.237, P < 0.001); IS in the PP-IR 24 group was smaller than in the NPP-IR 24 group (40 ± 9% vs. 56 ± 7%, t = 4.135, P = 0.002).
Conclusion: Transfusion of PP collected at late phase after remote ischemic preconditioning could reduce IS, suggesting that late-phase cardioprotection was transferable *in vivo*.

Key words: Ischemia; Ischemic Preconditioning; Myocardial Infarction, Myocardial Reperfusion Injury; Plasma

Introduction

Myocardial ischemia-reperfusion (IR) injury occurs frequently in a variety of clinical settings.[1,2] Remote ischemic preconditioning (RIPC) induced by transient limb ischemia has been shown to be a feasible and noninvasive approach for cardioprotection and offers both early-phase and late-phase protection.[3-6] The early-phase protection lasted for up to 3 h after RIPC, whereas the late-phase protection started after 24 h and lasted for up to 72–96 h, or

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sometimes for weeks.[6] However, the mechanism underlying the protective effects of RIPC is unclear.

Recently, some studies showed that humoral factors were responsible for the infarct-sparing effects.[7-11] Identifying these protective factors has been without success as humoral components comprise a reservoir of cardioprotective factors.[12,13] Current data suggest that preconditioned plasma (PP) (obtained from individuals after RIPC) contains cardioprotective factors released after RIPC, and that transfer of protection induced by RIPC between individuals through plasma transfusion may be possible.[7,8] Previous studies[7,14] focused on whether early-phase protection could be transferred, but the mechanism involved in the late phase of protection suggested that new proteins may be produced and that protective factors may be more persistent than those in the early-phase; therefore, transfer of late-phase protection may be more clinically applicable.[15]

We hypothesized that PP might be a reservoir of cardioprotective factors and that late-phase protection induced by RIPC could be transferred through plasma transfusion between individuals *in vivo*. Therefore, this study aimed to investigate whether transfusion of plasma collected at the late-phase of protection could reduce infarct size (IS) in an *in vivo* IR rat model.

Methods

Ethics

All animal protocols were approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University (No. LAEC-2012-0602).

Animals and grouping

Eighty 10- to 12-week-old, average weight 260 g (mean: 260 ± 9 g) male Lewis rats (Vital River Company, Beijing, China) were completely randomized to eight groups ($n = 10$ for each group) according to a computer-generated randomization list, including two groups of plasma donor rats (PD groups) and six groups of myocardial IR rats (study groups). The PD groups were divided into two subgroups according to whether transient limb ischemia was induced (PDLI) or not (PD control [PDC]). Depending on the type of fluid transfused and the transfusion time before ischemia, the study groups were divided into six subgroups: received normal saline (NS) 1 h before ischemia, NS-IR 1 group; received NS 24 h before ischemia, NS-IR 24 group; received nonpreconditioned plasma (NPP) 1 h before ischemia, NPP-IR 1 group; received NPP 24 h before ischemia, NPP-IR 24 group; received PP 1 h before ischemia, PP-IR 1 group; and received PP 24 h before ischemia, PP-IR 24 group.

Transient limb ischemia

The rats of PDLI group were anesthetized with intraperitoneal pentobarbital (50 mg/kg) and then underwent transient limb ischemia. Transient limb ischemia was induced by tying elastic rubber bands around both proximal hind limbs for 5 min, followed by 5 min of reperfusion by releasing the noninvasive ligature. Management of the rats in PDC group was identical to that in PDLI group, except that the elastic bands placed on both hind limbs were not tied.

