ORIGINAL ARTICLE

# Limb Ischemic Preconditioning Reduces Heart and Lung Injury After an Open Heart Operation in Infants

**Zhou Wenwu · Zeng Debing · Chen Renwei ·
Liu Jian · Yang Guangxian · Liu Pingbo ·
Zhou Xinmin**

**Abstract** Open heart surgery supported by cardiopulmonary bypass is associated with heart and lung ischemia–reperfusion injury (IRI). Limb remote ischemic preconditioning (RIPC) reduces injury caused by ischemia–reperfusion in multiple distant organs. We conducted a prospective clinical trial (randomized and controlled) to test the feasibility and safety of limb RIPC, as well as its protective effects against myocardial and pulmonary IRI for infants undergoing repair of simple congenital heart defects. Infants undergoing repair of ventricular septal defects were enrolled in our study and randomly assigned to one of two treatment groups: limb RIPC or control. RIPC was induced twice (24 h and 1 h preoperatively) via three 5-min cycles of ischemia and reperfusion on the left upper arm using a blood pressure cuff. Lung compliance, respiratory index (RI), and cardiac inotropic score (IS) were calculated for each patient. Serum concentrations of the following factors were measured perioperatively: interleukin (IL)-6, IL-8, IL-10, and tumor necrosis factor (TNF)-α; lactate dehydrogenase (LDH), creatine kinase (CK), and its isoenzyme (CK-MB), and troponin I (TnI); malondialdehyde (MDA) and superoxide dismutase (SOD). The expression of heat shock protein 70 (HSP 70) in cardiomyocytes was analyzed by Western blot. Surgical outcomes, including limb movement and sensory function, were recorded in detail. Sixty infants weighting less than 7 kg were studied, with 30 patients in the RIPC group and 30 in the control group. Within 6 months of discharge from the hospital, no limb disability, sensory disturbance, or other surgical complications were found in any patient. Compared with the control group, patients in the RIPC group had higher Cs and Cd, along with lower RI and IS at various postoperative phases. At the beginning of the operation, serum concentrations of IL-6, IL-8, IL-10, TNF-α, LDH, CK, and TnI were higher in the RIPC group than the control group. Postoperatively, release of cytokines and leakage of heart enzymes were attenuated in the RIPC group; serum concentrations of cytokines and heart enzymes were lower in the RIPC group at some, but not all, postoperative time points. Furthermore, the RIPC group had lower coronary sinus venous concentrations of MDA and higher concentrations of SOD. Similarly, the expression of HSP 70 was upregulated in cardiomyocytes from the RIPC group. Limb RIPC can be applied safely and easily in infants, can attenuate systemic inflammatory response syndrome, and can increase systemic tolerance to IRI, imparting a protective effect against myocardial and pulmonary IRI. The expression of HSP 70 has an important role in the mechanism of action for RIPC.

**Keywords** Limb · Remote ischemic preconditioning · Infant · Congenital heart disease · Surgical repair

Z. Wenwu · Z. Debing · C. Renwei · L. Jian · Y. Guangxian · L. Pingbo
Department of Cardiothoracic Surgery, Hunan Children's Hospital, Changsha, Hunan, China
e-mail: acrophober@163.com

Z. Xinmin (✉)
Department of Cardiothoracic Surgery, The 2nd Xiangya Hospital, Center South University, Changsha, Hunan, China
e-mail: mark037100@163.com

Open heart surgery in infant results in a predictable ischemia–reperfusion injury (IRI) to multiple organs with a well-documented systemic inflammatory response syndrome (SIRS), which accounts for significant mortality and morbidity in the surgical outcomes of congenital heart disease [4]. Ischemic preconditioning (IPC) is an innate

protective mechanism that markedly reduces IRI in most human tissues. Remote ischemic preconditioning (RIPC), initially described by Pryzklenk et al. [15], protects target organs against sustained IRI through the application of nonlethal stress. More specifically, inducing transient ischemia to an organ distant from the target organ results in systemic and local tolerance to subsequent IRI.

Using a simple and noninvasive technique, the application of four 5-min cycles of limb ischemia and reperfusion with a blood pressure cuff, Cheung et al. recently conducted a randomized controlled trial to study the effects of limb RIPC in children undergoing repair of congenital heart defects [3]. As a result, they were first to demonstrate the myocardial protective effects of RIPC in the pediatric population. By means of a similar technique, Liu et al. reported that limb RIPC prior to the aorta clamping can significantly reduce postoperative ventricular arrhythmias, leakage of myocardial enzymes, and injury to the myocardial ultrastructure in adult rheumatic heart disease patients undergoing cardiac valve replacement [11]. Likewise, by wrapping a blood pressure cuff around one arm to induce limb RIPC, we previously demonstrated improved endothelial function in the contralateral arm of infants [18]. However, we failed to detect the heart protective effects of limb RIPC in adult patients undergoing mitral valve replacement (data unpublished). In order to further investigate the protective utility of RIPC and to explore its underlying mechanisms, we designed this randomized clinical trial. Based on previous experience, we planned to induce limb RIPC twice (24 and 1 h before surgery) to test whether RIPC would provide early and/or late protection against heart and lung IRI in infants undergoing repair of simple ventricular septal defects (VSDs).

