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ORIGINAL ARTICLE

Improved Long-term Survival with Remote Limb Ischemic Preconditioning in a Rat Fixed-Pressure Hemorrhagic Shock Model

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Abstract

Purpose We investigated whether bilateral, lower limb remote ischemic preconditioning (RIPC) improved long-term survival using a rat model of hemorrhagic shock/resuscitation.

Methods Rats were anesthetized, intubated and ventilated, and randomly assigned to RIPC, induced by inflating bilateral pressure cuffs around the femoral arteries to 200 mmHg for 5 min, followed by 5-min release of the cuffs (repeated for 4 cycles), or control group (cuffs were inflated to 30 mmHg). Hemorrhagic shock was induced by withdrawing blood to a fixed mean blood pressure of 30 mmHg for 30 min, followed by 30 min of resuscitation with shed blood. Rats remained anesthetized for 1 h during which hemodynamics were monitored then they were allowed to survive for 6 weeks.

Results The percentage of estimated total blood volume withdrawn to maintain a level of 30 mmHg was similar in both groups. RIPC significantly increased survival at 6 weeks: 5 of 27 (19%) rats in the control group and 13 of 26 (50%; $p = 0.02$) rats in the RIPC group survived. Blood pressure was higher in the RIPC group. The diastolic internal dimension of the left ventricle, an indicator of circulating intravascular blood volume, was significantly larger in the RIPC group at 1 h after initiation of resuscitation compared to the control group ($p = 0.04$). Left ventricular function assessed by fractional shortening was comparable in both groups at 1 h after initiation of resuscitation. Blood urea nitrogen (BUN) was within normal range in the RIPC group (17.3 ± 1.2 mg/dl) but elevated in the control group (22.0 ± 1.7 mg/dl) at 48 h after shock.

Conclusions RIPC significantly improved short-term survival in rats that were subjected to hemorrhagic shock, and this benefit was maintained long term. RIPC led to greater circulating intravascular blood volume in the early phase of resuscitation and improved BUN.

Keywords Hemorrhagic shock · Remote ischemic preconditioning

Introduction

Traumatic injury remains the leading cause of death among North Americans aged 1–44 years, causing over one-third of deaths in 1–19 year olds [1]. Hemorrhagic shock followed by resuscitation represents a pathophysiologic process of total body global ischemia and reperfusion injury [2]. A number of homeostatic mechanisms are involved to maintain perfusion to vital organs. To preserve perfusion to the brain and heart during hypovolemia, vasoconstriction occurs in the kidneys, liver, intestine, and skeletal muscle. This can result in hypoperfusion of these organs, leading ultimately to multi-organ injury and failure. In extreme hemorrhage with exsanguination, vasoconstriction in non-vital tissues no longer prevents hypoperfusion of the brain and myocardium: the reduction in intravascular volume causes brain and cardiovascular compromise, resulting in rapid cerebral anoxia and fatal arrhythmias [3]. Death will follow if the hemorrhage is not rapidly controlled, and the resuscitation efforts are not adequate. In patients with traumatic hypovolemic shock, the median time from onset of hemorrhagic shock to death is 2 h [4].

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In the clinical setting, lifesaving approaches to treat hemorrhagic shock include early recognition of shock, rapid control of the source of hemorrhage, and restoration of volume and oxygen-carrying capacity, in the attempt to limit the depth and duration of the shock state before it becomes irreversible [5]. Resuscitation treatment focuses primarily on fluid replacement using oxygen-carrying blood substitutes, hypertonic solutions, colloid solutions, and crystalloids [6].

Many experimental studies have tested the administration of various fluids as treatment for hypovolemic shock in a variety of models (see [7, 8] for reviews). However, a different approach would be to increase tissue tolerance to hemorrhagic shock. Until recently, this strategy has remained largely unexplored.

One such approach might be to enhance ischemic tolerance using remote ischemic preconditioning (RIPC). Over the past 2 decades, numerous experimental and clinical studies have demonstrated that RIPC is a therapeutic, noninvasive intervention capable of conferring multi-organ protection against acute ischemia/reperfusion injury [9]. RIPC, which was originally characterized as an interaction between two separate coronary vascular territories [10], is the phenomenon whereby the controlled induction of nonlethal and transient ischemia in one organ or tissue increases ischemic tolerance in a distant vital organ or tissue against prolonged ischemia-reperfusion-induced injury [11]. Although the underlying molecular mechanism of the protective effects remains unknown, remote limb ischemic preconditioning is well known to provide cardioprotection [10, 12], neuroprotection [13], pulmonoprotection [14], hepatoprotection [15], and gastrointestinal protection [16, 17]. Therefore, preconditioning might be a potential therapeutic option to provide benefit against hemorrhagic shock-induced multi-organ injury.