Plasma preparation and transfusion

Forty-eight hours after completing the ischemia or control protocol, blood was drawn from the PD rats. PP was obtained from the rats undergoing transient limb ischemia, while NPP was obtained from the rats without transient limb ischemia. According to the grouping, 2 ml of NS, or NPP or PP was immediately transfused into the assigned IR rats through the caudal vein at a rate of 1 ml/min, either one or 24 h before inducing IR. The detailed protocols of transient limb ischemia, plasma preparation, and transfusion were as described in our previous paper.[9]

Myocardial ischemia and reperfusion

A myocardial IR model was established as previously described.[9] Briefly, IR rats were anesthetized with intraperitoneal pentobarbital (60 mg/kg), intubated, and ventilated (Harvard Rodent Ventilator, Holliston, USA) with room air, at a tidal volume of 8–10 ml/kg and a respiratory rate of 70–80/min. The right jugular vein was cannulated for fluid administration, and the right carotid artery was cannulated for blood pressure monitoring (Harvard Transducer, Holliston, USA). The rats were monitored closely for oxygenation by arterial blood gas analysis. The electrocardiogram was monitored continuously by a BIOPAC system (BIOPAC, Goleta, USA) throughout the experiment. The rectal temperature was maintained at 36.8–37.2°C by placing the rat on a heating pad (Nuanfeng Heating Element Company, Suzhou, China) and carefully adjusting the levels of heating. Left thoracotomy was performed between the third and fourth ribs, and the LAD coronary artery was identified and ligated with 7-0 polypropylene suture tunneled under the LAD. A slipknot was tied over a section of cotton thread placed directly over the vessel to create the occlusion. Occlusion was deemed successful when the myocardium supplied by the vessel turned pale. After 30 min of ischemia, the slipknot was released by gently pulling the slipknot suture in the opposite direction, and the cotton thread was then pulled out. At this time, reperfusion began for 180 min.

Infarct size analysis

Ischemic area (IA) was defined as the percentage of area at risk (AAR) in the entire left ventricle (LV), and IS was defined as the percentage of IA in the AAR. AAR and IA were determined by double-staining with Evans blue and triphenyltetrazolium chloride (TTC). After 180 min of reperfusion, the polypropylene suture tunneled under the LAD was retied, and 4 ml of 2% Evans blue (Sigma, St. Louis, USA) was given intravenously to distinguish the AAR (unstained portion of myocardium) from the area not at risk (Evans blue-stained portion of myocardium). The heart was excised under pentobarbital anesthesia, and the rat was sacrificed after the heart excision under anesthesia. The right ventricle was removed on ice. The LV including the septum was held at −20°C for 30 min and then cut into slices of about

2 mm from apex to base. The slices were incubated in 1% TTC (Sigma, St. Louis, USA) solution at 37°C for 15 min to distinguish dead tissue (pale color) from viable tissue (Evans blue-stained portion of myocardium appeared to be slightly blue-brown, and Evans blue-unstained portion of myocardium appeared to be strongly red). The slices were then fixed in 10% formalin for 20 min. The stained slices were placed on a glass slide and covered by another glass slide. Two-millimeter shims at the four corners held the glass away from the bottom sheet. The slices were then compressed to 2 mm by pressing the upper glass down against the shims using spring clamps. Images of both sides of each slice were taken with a Leica M205 FA microscope (Leica Microsystems, Solms, Germany) using a Leica DFC 420 camera, and AAR and IA were then determined via planimetry using Image J 1.46r (National Institutes of Health, Bethesda, USA). IS and IA were calculated according to the above-mentioned method.

Statistical analysis

Based on previous literature,[7,16] we preliminarily set the sample size as $n = 10$ in each group and then determined the mean and standard deviation (SD) in each group ($n = 8$, because 2 rats died in each group). The sample size was then calculated based on the mean, SD, and the set statistical power ($[1-\beta] = 0.8$, $\alpha = 0.05$), resulting in a required sample size of $n = 6$ in each group. Our sample size satisfied the requirement.

The data were analyzed with SPSS 16.0 (SPSS, Inc., Chicago, IL, USA). All values were expressed as mean ± SD. This study had a 3 × 2 factorial design. Factorial analysis was used to test the main effects of fluid type and transfusion time on IS and to determine whether an interaction between the fluid and transfusion time existed. If an interaction existed, one-way analysis of variance with Bonferroni correction for multiple comparisons was used to analyze the single effects of fluid type when the transfusion time was fixed. Differences were regarded as statistically significant when $P < 0.05$.

