ANNALS of Clinical and Translational Neurology
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RESEARCH ARTICLE

Short-term remote ischemic conditioning may protect monkeys after ischemic stroke

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Funding Information
This study was supported by the National Key R&D Program [2017YFC1308401], the National Natural Science Foundation [81371289], and the Project of Beijing Municipal Top Talent for Healthy Work [2014-2-015] of China.

Received: 20 September 2018; Revised: 1 November 2018; Accepted: 7 November 2018

Annals of Clinical and Translational Neurology **2019; 6(2): 310–323**

doi: 10.1002/acn3.705

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Abstract

Objective: We aimed to evaluate the safety and effectiveness of short-term remote ischemic postconditioning (RIPC) in acute stroke monkey models. **Methods**: Acute stroke monkeys were allocated to four groups based on the number of limbs exposed to RIPC. RIPC was initiated by 5-min cuff inflation/deflation cycles of the target limb(s) for 5–10 bouts. Vital signs, skin integrity, brain MRI, and serum levels of cardiac enzymes (myoglobin, creatine kinase [CK], CK-muscle/brain [CK-MB]), one inflammatory marker (high-sensitivity C-reactive protein [hsCRP], and one endothelial injury marker (von Willebrand factor [vWF]) were assessed. Spetzler scores were used to assess neurological function. **Results**: No significant differences in vital signs or local skin integrity were found. Short-term RIPC did not reduce infarct volume under any condition at the 24th hour after stroke. However, neurological function improved in multi-limb RIPC compared with sham and single-limb RIPC at the 30th day follow-up after stroke. Myoglobin, CK, and CK-MB levels were reduced after multi-limb RIPC, regardless of the number of bouts. Moreover, multi-limb RIPC produced a greater diminution in CK-MB levels, whereas two-limb RIPC was more effective in reducing serum CK levels at the 24th hour after stroke. hsCRP increased after 5 bouts of multi-limb RIPC before decreasing below baseline and single-limb RIPC levels. Serum vWF was decreased at later time points after RIPC in all RIPC groups. **Conclusions**: Stroke monkeys in hyperacute stage may benefit from short-term RIPC; however, whether this intervention can be translated into clinical use in patients with acute ischemic stroke warrants further study.

Introduction

Stroke is a leading cause of mortality and morbidity worldwide. Elevations in several biomarkers of inflammation and endothelial injury, such as high-sensitivity C-reactive protein (hsCRP) and von Willebrand factor (vWF) have been reported to be associated with poor stroke outcomes.[1–4] Meanwhile, studies showed that stroke-related cardiac disorders, which often occur in the hyperacute stage (brain–heart syndrome), could also worsen the prognosis of stroke.[5–8] Stroke-related cardiac dysfunction is characterized by abnormal elevation of cardiac enzymes, such as creatine kinase (CK), creatine kinase-muscle/brain (CK-MB), myoglobin, and cardiac troponin T (cTnT). It has been suggested that activation of the sympathetic nervous system after stroke leads to a hyperdynamic cardiovascular state and an elevation in myocardial enzymes.[9] Moreover, sympathetic activation in acute ischemic stroke may pose an undesirable negative impact on cardiac functions.[10] In turn, cardiac complications are supposed to increase the risk of stroke-related death.[11] At present, there are few effective approaches for

stroke-related cardiac dysfunction. Hence, there is an urgent need to develop and explore novel, safe and effective treatment strategies for clinical application.

Remote ischemic postconditioning (RIPC), a nonpharmacological approach performed by intermittently and noninvasively blocking the blood flow of a limb (or limbs), has been reported to ameliorate brain injury when used persistently after ischemic stroke.[12–14] The underlying mechanisms of RIPC-induced protection against brain and heart injury are far from clear but may include alleviation of inflammation, inhibition of necrosis, and regulation of protein synthesis.[15–17] Animal research indicated that short-term RIPC could attenuate brain or heart injury by reducing the concentration of cardiac enzymes, such as CK,[18] in peripheral blood circulation. Additionally, clinical studies demonstrated that short-term RIPC decreased the levels of cTnT after percutaneous coronary intervention (PCI) and improved postintervention clinical outcomes.[19–21] In contrast, some other studies claimed that no myocardial protection was conferred by RIPC, and it might even worsen myocardial cell injury and increase inflammatory response after PCI.[22,23] Thus, the effects of short-term RIPC on ischemic brain and heart injury are still equivocal. The inconsistencies in the benefit of RIPC among studies may be related to the lack of a standardized RIPC protocol. Notably, a former study concluded that one- and two-limb RIPC shared equal protective effects, and the cycle rather than the size of tissue mass exposed to RIPC, determined the efficacy.[24] However, whether the localization or "dose" of RIPC affects the extent of protection remains unclear.

Although it has been well established that the most effective therapeutic strategy for hyperacute ischemic stroke is intravenous thrombolysis, its clinical use is largely limited by a strict time window and some contraindications. According to current guidelines, intravenous thrombolysis is used only if the duration since the onset of stroke symptoms is within 4.5 hours. Although there are a few clinical trials regarding the application of RIPC in acute ischemic stroke, the conclusions are not entirely consistent or even contradictory. For instance, England et al. found that short-term RIPC may improve poststroke outcomes,[25] while Hougaard et al. concluded that short-term RIPC combined with rt-PA was not superior to rt-PA alone for treating acute stroke.[26] Besides, the in-hospital mortality rate has been reported up to 5–8% during acute phase in brain–heart disorders.[27] Therefore, further preclinical and clinical studies about the efficacy of short-term RIPC on brain–heart protection in acute stroke are still needed.

Herein, we used ischemic stroke monkey models to evaluate the safety and efficacy of short-term RIPC on stroke-related cardiac dysfunction in the hyperacute stage. Furthermore, the optimal parameters of the RIPC regimen by altering the number of limbs undergoing RIPC and the number of bouts of inflation/deflation in each RIPC training cycle were assessed.

Materials and Methods

The study protocol was approved by the Animal Care and Use Committee of Capital Medical University (Beijing, China). All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council), the Ministry of the Environment Guidelines for the Care and Use of Animals in Research, and Guidelines for Proper Conduct of Animal Experiments (2006). All the animal experiments were performed in accordance with the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines.

The monkey model of ischemic stroke

Fourteen healthy male rhesus monkeys, with an average age of 2.3 ± 0.42 years and weight of 8.25 ± 0.65 kg, were screened 1 month prior to the experiment and observed for further confirmation of no potential systemic diseases or neurological disorders.