Some experimental studies have been performed to test this hypothesis [18–23], and data showed a benefit from preconditioning in models of hemorrhagic shock. However, short-term survival was assessed in only a few of these studies and long-term survival was not assessed at all. Therefore, the purpose of the present study was to investigate whether RIPC could improve long-term survival in a rat model of hemorrhagic shock/resuscitation.

Methods

The present study was approved by the Institutional Animal Care and Use Committees at Huntington Medical Research Institutes and was performed in accordance with the Guidelines for the Care and Use of Laboratory Animals (NIH publication No. 85-23, National Academy Press, Washington DC, revised 2011). All animals were housed in environmentally controlled rooms with temperature at 22 ± 2 °C and 12-h light-dark cycles, and allowed free access to standard laboratory chow and water. Prior to surgery, the rats were acclimatized for a minimum of 5 days after arrival at the laboratory. We specifically chose the rat model of hemorrhagic shock as there is already a body of literature based on this model [24], and in pilot studies, we could show that a duration of 30 min of blood pressure lowered to 30 mmHg resulted in significant mortality of about 20–30%, which allows for room for therapeutic improvement. In addition, many of the kinds of studies that previously required larger animals can be done in the rodent model including hemodynamic measurements and echocardiography.

Surgical Procedures for Hemorrhagic Shock Model

Age-matched Sprague-Dawley rats (both genders, mean weight 217 ± 3 g for females and 337 ± 5 g for males), obtained from Charles River, Inc., were anesthetized with intraperitoneal ketamine (90 mg/kg) and xylazine (10 mg/kg). A cannula was inserted into the trachea and the cannula attached to a respirator; the rats were ventilated with room air at 60 strokes/min and 10 ml/kg tidal volume. Body temperature was maintained at 37 °C with a heating pad. Under aseptic conditions, the left femoral artery, carotid artery, and jugular vein were dissected using a minimal dissection technique, distally ligated, and cannulated with a polyethylene catheter (PE-50) filled with heparinized saline (8 IU/ml) to avoid local coagulation. The femoral artery catheter was connected to a pressure transducer to continuously monitor the arterial blood pressure and heart rate, and the carotid artery catheter was used for shed blood withdrawal to induce pressure-controlled hemorrhagic shock. The jugular vein catheter was used to reinfuse the shed blood. Lead II of the electrocardiogram (ECG) was continuously recorded. After the rats were heparinized with intravenous heparin (500 U/kg), the shed blood was withdrawn into a heparinized syringe over a period of 10 min using a syringe pump and kept at room temperature. The mean blood pressure was reduced to a target value of ~ 30 mmHg and maintained at that pressure for 30 min by withdrawing or infusing small amounts of blood. Shed blood volume was expressed as a percentage of calculated total blood volume [estimated from 6.12 ml/100 g body weight] [18]. After 30 min of shock, the rats were resuscitated with the reinfusion of total shed blood over a 30-min period, and the rats were continuously monitored for another 30 min. At 1 h after initiation of resuscitation, the catheters were removed from the femoral artery, carotid artery, and jugular vein and the blood vessels tied off. The neck and groin incisions were closed in layers. The rats were returned to their cages to allow recovery from anesthesia. Postoperative analgesia (buprenorphine, 0.01 mg/kg body weight, subcutaneous) was maintained for 2 days. They were observed 6 weeks for survival.

Experimental Design

Fifty-three rats were randomized into RIPC and control groups. Randomization was achieved by placing folded pieces of paper with group assignment in a jar and drawing blindly after surgical preparation was complete. Rats in the RIPC group underwent bilateral hind-limb ischemia/reperfusion for 4 consecutive cycles of 5 min of ischemia followed by 5 min of reperfusion, prior to blood withdrawal to induce hemorrhagic shock. After the last 5 min of reperfusion, shock (30 mmHg) was achieved in about 10 min of bleeding in both groups. Bilateral conditioning was used because it has been shown to be effective in various models [11, 25–27]. RIPC was delivered by placing the blood pressure cuffs (one cuff on each hind leg) at the inguinal level, and ischemia was induced by inflating the cuff with air to 200 mmHg. Reperfusion was initiated by deflating the cuffs. In the control group, all procedures were followed as in RIPC group except the cuff pressures were inflated only to 30 mmHg.