Results

Rats excluded from study

There was no dropout in the PD groups. No study rats had heart failure after 2 ml of plasma transfusion. Two rats died in each study group during the IR procedure, so the sample size was 8 in each study group [Table 1].

Myocardial ischemic area

IA (AAR/LV) did not differ significantly among the NS-IR 1, NPP-IR 1, and PP-IR 1 groups (NS-IR 1: 37 ± 10%, NPP-IR 1: 35 ± 11%, PP-IR 1: 35 ± 11%; $F = 0.196$, $P = 0.824$) or among the NS-IR 24, NPP-IR 24, and PP-IR 24 groups (NS-IR 24: 39 ± 10%, NPP-IR 24 35 ± 11%, PP-IR 24 35 ± 7%; $F = 0.337$, $P = 0.718$) [Figure 1].

Effect of preconditioned plasma on myocardial infarct size

Transfusion 1 h before ischemia

IS in the NPP-IR 1 or PP-IR 1 groups was significantly reduced compared to that in the NS-IR 1 group ($F = 6.838$, $P = 0.005$; NPP-IR 1: 57 ± 8% vs. NS-IR1: 68 ± 6%, $t = 2.843$, $P = 0.020$; PP-IR 1: 56 ± 8% vs. NS-IR 1: 68 ± 6%, $t = 3.102$, $P = 0.009$). However, IS did not differ between the NPP-IR1 and PP-IR1 groups (57 ± 8% vs. 56 ± 8%, $t = 0.069$, $P = 1.000$) [Figures 2 and 3].

Table 1: The causes of death in each study group of rat (n = 10 per group)

Group	Massive bleeding, *n*	Obstruction of trachea, *n*
NS-IR 1	1	1
NS-IR 24	2	0
NPP-IR 1	2	0
NPP-IR 24	2	0
PP-IR 1	1	1
PP-IR 24	2	0

IR: Ischemia-reperfusion; NPP: Nonpreconditioned plasma; PP: Preconditioned plasma; NS: Natural saline; IR 1: 1 h before IR; IR 24: 24 h before IR.

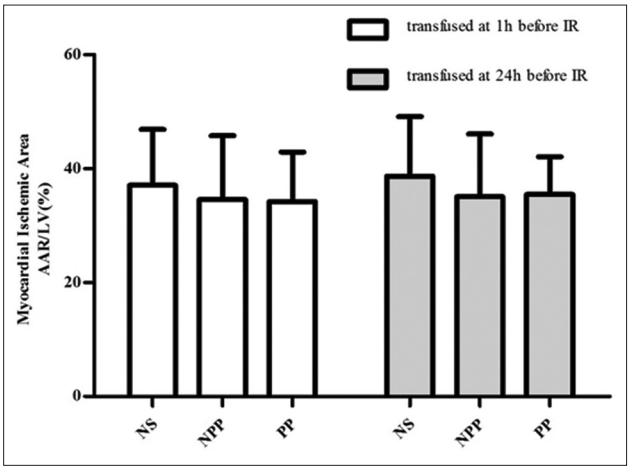

Figure 1: Myocardial IA in different groups (n = 8 for each group). IA (AAR/LV) did not differ significantly among the NS-IR 1, NPP-IR 1, and PP-IR 1 groups or the NS-IR 24, NPP-IR 24, and PP-IR 24 groups ($P > 0.05$). Bars represent the standard deviation in each group. IA: Ischemic area; AAR: Area at risk; LV: Left ventricle; IR: Ischemia-reperfusion; NPP: Nonpreconditioned plasma; PP: Preconditioned plasma; NS: Natural saline.