## Methods

The study protocol was approved by the ethics committee of the Hunan Children's Hospital. Written informed consent was obtained from the infants' custodians before study inclusion. The trial was monitored by an independent data and safety monitoring board. Staff involved in clinical care and members of the study group obtaining functional data were blinded to randomization for the period of data acquisition and analysis. Group allocation was not revealed until the final statistical analysis.

Sixty infants undergoing surgical repair of VSDs were enrolled in this study and divided into the RIPC group or the control group at random (30 infants for each group). The inclusion criteria were as follows: (1) diagnosis of simple VSD with mild pulmonary hypertension or without any pulmonary hypertension; (2) weight less than 7 kg; (3)

prior to the operation, pneumonia and heart failure were well controlled and patients were water-electrolyte balanced and without acidosis; (4) no history of limb trauma; and (5) no other systemic diseases such as a chromosomal defect, airway and parenchymal lung disease, immunodeficiency, or blood disorders.

### Limb RIPC Protocol

Infants were sedated by oral administration of chloral hydrate (0.01 g/kg) 5 min before preconditioning. The limb RIPC protocol was carried out as previously described [18] with little modification. Briefly, a blood pressure cuff (with a width of 4 cm) was wrapped around the left upper arm approximately 1 cm proximal to the elbow joint. Next, the cuff was inflated to a pressure of 240 mm Hg, maintaining ischemia for 5 min, and then deflated to allow reperfusion for 5 min. The inflation–deflation process was repeated for two additional cycles. Limb RIPC was induced twice for each infant in the RIPC group: at 24 and at 1 h prior to the start of the corrective operation. In contrast, infants in the control group were not pretreated.

### Surgical Techniques and Postoperative Managements

All children underwent surgical repairs using standard cardiopulmonary bypass (CPB) techniques with cardioplegic arrest. No patients required direct measurement of pulmonary artery pressure during the operations. Modified ultrafiltration was carried out in all infants. The duration of cardiopulmonary bypass and aortic cross-clamp time were recorded.

After surgery, ventilation was continued in the intensive care unit with a volume-cycled respirator that automatically records and stores ventilator parameters. Each patient's ventilator support time and duration of intensive care unit (ICU) stay were also recorded.

### Data Acquisition and Sample Preparation

#### Lung Function

Lung function data was obtained at baseline prior to sternotomy and then at 2, 4, 12, and 24 h after closure of the sternum incision. The tidal volume (VT), fraction of inspired oxygen ($FiO_2$), peak pressure of airway ($P_{max}$), mean pressure of airway ($P_{mean}$), and positive end expiratory pressure (PEEP) were recorded and stored as a computer file for later analysis. Data for arterial blood gas analysis were also recorded. The following equations were used to determine lung function:

Static lung compliance (Cs) = VT/($P_{mean}$ − PEEP);
Dynamic lung compliance (Cd) = VT/($P_{max}$ − PEEP);
Respiratory index (RI) = $P$(A − $aDO_2$)/$PaO_2$,

where P(A − $aDO_2$) = $713FiO_2$ − $PaCO_2$ − $PaO_2$.

### Heart Function

Heart function was evaluated before the operation by left ventricular fractional shortening (LVFS) and left ventricular ejection fraction (LVEF) derived from echocardiography. Two hours, 4 h, 12 h, and 24 h after the operation, heart function was assessed by inotropic support requirement rather than data from echocardiography. The inotropic support requirement was quantified by calculating the inotropic score from the dosage of inotropic drugs (IS = dopamine × 1 + dobutamine × 1 + amrinone × 1 + milrinone × 10 + adrenalin × 100 + isoprenaline × 100).

### Inflammatory Mediators and Enzymes

Five minutes before the sternotomy and 2, 4, 12, and 24 h after surgery, venous blood was sampled from the jugular venous line for measurement of the cytokines interleukin (IL)-6, IL-8, and IL-10, tumor necrosis factor (TNF)-α, lactate dehydrogenase (LDH), creatine kinase (CK) and its isoenzyme (CK-MB), and troponin I (TnI). Venous blood from the coronary sinus was also obtained twice (once before the administration of aortic cross-clamp and once 5 min after clamp removal; once cardiopulmonary bypass began, hypothermic temperatures usually resulted in low heart rates, which made it possible to obtain venous blood from the coronary sinus via an urethral catheter placed through a small incision in the right atrium before the heart arrested) for measurement of malondialdehyde (MDA) and superoxide dismutase (SOD). Samples were collected and immediately centrifuged. The resulting plasma and serum were frozen at –70°C for later analysis by commercially available kits. To rule out the influence of hemodilution, all values were normalized to real-time hematocrit (HCT) by the following equation: final value = measured value × preoperative HCT/real-time HCT.