Cardiac Function

Transthoracic echocardiographic imaging was performed using a 15-MHz linear array transducer of a Philips ultrasound system at baseline prior to bleeding, 5 min before resuscitation with shed blood, and 1 h after initiation of resuscitation. Echocardiograms were analyzed and measurements calculated post hoc in a blinded fashion. Two-dimensional parasternal short-axis views and two-dimensional targeted M-mode tracings were taken to determine diastolic and systolic internal dimensions of the left ventricle (LVIDd and LVIDs, mm) and left ventricular fractional shortening (LVFS, %), respectively.

Blood Gas Analyses

Blood samples of 0.3 ml were collected from the carotid arterial catheter at 1 h after initiation of resuscitation. Blood pH, arterial partial pressure of carbon dioxide ($PaCO_2$), arterial partial pressure of oxygen (PaO_2), Na, K, Cl, iCa, glucose, and lactate concentration (Lac) were measured with a blood gas analyzer. In addition, blood was collected for cell counts, platelet count, and other electrolytes and chemistries.

Analyses of C-reactive Protein and Cytokines

At 48 h and 6 weeks resuscitation, serum c-reactive protein (CRP) levels were determined using the Rat CRP ELISA Kit (RayBiotech, Norcross, GA, USA). Serum TNFα, IL-1α, IL-1β, and IL-6 were measured using the multiplex cytokines kit (IDEXX laboratories, Columbia, MO, USA) according to the manufacturer's instructions.

Tissue Histological Analysis

At 6 weeks after hemorrhagic shock, all surviving rats were deeply anesthetized. To detect areas of microvascular damage in the tissues, thioflavin S was injected into the jugular vein. The rats were euthanized by an intravenous injection of KCl. Following euthanasia, heart, brain, lungs, liver, and kidney were isolated, weighed, and photographed under white light, and under ultraviolet light to detect thioflavin S perfusion defects that demarcate microvascular obstruction. Triphenyltetrazolium chloride staining was used to identify necrosis (white areas) versus viable tissue (brick red areas). The tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. The processed tissues were sectioned into 5-μm slices and were used for hematoxylin and eosin staining for tissue structure, and picrosirius red staining to assess fibrosis.

Statistical Analyses

Statistical analyses were performed with SAS v.9.4 software. Parametric data are reported as mean ± SEM. Non-parametric data (cytokines) were analyzed using rank sum testing and expressed as median values. *T* tests were used for blood gas and chemistry variables. A linear mixed-models analysis for repeated measures was used to analyze hemodynamic variables, testing for a group × time interaction. Differences between specific time points of hemodynamic variables were determined by post hoc contrasts from the same mixed models. Survival analysis was performed by log-rank testing. Values were considered significant at $p < 0.05$.

Results

Survival

A total of 14 female and 13 male rats in the control group and 12 female and 14 male rats in the RIPC group were used in this study. At 6 weeks, 5 of 27 (19%) rats in the control group and 13 of 26 (50%; $p = 0.021$) rats in the RIPC group survived. The survival curves for the two groups diverged within the first day (Fig. 1), indicating that RIPC improved early survival. This benefit on survival was maintained long term: RIPC significantly increased survival at 6 weeks. Survival data, analyzed by Log-rank test: $\chi^2(1) = 8.1$, $p = 0.004$, indicated that the two groups are significantly different from one another in their survival distributions. Most rats that failed to survive died within 24 h, but 1 rat died at 29 h and 1 at 10 days in the control group; 2 rats died at 2 days in the RIPC group. No seizures were observed in any of the animals. There was no significant difference in survival by gender at 6 weeks in either control or RIPC groups.