Transfusion 24 h before ischemia

IS in the NPP-IR 24 and PP-IR 24 groups was significantly reduced compared to that in the NS-IR 24 group ($F = 24.796$, $P < 0.001$; NPP-IR24: 56 ± 7% vs. NS-IR24: 68 ± 7%, $t = 3.102$, $P = 0.026$; PP-IR24: 40 ± 9% vs. NS-IR24: 68 ± 7%, $t = 7.237$, $P < 0.001$). Compared to the IS in the NS-IR24 group, the IS in the NPP-IR24 group was reduced by 17% and that in the PP-IR24 group was reduced by 40%. Moreover, IS in the PP-IR 24 group was reduced by 28% compared to that in the NPP-IR24 group (40 ± 9% vs. 56 ± 7%, $t = 4.135$, $P = 0.002$) [Figures 2 and 3].

Discussion

Our study found that transfusion of PP collected at the late-phase of protection into recipients 24 h before

myocardial IR could reduce IS. However, PP did not significantly reduce IS when transfused 1 h before IR, compared to the result with NPP.

Myocardial IS is recognized as a determining factor in myocardial infarction prognosis.[17] Previous studies[18,19] on animals and patients showed that the lager the IS, the more severe the LV dysfunction. Reducing IS is a therapeutic goal for myocardial infarction. The present study showed that transfusion of PP 24 h before ischemia could reduce the IS by about 40%, compared to the result with transfusion of NS, and by nearly 30%, compared to that with NPP. IS reduction in our study was smaller than that reported in previous studies, in which IPC or RIPC could reduce IS by at least 50%.[20,21] This difference may be due to different study regimens. The total blood volume of the rats in this study was about 16 ml.[22] The 2 ml of transfused plasma only accounted for about 13% of total blood volume. This suggests that a small amount of PP transfusion can achieve a protective effect.

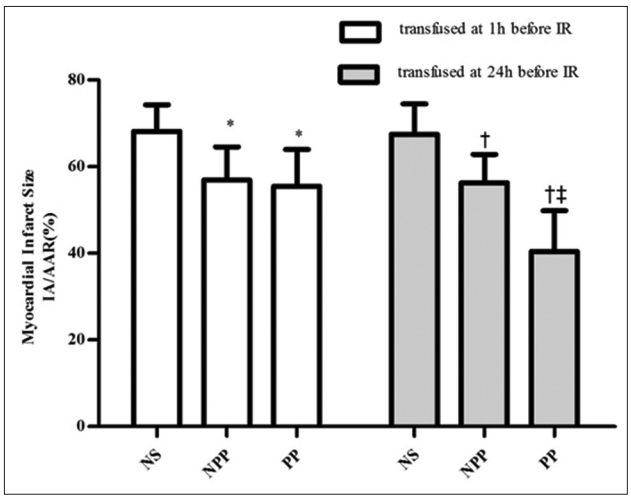

Figure 2: Myocardial infarct size in different groups (n = 8 for each group). Bars represent the standard deviation in each group. *Compared to NS-IR1, $P < 0.05$, †Compared to NS-IR24, $P < 0.05$, ‡Compared to NPP-IR 24, $P < 0.05$. IA: Ischemic area; AAR: Area at risk; IR: Ischemia-reperfusion; NPP: Nonpreconditioned plasma; PP: Preconditioned plasma; NS: Natural saline.

In previous *in vitro* studies, the transfer of preconditioned humoral fluid (coronary effluent, whole blood, or plasma) occurred just before ischemia; a larger amount of humoral fluid was transferred, and the transferring process took a long time.[23,24] In Dickson's study,[24] donor preconditioned hearts underwent repeated brief ischemia on a Langendorff apparatus, and coronary effluent was collected during the preconditioning period; then, recipient hearts were perfused with the effluent for 30 min before being subjected to a long-term IR episode. In another study[24] by the same research group, 1 min prior and after each of 5 IPC episodes, 5 ml of whole blood was exchanged between the preconditioned rabbit and the matched rabbit, and all rabbits underwent blood exchange 10 times. In Shimizu's study,[7] plasma dialysate was obtained at the end of the RIPC protocol from preconditioned rabbits or preconditioned human volunteers and then added to the buffer to perfuse rabbit hearts for 35 min before subjecting the heart to a long period of IR. These studies have important scientific implications: they confirmed that the protective effects of IPC could be transferred by humoral fluid. However, all the procedures seemed very complicated. Our study showed that transfusing a small volume of PP in a relatively short period can limit IS to a large extent.