Anesthesia was induced in monkeys by intramuscular injection of ketamine (10 mg/kg, Sanofi, Shanghai, China), and maintained with intravenous infusion of propofol (0.5 mg/kg per hour, Astra Zeneca, Caponago, Italy). Afterwards, all monkeys underwent acute right middle cerebral artery occlusion of the M2 segment (MCA-M2), as previously described.[28] Briefly, during the process of digital subtraction angiography (DSA), an autologous blood clot was injected via a microcatheter into the MCA-M1 segment and flushed into the superior division of MCA-M2 segment. The MCA-M2 occlusion and newly formed ischemic stroke lesions at the M2 segment were confirmed by MRI scanning immediately after the operation. MRI scanning was performed on a Magnetom Trio MRI Scanner (3.0T; Siemens AG, Siemens Medical Solutions, Erlangen, Germany) with the following scan sequences: (1) T2-weighted imaging used fast-spin echo method, TR = 4000 msec, TE = 100 msec, bandwidth = 200 Hz/pixel, FOV = 180 mm, slice thickness = 2 mm, 4 averages); (2) DWI, single-shot EPI, TR = 6600 msec, TE = 100 msec, bandwidth = 1002 Hz/pixel, FOV = 220 mm, slice thickness = 2 mm, 4 averages; (3) MRA, TR = 20 msec, TE = 3.6 msec, bandwidth = 186 Hz/pixel, FOV = 220 mm, slice thickness = 1 mm, 1 average). The stroke lesions confirmed by MRI/DWI were consistent with results of the autopsy (Fig. 1A, B). Stroke volumes were calculated using the ITK-Snap contouring software (Philadelphia, PA) with stacks of average diffusion images reconstructed in three

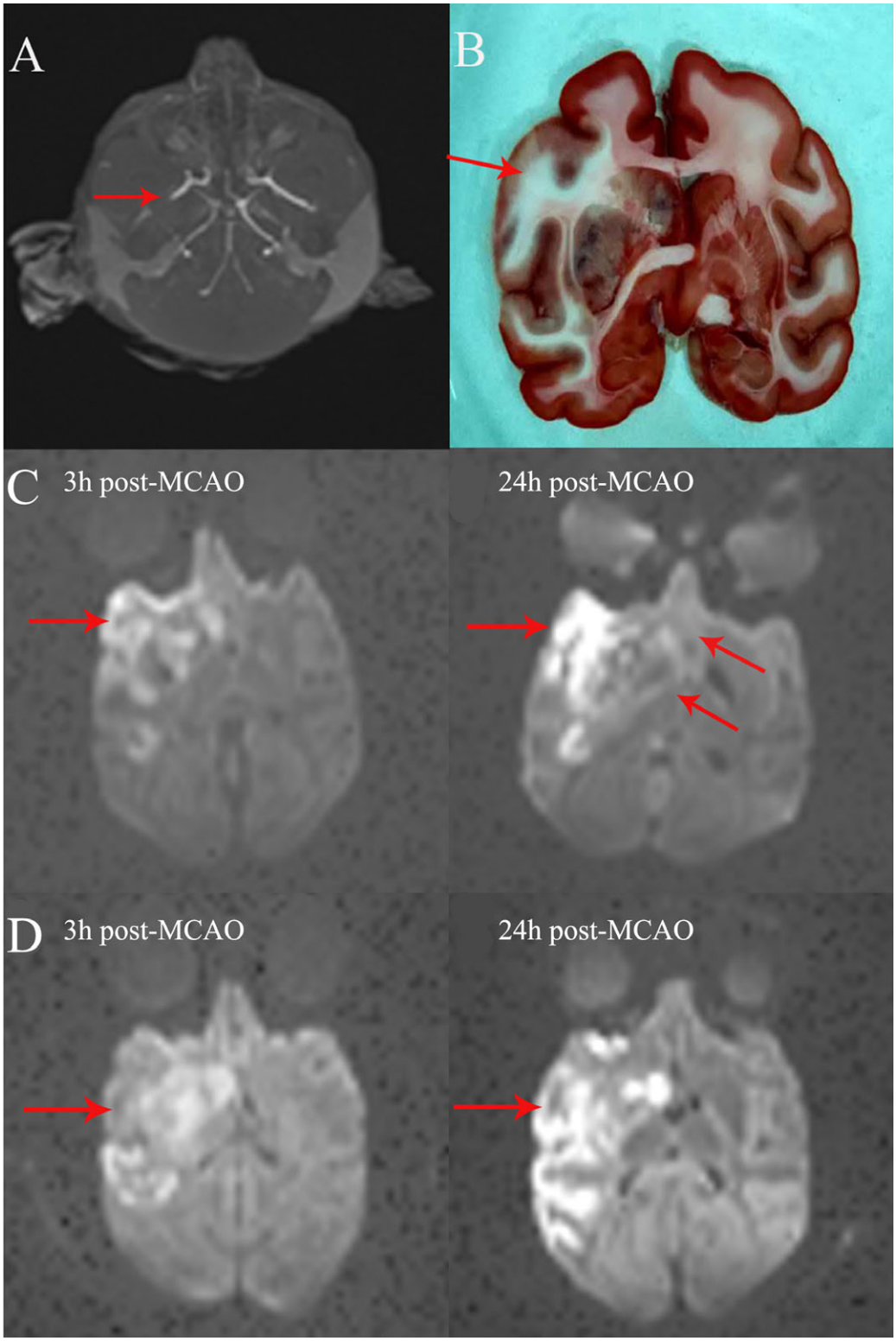


Figure 1. Images of rhesus monkey with acute right middle cerebral arterial occlusion-mediated stroke: (A) occlusion at MCA-M2 segment (MRA, arrow), (B) infarcted volume in the right fronto-temporal lobe (brain autopsy slide), (C) newly temporal lobe infarctions at 3 h and 24 h after MCA-M2 occlusion of a monkey underwent sham RIPC (DWI). (D) newly temporal lobe infarctions at 3 h and 24 h after MCA-M2 occlusion of a monkey underwent four-limb RIPC (DWI). RPIPC, remote ischemic postconditioning