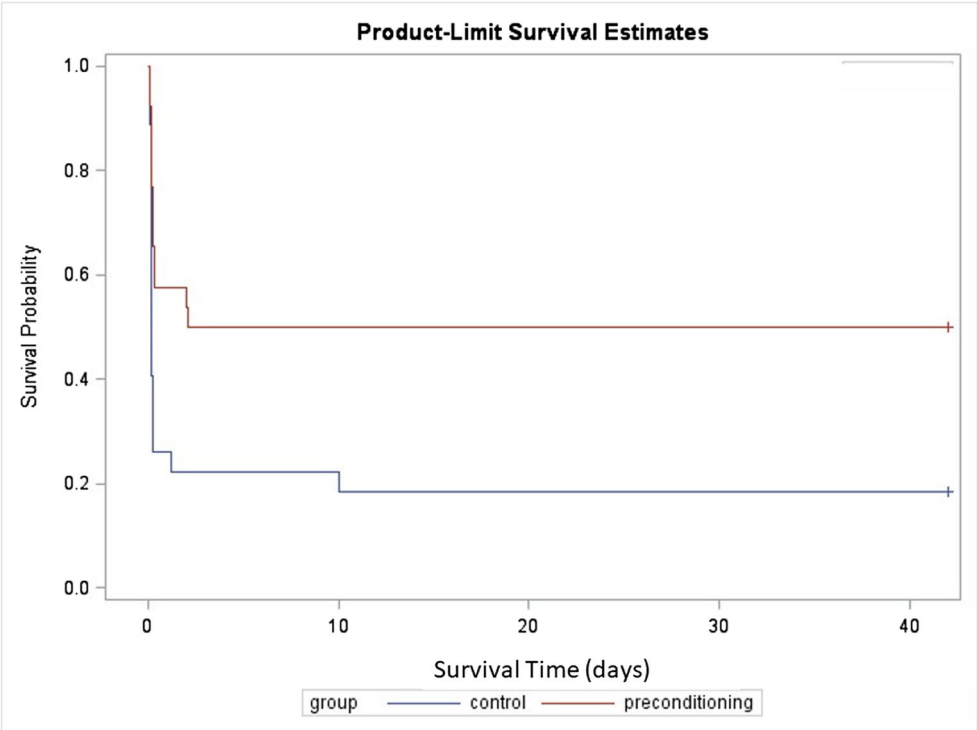

Fig. 1 Kaplan-Meier curve of survival in the two groups, showing that the two groups were significantly different from one another in their survival distribution ($p = 0.004$). Survivors, $n = 5$ of 27 rats in the control group and $n = 13$ of 26 rats in the RIPC group

Shed Blood Volume

There was no difference in rat body weights between the two groups on the day of hemorrhagic shock (275 ± 13 g in control group and 281 ± 13 g in the RIPC group; $p = 0.72$). The total withdrawn blood volume (expressed as percentage of estimated total blood volume) was 42 ± 1% in the control group ($n = 27$), and 49 ± 19% in the RIPC group. There was no difference in the shed blood volume withdrawn to maintain the mean blood pressure ~ 30 mmHg during the shock phase ($p = 0.84$).

Hemodynamics During Shock and Resuscitation

There was a significant overall difference in mean blood pressures in the two groups (group × time interaction term, $p < 0.0001$) (Fig. 2). During the first 15 min after initiation of resuscitation, mean blood pressure increased in both groups and tended to be higher in the RIPC group. Then, the blood pressure gradually decreased in both groups. Overall, there was no significant difference in heart rate (group × time interaction term, $p = 0.6$). Pulse pressure was different between the two groups determined by the group × time interaction term ($p = 0.01$), but there was no specific time points showing a group difference (all p values greater than 0.4).

Cardiac Function

The diastolic and systolic internal dimensions of the left ventricle (LVIDd and LVIDs, mm) and percentage of left ventricular fractional shortening (LVFS, %) were similar in both groups at baseline. Blood withdrawal significantly decreased LVIDd and LVIDs and increased the LVFS in both groups. LVIDd, LVIDs, and the LVFS were comparable in the two groups 5 min before resuscitation with the shed blood. Shed blood reinfusion increased the LVIDd and LVIDs at 1 h after initiation of resuscitation in both groups, but LVIDd was significantly higher in the RIPC group suggesting that there was more circulating intravascular blood volume in the RIPC group than in the control group (Table 1). LVFS was not depressed during any of the measures in either group.

Arterial Blood Analyses

There were no statistically significant differences at 1 h after initiation of resuscitation in arterial blood gas values (Supplemental Data Table 1). PaO_2 and $PaCO_2$ levels were lower than those reported in normal rats, but there were no differences between controls and RIPC.

Blood counts and chemistries were similar in both groups measured at 1 h after initiation of resuscitation. Magnesium levels were slightly but significantly lower in the RIPC group; otherwise, there were no differences in other parameters between groups (see Supplemental Data Table 2).

Blood sampled at 48 h after resuscitation through a catheter in the tail vein (see Supplemental Data Table 3) demonstrated liver injury in both groups as indicated by elevated AST and ALT, but there were no significant differences between groups. Blood urea nitrogen (BUN) was within normal range