Previous studies[7,14] focused on whether protection in the early phase could be transferred. In the present study, we selected 48 h after transient limb ischemia as the time point of plasma collection, which was clearly in the late phase of protection, to explore whether protection in the late phase could be transferred via plasma. We also explored the transfer of protective effects of RIPC based on when the plasma was transfused. Our results showed that PP transfused at 24 h but not 1 h before ischemia could reduce IS. We speculated that the PP may contain cardioprotective substances acting

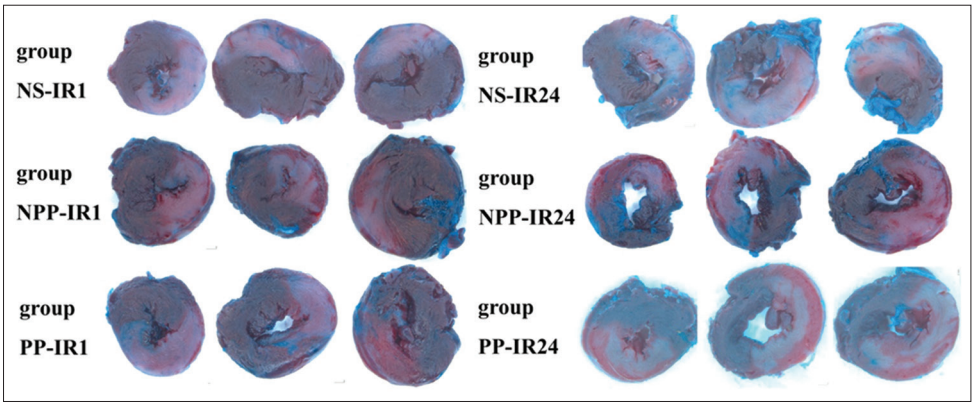

Figure 3: Representative images of Evans blue and triphenyltetrazolium chloride double-stained myocardial slices. Each line shows the representative images from one left ventricle in each study group. The Evans blue positive-staining region (blue-brown color) is the nonischemic region. The pale-appearing triphenyltetrazolium chloride negative-staining region is the infarct area. The Evans blue negative-staining region, including the strongly red-appearing triphenyltetrazolium chloride positive-staining region and the infarct area, are the areas at risk. IR: Ischemia-reperfusion; NPP: Nonpreconditioned plasma; PP: Preconditioned plasma; NS: Natural saline; IR 1: 1 h before IR; IR 24: 24 h before IR.

as an initiator, and the initiator may need time to activate the protective signaling system. However, further studies are needed to verify this hypothesis.

Interestingly, this study also showed that transfusion of NPP could reduce IS, compared to the result with transfusion of NS. The mechanism may involve that the plasma of donor rats could maintain blood volume more effectively than NS and contain the possible residual pentobarbital from anesthesia administration. Moreover, whether the stretch-activated mechanism was involved in the protection induced by NPP transfusion remains to be further explored.

This study was started in 2012 but was published later than a similar study by Skyschally *et al*.[14] However, our study examined the protective effect of PP on individuals of the same species and focused on the late phase of protection; in contrast, Skyschally's study explored the effect between species and focused on the early phase of protection.

This study examined the myocardial protective effect of PP transfusion at 1 or 24 h before ischemia, but the effect of transfusion at more time points before ischemia needs further investigation. Furthermore, whether there is a dose-dependent effect and the mechanism underlying the transfer of the protective effect should be studied in the future.

In conclusion, this study reported the use of an *in vivo* model to show that transfusion of late-phase PP into IR rats 24 h before ischemia could reduce IS. These findings suggest that cardioprotection by RIPC is transferable via plasma *in vivo*.

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Conflicts of interest

There are no conflicts of interest.

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