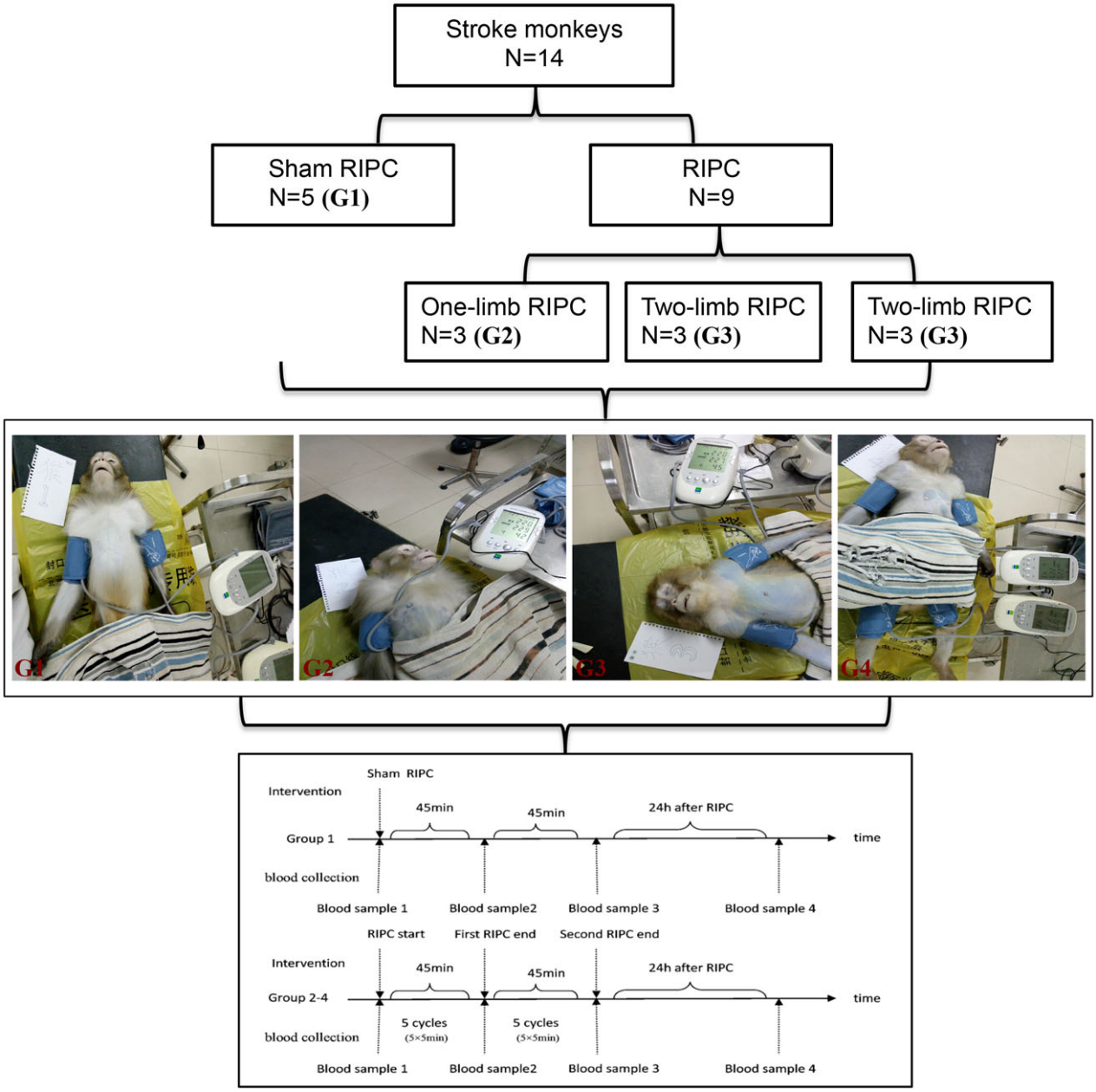


Figure 2. Flowchart illustrating study design and timeline.

dimensions. Following verification of the ischemic lesions, monkeys were divided into four groups based on the number of limbs undergoing RIPC: (1) sham (group-1, $n = 5$), (2) single-limb (group-2, $n = 3$), (3) two-limb (group-3, $n = 3$) and (4) four-limb (group-4, $n = 3$), see Figure 2.

Limb RIPC performance

Both sham RIPC and RIPC were performed under propofol anesthesia (0.5 mg/kg per hour, Astra Zeneca, Caponago, Italy). RIPC was achieved by blocking arterial and venous blood flow of one or more limb(s) for 5 min with 200 mmHg cuff pressure, followed by 5 min of deflation to restore perfusion. This cycle was automatically repeated for 5 bouts by the RIPC device (patent number ZL200820123637.X, China). A total of two consecutive cycles (5 min × 10 bouts) were initiated immediately after stroke. Sham RIPC was performed with the cuffs placed around the bilateral upper limbs without inflation/deflation (i.e., the inflation pressure was 0 mmHg). MRI/DWI follow-ups were completed at 3 and 24 h after RIPC/sham intervention.

Blood samples were collected from monkeys through the femoral vein on the nonoperative side at baseline (prior to RIPC/sham), immediately after 5 min × 5 bouts, immediately after 5 min × 10 bouts of RIPC/sham, and at 24 h after RIPC/sham. The experimental schema is shown in Figure 2.

Safety of RIPC

To evaluate the safety of RIPC during acute ischemic stroke, we monitored vital signs, including blood pressure, heart rate, and respiratory frequency at the same time points when blood samples were collected. Local skin integrity was assessed by monitoring any signs of swelling, erythema, and ecchymosis. Infarct volume and hemorrhagic transformation were detected using MRI/DWI at 3 and 24 h (under mild anesthesia) after RIPC/sham intervention. Finally, measurement of mortality rate was completed in the 14 stroke monkeys during 2 months following transient RIPC.

Effectiveness of RIPC

To evaluate the effectiveness of RIPC on acute stroke-related cardiac dysfunction, we assessed inflammatory responses, vascular endothelial injury, coagulation parameters, and platelet aggregation status after stroke. Serum levels of myoglobin, CK-MB, CK, hsCRP, vWF, and fibrinogen (Fib) were measured using enzyme-linked immunosorbent assay (ELISA) kits (Jingmei Biotech Co., Ltd., Shenzhen, China). Coagulation parameters including prothrombin time (PT), thrombin time (TT), activated partial thromboplastin time (APTT), International Normalized Ratio (INR), and platelet aggregation rates were detected with the automatic blood cell analyzer-XFA6100 (Perlong Medical Equipment Co., Ltd, Nanjing, China). Experimenters were blinded to the study protocol.

The Spetzler neurological deficit rating scale was used to evaluate neurological function.[29] This scale is weighted heavily not only on motor function (70 points), but it also takes into account behavioral changes (mental status, 20 points) and cranial nerve impairment (10 points). Spetzler scores were evaluated by two separate observers at 3 h, 24 h, 30 days, and 60 days after stroke for the sake of assessing the long-term effect of transient RIPC on stroke outcomes. Meanwhile, the survival rate was also recorded.