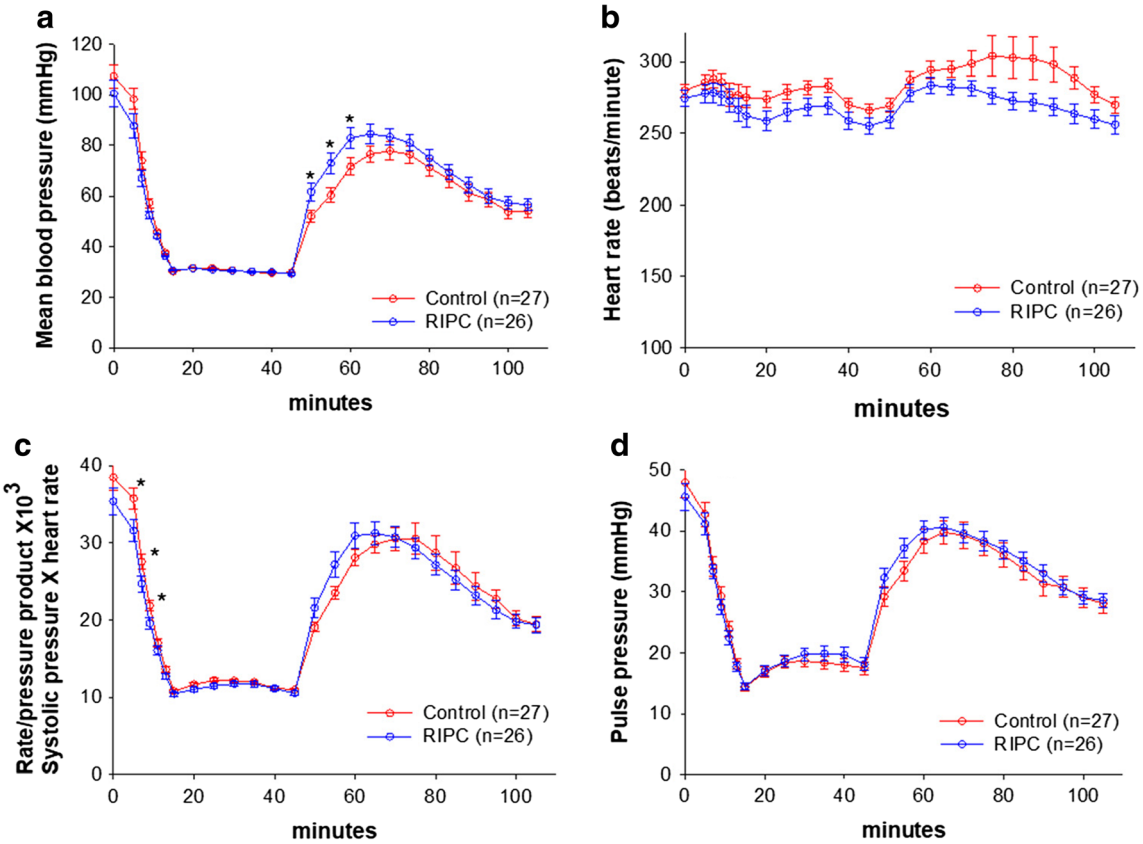

Fig. 2 Hemodynamic variables at baseline and during blood withdrawal, the shock phase, resuscitation, and observation phases. **a** The time course of mean arterial pressure is significantly different for the two groups (group × time interaction term, $p < 0.0001$; an asterisk indicates the time points, at which there is significant difference between the two groups). **b** The time course of heart rate is not significantly different for the two groups (group × time interaction term, $p = 0.6$). **c** The time course of rate/pressure product is significantly different for the two groups (group × time interaction term, $p = 0.0002$; an asterisk indicates the time points, at which there is significant difference between the two groups). **d** The time course of pulse pressure is significantly different for the two groups (group × time interaction term, $p = 0.01$), but there was no specific time points showing a group difference (all p values > 0.4 at different time points)

in the RIPC group (17.3 ± 1.2 mg/dl) but elevated in the control group (22.0 ± 1.7 mg/dl).

Table 1 Cardiac function (echocardiography)

	Control ($n = 27$)	RIPC ($n = 26$)	p value
Baseline			
Diastolic ID (mm)	6.7 ± 0.1	6.7 ± 0.1	0.83
Systolic ID (mm)	3.8 ± 0.1	3.8 ± 0.1	0.84
LVFS (%)	43.4 ± 1.1	44.2 ± 1.1	0.59
25 min of shock			
Diastolic ID (mm)	4.1 ± 0.1	3.9 ± 0.1	0.28
Systolic ID (mm)	2.2 ± 0.2	1.9 ± 0.2	0.19
LVFS (%)	47.8 ± 2.6	52.8 ± 2.8	0.20
1 h after blood resuscitation			
Diastolic ID (mm)	5.4 ± 0.1	5.8 ± 0.1	0.04
Systolic ID (mm)	2.7 ± 0.1	2.9 ± 0.1	0.46
LVFS (%)	49.6 ± 1.8	50.9 ± 1.9	0.64

ID, internal dimension; *LVFS*, left ventricular fractional shortening

C-reactive Protein and Cytokines

CRP levels were higher (non-statistically) in the RIPC group at 48 h (1118 ± 383 μg/ml) than at 6 weeks (448 ± 84). In the control groups, values were similar both at 48 h and 6 weeks (784 ± 416 and 761 ± 79 μg/ml, respectively). Median values for IL-1a, IL-1b, and IL-6 were similar in both groups at 48 h and 6 weeks. Serum TNF alpha levels were undetectable in both groups at 48 h and 6 weeks.