Statistical analyses

All data followed a normal distribution and were presented as mean ± SD. We performed an analysis of variance with repeated measures to determine differences among groups over time using a conservative Greenhouse–Geisser correction. Post hoc analyses were performed using the Tukey's honestly significant difference tests. Statistical significance was measured at $P < 0.05$. All analyses were performed using IBM SPSS Statistics software for Windows (version 19.0, IBM Corp., Armonk, NY). Detailed statistical analyses are presented in the figure legends.

Results

Safety of transient RIPC

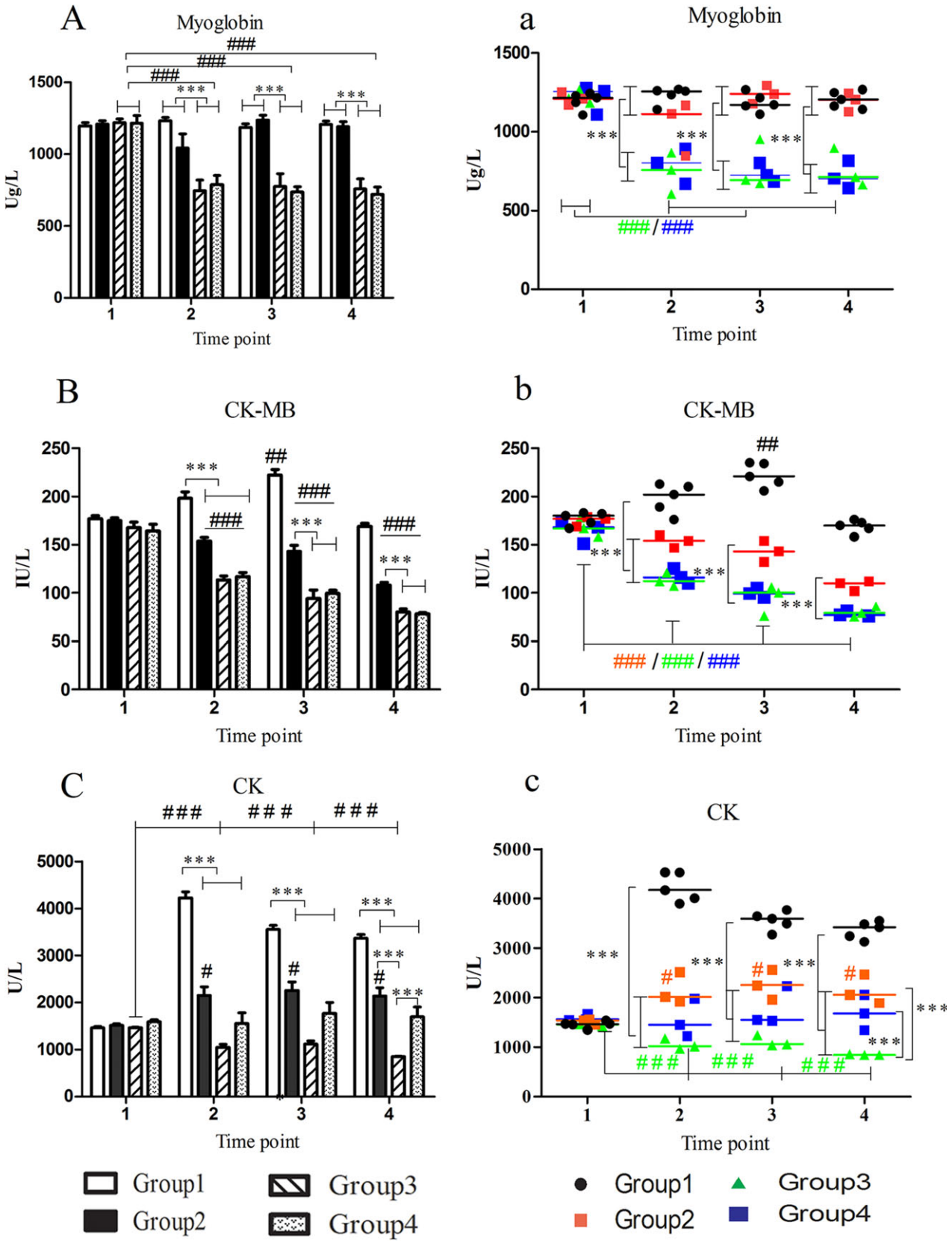
RIPC was well tolerated by all stroke monkeys in the hyperacute stage. Blood pressure, heart rate, and respiratory frequency fluctuated within the normal range during the whole process of RIPC intervention (Table S1). No swelling, erythema, or ecchymosis of the local skin was observed. The infarct volumes in the four groups at 24 h after RIPC/sham showed no significant difference when compared with their baseline levels. There was no indication of hemorrhagic transformation at both 3 h and 24 h after sham RIPC (Fig. 1C) or RIPC (Fig. 1D). All stroke monkeys that underwent RIPC (groups-2 to -4, $n = 9$) were alive during the hyperacute stage and the follow-up period after RIPC. Nonetheless, in the control group ($n = 5$), one out of the five monkeys died at the 33rd day after sham RIPC, while the remaining monkeys were alive till the end of the study.

Effectiveness of transient RIPC

Serum myoglobin

Myoglobin levels at baseline in the four groups ranged from 1105.5 to 1277 μg/L (Fig. 3A–a), which were substantially higher than the normal range (0–70 μg/L). Despite no statistical difference in myoglobin levels among the four groups were detected ($F = 0.025$, $P = 0.98$), variations were noticed between pre- and post-RIPC intervention. There was a trend toward an elevation in the level of myoglobin in the sham RIPC group (group-1) at 24 h ($P = 0.09$). Single-limb RIPC (group-2) decreased the myoglobin level slightly after 5 min × 5 bouts of RIPC ($P = 0.067$), after which it rebounded to baseline value. Myoglobin levels in the two- (group-3) and four-limb (group-4) RIPC groups were significantly reduced after 5 min × 5 bouts and 5 min × 10 bouts of RIPC, and at 24 h post-RIPC, when compared to their baseline values (group-3 vs. baseline, $P < 0.001$; group-4 vs. baseline, $P < 0.001$).

Comparisons among the four groups showed that the levels of myoglobin were significantly higher in the sham and single-limb RIPC groups compared to the two- and


A
Myoglobin
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Group1
Group2
Group3
Group4

Figure 3. Bar graphs showing mean values of serum levels of (A) myoglobin, (B) CK-MB, (C) CK measured at 1 = baseline prior to RIPC, 2 = after 5 min × 5 bouts of RIPC, 3 = after 5 min × 10 bouts of RIPC, 4 = 24 h after RIPC. Color Dot graphs showing individual data points for each animal in the different experimental groups at each time point. There is a significant effect of RIPC numbers of bouts and limbs (by repeated ANOVA). The symbol * represents significant differences among groups at each time point by post hoc comparison with Tukey tests. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. The symbol # denotes significant differences in each group at different time points when compared with the baseline (#$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$). CK, creatine kinase; RIPC, remote ischemic postconditioning

four-limb RIPC groups at all time points examined after intervention (all $P < 0.001$). Additionally, there was no statistically significant difference between two- and four-limb RIPC at any time point.

Creatine kinase MB

CK-MB increased in all monkeys after stroke. After sham RIPC, CK-MB increased and peaked at 5 min × 10 bouts ($P < 0.01$) before returning to baseline value at 24 h post-RIPC (Fig. 3B–b). RIPC, regardless of the number of limbs and bouts, significantly reduced CK-MB levels at all time points, when compared to sham RIPC (all $P < 0.001$). Notably, although greater reductions were observed in the two- and four-limb RIPC groups relative to the other two groups, there was no significant difference between groups subjected to two- and four-limb RIPC at 24 h post-RIPC ($P = 0.67$).

Creatine kinase

The levels of CK prior to RIPC/sham in all stroke monkeys (1346-1669 IU/L) were markedly elevated beyond the normal range (24-195 IU/L). For the sham RIPC group, CK levels remained elevated for 24 h compared to the values of baseline ($P < 0.001$) (Fig. 3C–c) and the remaining groups ($P < 0.001$). Single-limb RIPC produced a small increase in CK over time compared to baseline ($P < 0.05$). Despite this increase, CK levels after single-limb RIPC were still substantially lower than sham RIPC monkeys ($P < 0.001$), but higher than those in two-limb RIPC group ($P < 0.001$). CK levels after two-limb RIPC were markedly decreased at all time points when compared to baseline, sham, single- and four-limb RIPC (all $P < 0.001$). Four-limb RIPC had no stronger effect on CK levels across time relative to baseline and sham RIPC ($P = 0.851$).

Inflammatory response

The levels of hsCRP increased in all monkeys after stroke. Serum hsCRP levels changed as a function of the number of limbs that underwent RIPC. In the sham group, hsCRP increased across time ($P < 0.01$). Single-limb RIPC had no effect on hsCRP levels compared to baseline ($P = 0.133$). Single-limb RIPC significantly decreased hsCRP after 10 bouts compared to sham ($P < 0.01$). Two- and four-limb RIPC initially increased hsCRP levels after 5 min × 5 bouts compared to baseline, sham, and single RIPC, but markedly decreased hsCRP levels below those measured at baseline, and at subsequent time points in both the sham and single-limb RIPC groups (all $P < 0.001$). There was no significant statistical difference in hsCRP between two- and four-limb RIPC at 24 h after RIPC intervention ($P = 0.14$), (Fig. 4A–a).

Von Willebrand factor

The levels of vWF in all stroke monkeys were elevated above the normal range (500–2000 U/L). Sham RIPC had no effect on vWF. Serum vWF levels in the sham group increased at 24 h after RIPC ($P < 0.05$). Single-, two-, and four-limb RIPC produced similar decreases in vWF after 10 bouts compared to baseline and sham RIPC (all $P < 0.001$; Fig. 4B–b). However, at 24 h after RIPC, vWF further decreased in the two- and four-limb RIPC groups compared to the single-limb RIPC group ($P < 0.001$). No differences in vWF levels were observed between monkeys in the two- and four-limb RIPC groups ($P = 0.348$).

Coagulation parameters and platelet aggregation rate

No statistically significant difference in coagulation parameters, including INR, PT, APTT, TT, and Fib, and platelet aggregation rates was detected among the four groups (all $P > 0.05$; Fig. 5A–C).

Infarct volume assessment

RIPC had no remarkable effect on infarct volumes, regardless of the duration of or the number of limbs exposed to RIPC (Fig. 1C–D and Table S1).

Spetzler neurological function scores and mortality rate assessment

Neurological function improved over time in all groups with respect to three main domains including motor function, behavior presentation (mental status), and