Histology of Tissues

At 6 weeks after hemorrhagic shock, gross pathology and light microscopy analyses were performed in the tissues from some surviving rats and showed brain infarction in 1 of 3 (33%) rats in the control group and 2 of 7 (29%) rats in the RIPC group. In brains without gross infarcts, the histology appeared normal in both groups (Fig. 3). Areas of microvascular damage (no-reflow) were not detected in the damaged brains or in other tissues. No evidence of myocardial, liver, or kidney damage were detected by either gross pathology or by microscopic

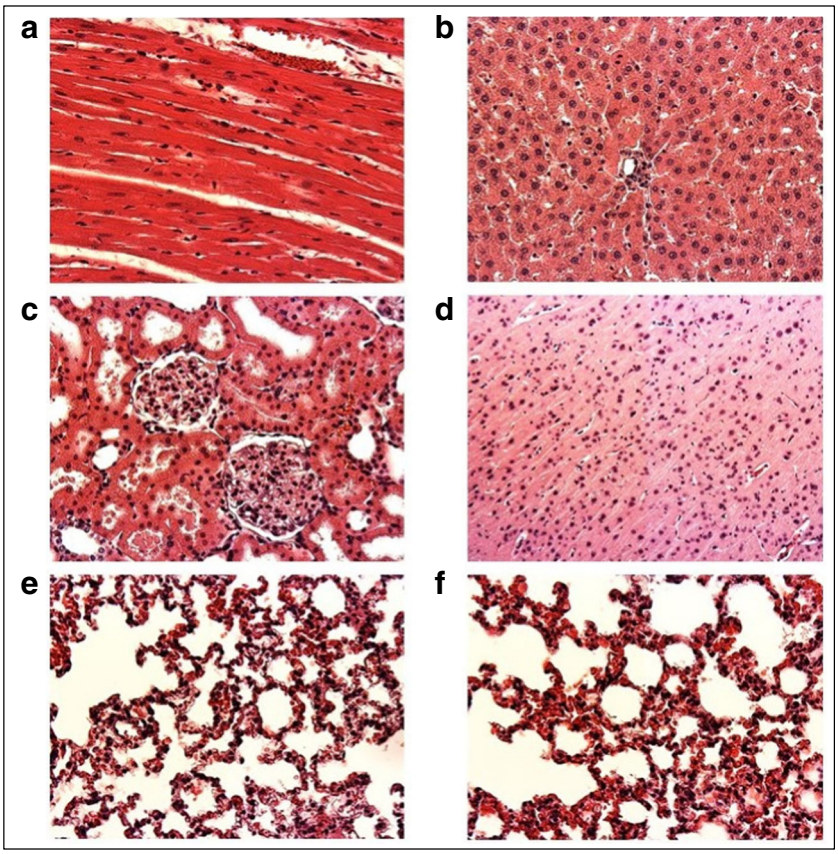

Fig. 3 Representative images of heart, liver, kidney, brain, and lung histology after H&E staining (original magnification × 20). **a** Section of heart in the control group. **b** Section of liver in the control group. **c** Section of kidney in the control group. **d** Section of brain in the control group. **e** Section of lung in the control group. **f** Section of lung in the RIPC group. The microscopic appearance of the heart, liver, and kidney was normal. In those brains without infarctions, the histology appeared normal. Lungs showed abnormalities in both groups as described in the "Histology of Tissues" section

analysis. Hematoxylin & eosin (H&E)-stained lung sections showed patchy lung lesions, including areas of thickened alveolar septa with mononuclear cell infiltration, disrupted alveolar morphology, and erythrocyte leaking into alveoli in control and RIPC groups. There was no difference in lung pathology between the control and RIPC groups. There were no architectural changes, inflammation, fibrosis, or necrosis in H&E-stained heart, kidney, or liver sections in either group.

Discussion

The major finding of the present study is that RIPC markedly improved short-term survival, after experimental hemorrhagic shock/resuscitation, and this benefit was maintained in the long term. Most deaths occurred within the first few days of shock, and animals that survived for the first few days survived long term. This suggests that if resuscitation is successful after acute bleeding, then there is usually no lethal consequences long term. While RIPC has been shown to be beneficial in numerous experimental and clinical studies including those of acute myocardial infarction and even stroke [28–31], there have been very few studies examining its effect in hemorrhagic shock and none that have assessed long-term survival.