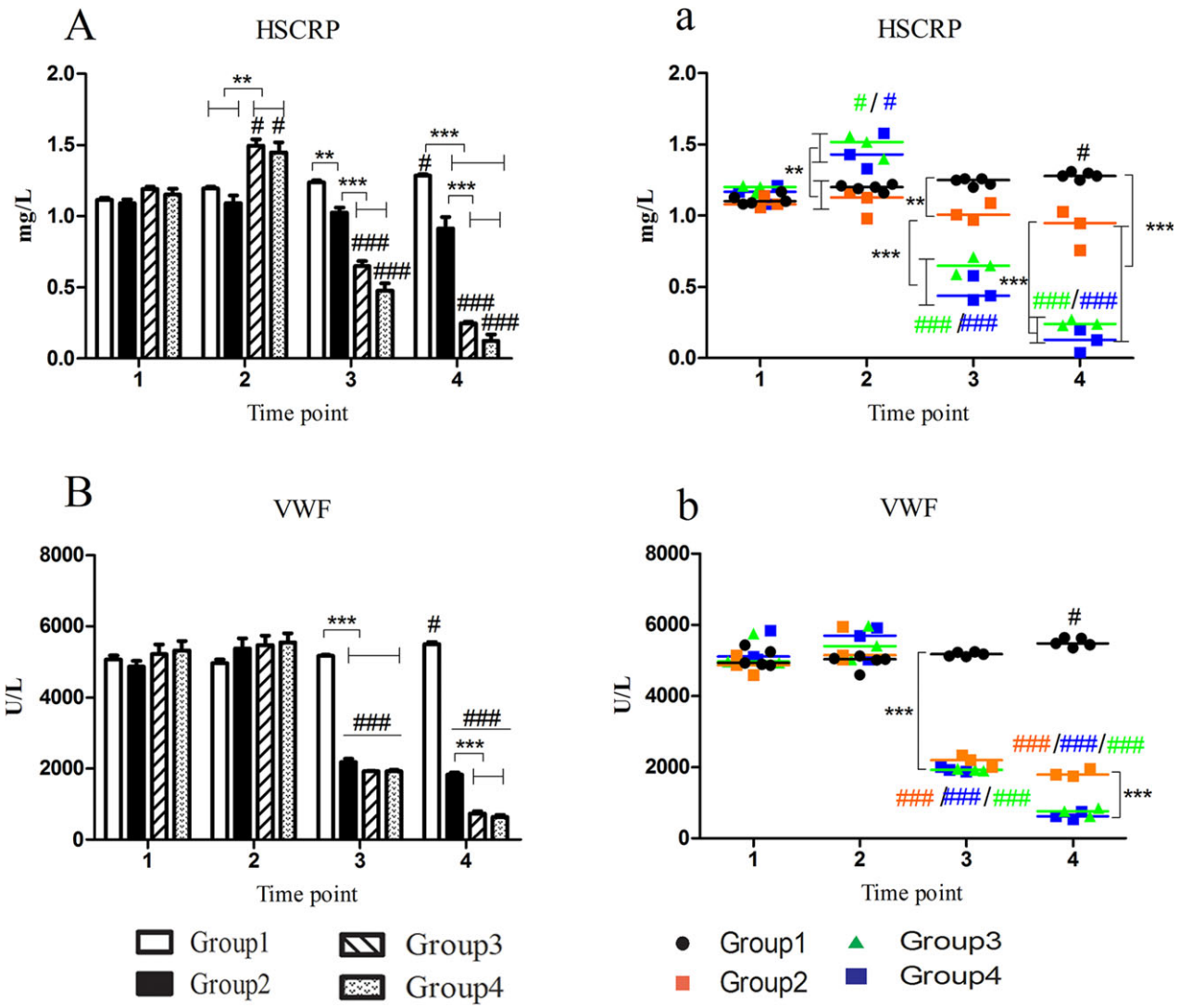

Figure 4. Bar graphs showing mean values of serum levels of (A) hsCRP and (B) vWF measured at 1 = baseline prior to RIPC, 2 = after 5 min × 5 bouts of RIPC, 3 = after 5 min × 10 bouts of RIPC, 4 = 24 h after RIPC. Color Dot graphs showing individual data points for each animal in the different experimental groups at each time point. There is a significant effect of RIPC numbers of bouts and limbs (by repeated ANOVA). The symbol * denotes significant differences among groups at each time point by post hoc comparison with Tukey tests. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. The symbol # represents significant difference in each group at different time points when compared with the baseline (#$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$). RIPC, remote ischemic postconditioning

cranial nerve function (Fig. 6A–C). No functional scores of the three aspects showed any improvement at 24 h after intervention in all groups. By day 30, significant improvements in neurological function were recorded in all groups, particularly in motor function. However, monkeys with either two- or multi-limb RIPC after stroke displayed a greater enhancement in motor function than sham RIPC and single-limb RIPC-treated monkeys (group-3 and 4 vs. group-1 and 2, $P < 0.001$). Additionally, the survival rates of stroke monkeys with RIPC and sham RIPC at 60 days follow-up were 100% (9/9) and 80% (4/5), respectively (Fig. 6D).

Discussion

To the best of our knowledge, this is the first study conducted to evaluate the safety and efficacy of short-term RIPC in non-human primates during the hyperacute stage of ischemic stroke. This study suggests that RIPC performed during hyperacute stroke appears to be safe and effective in

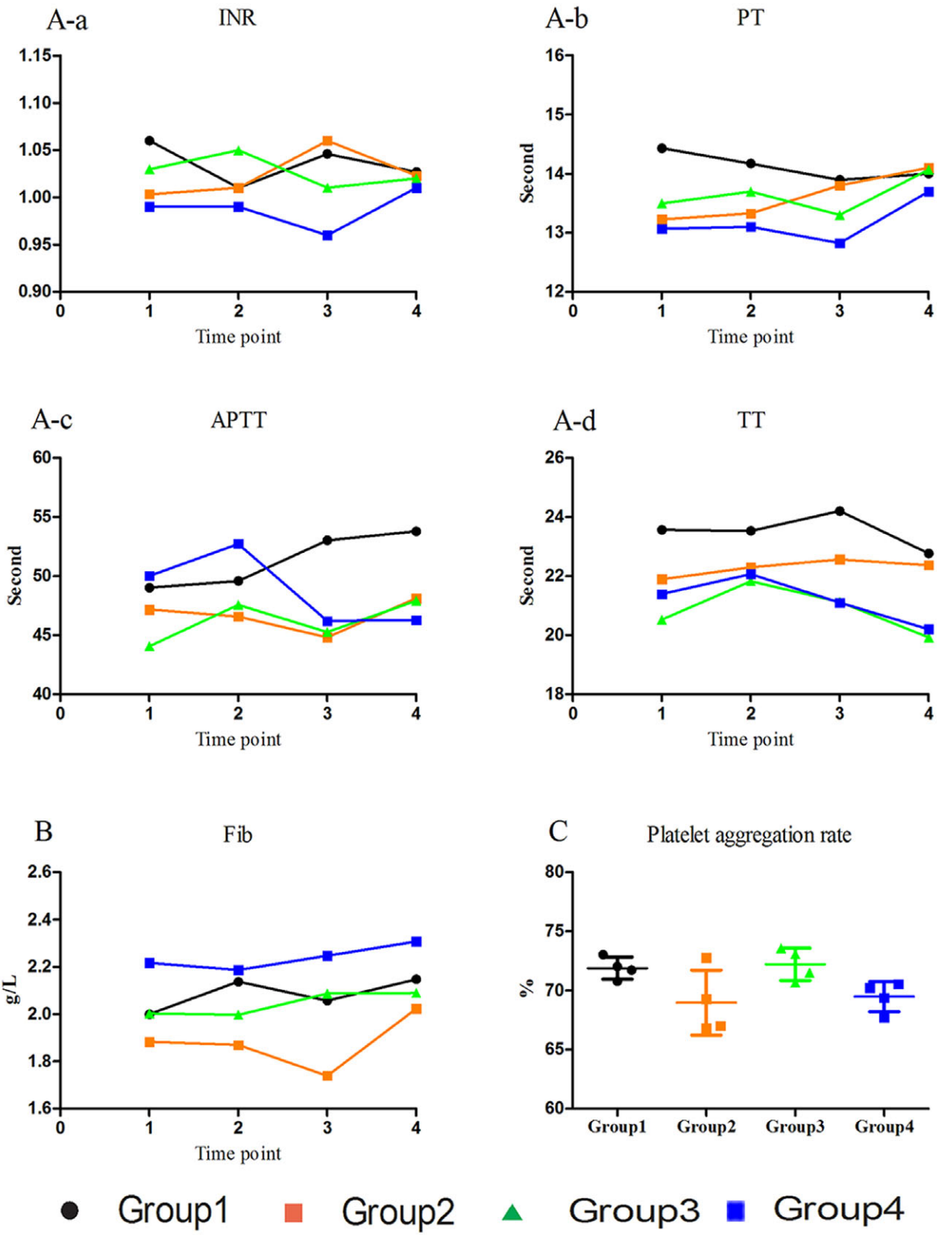

Figure 5. Coagulation parameters of (A) INR (A–a), PT (A–b), APTT (A–c), TT (A–d), (B) Fib, and (C) platelet aggregation rates.