This finding of a long-term benefit parallels data from a study performed in coronary artery bypass grafting patients who received RIPC prior to undergoing heart surgery [32]. In a follow-up study, all-cause mortality was significantly lower in the RIPC group at 5.83 years compared with the control group [33].

Numerous experimental and clinical studies have demonstrated that RIPC can confer multi-organ protection against acute ischemia/reperfusion injury in such organs as kidneys, intestine, liver, and lungs [9]. In addition, RIPC can reduce myocardial damage in patients undergoing surgery interventions [34]. In the present study, we did not find major differences in organ protection between the two groups. However, a limitation of our study is that the total number of survivors was relatively small; thus, we might not have been able to detect differences in organ protection. In the rats that survived 6 weeks, organ anatomy and function was similar between the controls and treated rats.

RIPC (brief transient episodes of ischemia separated by reperfusion) of remote tissues has been recognized as one of the most potent anti-ischemic interventions and has been extensively studied in the heart. However, in the setting of severe hemorrhagic shock, its protective effects have been investigated only to a limited extent.

Leung et al. [21] evaluated the efficacy of RIPC on organ protection after hemorrhagic shock/resuscitation in an

isoflurane-anesthetized C57Bl/6 mouse model. RIPC consisted of one instance of 10 min of left femoral artery occlusion followed by 10 min of reperfusion before the initiation of hemorrhagic shock. Control animals did not have femoral artery occlusion. Hemorrhagic shock was achieved by withdrawing blood over 15 min to lower the mean arterial blood pressure to 30 mmHg; the hypotensive period was maintained for 60 min. The animals were resuscitated with 0.9% normal saline equivalent to twice the volume of blood withdrawn. Liver and lung tissues and blood samples were collected for analysis at 1 and 2 h after resuscitation. In a second severe injury model, mice were subjected to the same procedure, but the hemorrhagic shock at 30 mmHg of mean arterial blood pressure was maintained for 2 h, and the RIPC was achieved by performing 4 cycles of alternating 5-min ischemia, followed by 5-min reperfusion. The results demonstrated that RIPC reduced levels of inflammation and injury in the liver and lung after shock/resuscitation in both mild and severe injury models. This study did not investigate survival.

In a volume-controlled hemorrhagic shock model, Hu et al. [18] tested pentobarbital-anesthetized male Sprague-Dawley rats and induced RIPC by 4 cycles of 5 min of limb ischemia followed by reperfusion for 5 min. An estimated 50% of the total blood volume was withdrawn during an interval of 60 min after performing RIPC. The mean blood pressure was maintained above 45 mmHg during the shock phase. Thirty minutes after the completion of bleeding, the shed blood was reinfused over the ensuing 30 min. These investigators found that mean blood pressure rapidly increased to near-baseline levels during the resuscitation phase and was significantly greater in the RIPC group. At 2 h after reinfusion, ejection fraction and myocardial performance index were significantly better, and the sublingual microvascular flow index and perfused vessel density were significantly greater in the RIPC group than in the control group ($p < 0.01$). At 72 h, survival in the RIPC group (7 of 7 rats) was significantly greater than that in the control group (1 of 7 survived, $p < 0.01$), and neurological deficit score was significantly better in the RIPC group than the control animals ($p < 0.01$). Our study confirms the general finding that RIPC improves acute survival but extends the previous study by showing that RIPC's benefit remains long term. Our study differs from Hu and coworkers' study in some respects: we assessed long-term survival (6 weeks), shed blood volume was less (42% versus 50%), rat mean blood pressure was lower (30 mmHg versus > 45 mmHg) during the shock phase, and in our study, rat survival was lower (50% versus 100%) in the RIPC group at 72 h after resuscitation. Other differences were anesthetic used, bleeding duration (10 min versus 60 min), and the different shock model (fixed-pressure versus fixed-volume hemorrhagic shock model).

Huang et al. [20] recently tested remote ischemic perconditioning (RIPerC, administered during the resuscitation period) and remote ischemic postconditioning (RIPostC, administered after the resuscitation period) in male rats. Hemorrhagic shock was induced by removing 45% of the estimated total blood volume, and remote ischemic conditioning was induced by four cycles of limb ischemia for 5 min followed by 5 min of reperfusion. Two hours after resuscitation, myocardial function indices such as ejection fraction and cardiac output were better in both preconditioned groups compared with a control group. Animals were monitored for 72 h before sacrifice, and survival was better in both conditioned groups compared with the control group.