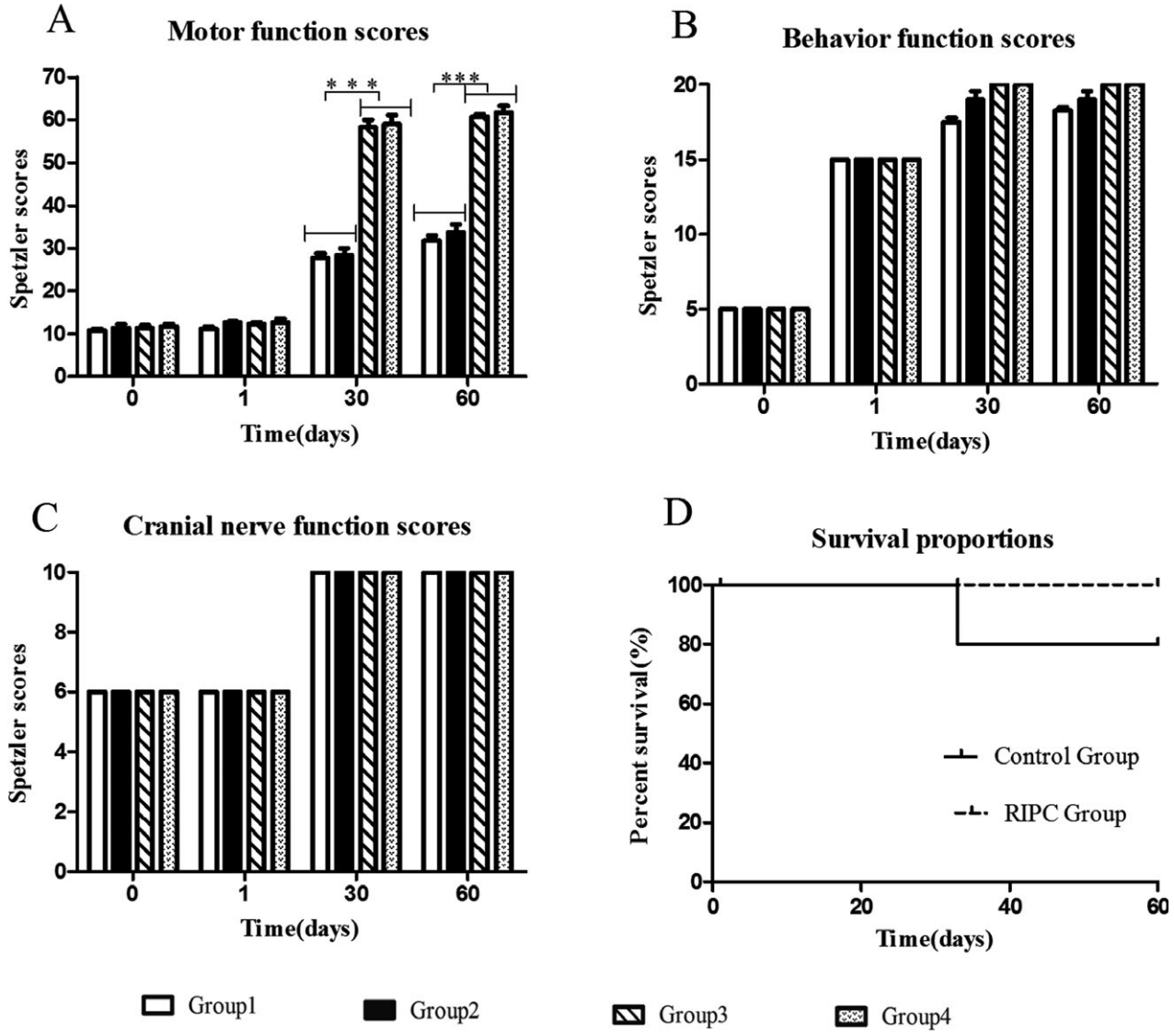

Figure 6. Bar graphs showing neurological function scores at 0, 1, 30, and 60 days after ischemic stroke, including motor function, behavior aspect (mental status), and cranial nerve function (A–C) (***$P < 0.001$ represents significant differences among groups); (D) the survival curve during 2-month observation.

improving neurological function, possibly via amelioration of stroke-related inflammation, endothelial injury, and serum parameters of cardiac dysfunction. Overall, two-limb RIPC with 5 min × 10 bouts of inflation/deflation may be considered as the optimal treatment regimen.

Safety of transient RIPC

In the present study, no obvious adverse events or intolerable discomfort were identified in response to short-term RIPC. Indeed, vital signs and skin integrity were not affected by the RIPC procedure. Neurological functional deficits and infarct volumes did not worsen after RIPC. Moreover, no hemorrhagic transformation occurred after RIPC. All monkeys subjected to RIPC were alive more than 2 months post-RIPC; whereas, one monkey out of the five monkeys died on the 33rd day following sham RIPC (directly related to stroke injury). Taken together, these findings indicate that short-term RIPC proves to be safe when performed during the hyperacute stage of ischemic stroke.

Effectiveness of transient RIPC

Neurological function spontaneously improved over the 2-month observation period. However, monkeys exposed

to RIPC displayed better neurological function, particularly in the two-limb and four-limb RIPC groups. These improvements occurred in the absence of any effects of RIPC on infarct volume. Poststroke prognosis is associated with a couple of factors, one of which is the inflammatory response. Inflammation during the acute phase of stroke is known to be detrimental to clinical outcomes.[30,31] hsCRP levels are expected to be higher in stroke than in non-stroke patients,[1] and further elevation in hsCRP levels is a risk factor for recurrent stroke and associated with poor functional outcome after stroke.[2] Previous studies have demonstrated that RIPC-mediated reductions in stroke risk and cerebral ischemic–reperfusion injury may be in relation to inhibition of inflammatory processes.[31–33] Consistent with these findings, our data also reveal that RIPC applied during the hyperacute stage of stroke can significantly reduce hsCRP at 5 min × 10 bouts of and 24 h after RIPC.

Endothelial injury is another crucial factor that correlates with increased risk of stroke and poor poststroke prognosis.[34,35] Using serum vWF as a marker of endothelial injury,[35–37] we demonstrate for the first time that stroke-induced elevation in serum vWF can be attenuated by transient RIPC, denoting that RIPC may ameliorate ischemic stroke-associated vascular endothelial injury. Hence, RIPC seems to be a promising therapeutic strategy to reduce stroke risk and improve functional outcome after stroke, possibly via attenuation of inflammation and endothelial injury.

Although a growing number of studies have investigated the effect of RIPC on reducing cardiac injury-related cardiac enzymes, research exploring the role of RIPC in stroke-related cardiac dysfunction, particularly during the hyperacute state after stroke (brain–heart syndrome), is lacking. Former literature demonstrated that myoglobin levels are higher in patients with severe stroke outcomes.[38,39] Furthermore, among cardiac enzymes, myoglobin levels at early clinical presentation possess a higher predictive value than other enzymes for stroke outcome.[38,39] In the present study, we found that RIPC reduced the levels of myoglobin, as well as CK and CK-MB. It is of great importance to note that short-term RIPC is able to decrease the level of abnormally elevated cardiac enzymes after acute stroke. We speculate that there are two potential underlying mechanisms accounting for the beneficial effects.[40] One is that RIPC may inhibit the sympathetic storm after acute stroke by ameliorating vascular inflammation and endothelial dysfunction, which result from overactivation of the renin–angiotensin–aldosterone system (RAAS). Another theory is that RIPC can help scavenge free radicals and attenuate oxidative stress induced by ischemic–reperfusion injury, thereby mitigating membrane damage and cardiac enzyme leakage. Given that, we propose that RIPC holds the potential of improving stroke-related cardiac complications.

Although several studies reported that RIPC might affect coagulation function, such as prolonging PT, and INR, as well as decreasing platelet aggregation rates in humans,[41,42] we did not document these effects in our stroke monkeys.

Optimization of RIPC strategy

At present, there is no standardized procedure for RIPC. Indeed, previously published literature underscored that the optimal regimen for RIPC-induced heart and brain protection varied, ranging from 1 to 12 bouts with single limb or double limbs.[43–45] The first ischemic tolerance phenomenon found in the brain showed that a single 2-min ischemia treatment conferred only partial protection, whereas two 2-min ischemia treatment exhibited complete protection against neuronal death.[46] Thus, the duration and interval of RIPC should be able to perturb cellular metabolism and stimulate protein synthesis as much as possible. The RIPC protocols used in our study included single-, two- and four-limb exposure with two training cycles (5 min × 5 bouts/cycle). With regard to the number of cycles, our results showed that two training cycles (5 min × 10 bouts) might be the best, as one cycle (5 min × 5 bouts) resulted in a mild inflammatory response indicated by an elevation of hsCRP (Fig. 4A–a). Moreover, two training RIPC cycles were superior to one cycle at decreasing biomarkers of endothelial injury.

In terms of the number of limbs transient RIPC performed on, our study also revealed that multi-limb RIPC, regardless of two- or four-limb, might be better than single-limb RIPC. Multi-limb RIPC produced more pronounced decreases in the levels of myoglobin, CK-MB, CK, hsCRP, and vWF, when compared to single-limb RIPC after two training cycles. However, two-limb RIPC was more prone to reduce CK than four-limb RIPC after two training cycles. In addition, two-limb RIPC is more practicable or reasonable in real clinical practice than four-limb RIPC in patients with acute stroke. Altogether, these results indicate that short-term two-limb RIPC with two 5-min ischemic–reperfusion cycles may be the optimal regimen for reducing stroke-related cardiac dysfunction, vascular endothelial injury, inflammation, and neurological deficits.

One limitation of this study is that we only examined the protective efficacy of RIPC after 5 and 10 bouts of RIPC. Thus, additional studies are warranted to gain a more comprehensive understanding of the impact of the number of bouts of inflation/deflation on RIPC-induced protection. Other limitations included the small number

of animals in each group and the lack of control groups used to measure the effect of RIPC on limb muscle injury in the absence of ischemic stroke. These limitations were partially due to economic issues and/or the difficulty in constructing the monkey stroke model. Regarding the study design, no cardiac function assessments were performed, and we will consider using more accurate assessing approaches such as electrocardiography, echocardiography and cardiac MRI in our next study. Last, but not least, we used young monkeys as the stroke model to explore the safety and efficacy of short-term RIC on brain–heart protection in acute stroke. As a matter of fact, brain–heart syndrome is one subtype of Takotsubo syndrome (TTS), which is characterized by a transient reversible left ventricular dysfunction, mimicking acute coronary syndrome. Clinically, stroke-related cardiac disorders are quite common in elderly, particularly male patients with preexisting cardiovascular risk factors. This could be explained by the theory that, the tolerance and adaptability to the sympathetic storm in this population are not strong enough. Nonetheless, an epidemiologic observation reported that TTS predominantly occurs in postmenopausal women other than elderly males.[47] In agreement with some previous literature demonstrating TTS in young individuals,[48,49] we also notice from our clinical practice that brain–heart syndrome can occur in young patients with severe stroke-induced brain injury. This may be supported by the evidence from our young stroke monkey model as well. Even so, we have to admit that using young monkeys as the stroke model is a limitation and future experimental or clinical studies enrolling subjects with different age, gender, and comorbid diseases are required.

Conclusions

In conclusion, this study suggests that short-term RIPC is safe and effective when performed during the hyperacute stage of ischemic stroke. RIPC improved neurological function 30–60 days after stroke in the absence of any effect on infarct volume. Two-limb RIPC in conjunction with two training cycles was more efficacious in reducing cardiac enzymes, vascular endothelial injury, and inflammatory responses than other treatment regimens. Given that, two-limb RIPC administered during the hyperacute stage of stroke appears to be a promising therapeutic strategy to reduce stroke-related cardiac dysfunction and neurological function deficits. So far, no unified approach for brain–heart syndrome has been established. It is of great importance that our study for the first time, shows that two-limb short-term RIPC may be a novel therapeutic method for attenuating acute cardiac dysfunction following stroke in non-human primates. This method has a tremendous value of clinical translation due to its noninvasive, inexpensive, convenient features. In the next step, our study group intends to enroll more patients with acute stroke-related cardiac disorder in attempt to further identify the safety and efficacy of short-term RIPC on improving clinical outcomes.

Author Contributions

Dr. Guo designed the study and drafted the manuscript; Dr. Zhou critical reviewed the manuscript; Dr. Wu, Dr. Ding, Dr. He, Dr. Duan, Dr. Shi, Dr. Yang acquired the data; Dr. Ji and Dr. Ding supervised and interpreted the study; Dr. Meng conceptualized, designed, analyzed and interpreted the study.

Conflict of Interests

No conflict of interests declared.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Basic data of all monkeys in four groups.