The studies above support the concept that RIPC is a promising anti-ischemic intervention for acute hemorrhagic shock but do not address the long-term effects of the treatment. Our results have shown for the first time that RIPC results in long-term improvement in survival in fixed-pressure hemorrhagic shock model. In addition, we found no evidence that left ventricular dysfunction either during hypotension or after restoration of flow contributed to the shock state. We found no evidence of myocardial necrosis by biomarkers or by histology in this study and no evidence of stunned myocardium.

Hemorrhage-induced ischemia/reperfusion contributes to endothelial cell injury, impaired microcirculation, vascular permeability, and vascular leakage [35]. Ischemic preconditioning has been demonstrated in experimental models to protect endothelial function and structure and protect tissues from injury by preserving microcirculation [36–38]. Our present study supports the concept that RIPC might have reduced vascular leakage because the LVIDd was significantly higher in the RIPC group than in the control group, possibly indicating more circulating intravascular blood volume in the RIPC group than in the control group.

Vascular reactivity is greatly reduced during hemorrhagic shock. Hemorrhage-induced vascular hyporeactivity can severely interfere with the treatment of shock and may be an important cause of death [39]. RIPC can increase endothelial reactivity [40] and improve shock-induced vascular hyporeactivity [19, 41]. In the present study, we observed that early hemodynamic responses after shed blood resuscitation were improved in the RIPC group, which was indicated by the significantly higher blood pressure during the blood reinfusion phase.

The ventilation parameters used during the procedure result in normal levels of PaO_2 and $PaCO_2$ in normal rats [42]. However, in the setting of hemorrhagic shock, these or similar settings are associated with lower levels of both PaO_2 and $PaCO_2$ as previously reported [43] and as observed in our present study. Explanation for the lowering of $PaCO_2$ might be due to a mismatch of fixed lung ventilation versus reduced perfusion, resulting in relative hyperventilation. An accompanying decrease in PaO_2 might be caused by poorer relative diffusion of O_2 compared to CO_2 in a lung with reduced diffusion capacity due to lung injury from shock [44].

The technique of RIPC could be easily adapted to a scenario of a soldier facing battle as this form of therapy is

inexpensive and easy to apply. It can be thought of as a "vaccination" against the ischemia/reperfusion injury that can occur with hemorrhagic shock and could potentially reduce mortality and morbidity associated with this injury. A soldier would simply inflate a blood pressure cuff above systolic pressure for 5 min, deflate the cuff for 5 min, and then repeat this three times. This therapy could be applied daily or just prior to high-risk combat missions and therefore would be a practical and clinically relevant approach to preventative therapy. Because the therapy can be applied prophylactically prior to the soldier going into battle, RIPC as a preventative for hemorrhagic shock represents a potential therapy that is truly preconditioning rather than per- or postconditioning.

Limitations

Hemorrhagic shock-induced global organ injury depends upon the hypotensive level and the duration of shock phase. A limitation to our study is that we only investigated the protective effects of RIPC at 30 mmHg for 30 min based on a pilot time-course study. The RIPC protection under various hypotension levels and durations will require future investigation. Other limitations of the study include the concept that some aspects may be inconclusive when applied to humans. It is known that RIPC works in humans and there are several studies showing that it reduces biomarkers of necrosis in acute myocardial infarction [29]. However, whether RIPC can protect humans after blood loss is not yet known. Certainly, the results of this study suggest that it would be worth investigating, especially because soldiers could apply RIPC themselves by simply inflating and deflating a blood pressure cuff BEFORE they go onto the battlefield, i.e., this therapy could be self-administered in a prophylactic format. Whereas soldiers going into battle may represent only a small fraction of patients who lose blood, there are other situations in which prophylactic RIPC might be useful, for example, law enforcement personnel such as police or SWAT teams, who know they are going into a potentially dangerous situation. While we applied RIPC before hemorrhage, remote conditioning has also been applied at the time of injury rather than before injury; in this case, the term perconditioning has been applied. This therapy was shown to work when blood pressure cuff inflation and deflation were instituted starting in the ambulance after the onset of acute myocardial infarction [28] therapy improved myocardial salvage and long-term clinical outcome [31]. Thus, inducing a conditioning protocol after onset of injury has the potential to work in situations such as car accidents and gunshot wounds but has yet to be tested in a clinical study.

One limitation of using the rodent model is that it is difficult to do serial blood draws and blood gas measurements without altering hemodynamics. However, we were able to examine blood gases in the early resuscitation phase and draw other chemistries at 48 h. Another limitation is that we did not measure interleukin levels earlier than 48 h.

In conclusion, our data suggest that RIPC is a promising therapeutic strategy to improve long-term survival from hemorrhagic shock injury. The underlying mechanisms involved in RIPC need to be further investigated. This understanding will facilitate the development of new therapeutic strategies to help treat hemorrhagic shock.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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