### Heat Shock Protein 70

The right atrial appendages were excised routinely for superior vena cava cannulation. These appendages were frozen at –70°C for later Western blot analysis for HSP 70 content.

Samples from cardiomyocytes were homogenized in lysis buffer. The total protein concentration in each sample was determined by the Bradford method. Forty micrograms of total protein from each sample was separated on a SDS-

polyacrylamide gel, transferred to a PVDF membrane, and then blocked in TBST (5% BSA) for 3 h. Primary antibody (mouse anti-human HSP 70 monoclonal antibody) and secondary antibody (horseradish peroxidase labeling) were incubated at room temperature for 2 and 1 h, respectively. After chemiluminescence detection, film images were scanned by a gel imaging system (Bio-RAD GelDOC 2000) and analyzed using Bio-Rad Quantity One software (version 4.03). HSP 70 protein concentration was quantified by densitometry and reported as a gray scale score. β-Actin was used as a control.

### Statistical Analysis

Data were expressed as mean ± SE. Statistical Product and Service Solutions 13.0 software (SPSS Institute) was used for all analyses. An independent-sample $t$-test was performed to check for differences in each variable at the same time points between the two groups. The critical alpha level for these analyses was set at $p < 0.05$.

## Results

### Patients and the Surgical Outcome

Sixty infants were enrolled in this study (30 in the RIPC group and 30 in the control group). The age and weight of the RIPC and control groups ranged from 81 to 270 days and from 4.3 to 7.0 kilograms, respectively. The mean age and weight were not significantly different between the two study groups. Limb RIPC procedures were carried out uneventfully in all patients in the RIPC group (Chinese infants are more sensitive to chloral hydrate and more tolerant to pain caused by the blood pressure cuff). Forty patients (21 in the RIPC and 19 in the control group) had mild pulmonary hypertension. The mean bypass and cross-clamp times were not significantly different. Concomitant heart lesions presented as atrial septal defects in 12 patients and mild right ventricular outflow tract obstructions presented in 4 patients. The septal defects were closed by patches with running sutures (in 22 infants) or interrupted sutures (in 38 infants). All operations were performed uneventfully and no patients were left with hemodynamically significant residual lesions or atrioventricular blocks. The ventilator support time and duration of in intensive care unit stay for the RIPC and control groups were not significantly different (Table 1). No infants died within 6 months after discharging from the hospital. No signs of limb pain, functional disability, or sensory disability were observed postoperatively. In addition, ultrasound findings of upper arm vessels were normal in both groups. Follow-

**Table 1** Patient and outcome data

|  | Control group | RIPC group |
|---|---|---|
| Gender (male/female) | 16/14 | 18/12 |
| Age (days) | 154.13 ± 55.82 | 160.83 ± 58.39 |
| Weight (kg) | 5.64 ± 0.92 | 5.75 ± 1.03 |
| VSD diameter (mm) | 10.40 ± 3.28 | 10.67 ± 2.66 |
| Pulmonary hypertension | 21 infants | 19 infants |
| Bypass time (min) | 33.77 ± 9.62 | 34.47 ± 10.88 |
| Aortic clamp time (min) | 24.17 ± 8.21 | 24.13 ± 9.83 |
| Ventilator support time (h) | 61.13 ± 30.14 | 66.23 ± 51.89 |
| Duration of ICU (days) | 5.47 ± 1.53 | 5.10 ± 2.43 |

*Note*: All variables compared between groups were not significantly different

up exams were performed on all patients 6 months after hospital discharge. In every case, radial artery pulse was normal and Allen's test was negative.

## Lung and Heart Functions, Enzymes and Inflammatory Mediators

Data on lung compliance, respiratory index, postoperative inotropic score, leakage of myocardial enzymes, and release of inflammatory cytokines are shown in Table 2.

Static lung compliance (Cs), dynamic lung compliance (Cd), and respiratory index (RI) were not significantly different between treatment groups at the preoperative baseline measurement. Postoperative differences in these measures

**Table 2** Data of lung and heart function, inflammatory mediators

|  |  | Baseline | 2 h postop | 4 h postop | 12 h postop | 24 h postop |
|---|---|---|---|---|---|---|
| Cs | Contr | 9.67 ± 3.44 | 7.06 ± 1.64 | 5.83 ± 1.04* | 6.34 ± 1.70* | 9.16 ± 2.95* |
|  | RIPC | 10.44 ± 4.41 | 7.40 ± 2.37 | 6.57 ± 1.68* | 8.55 ± 2.23* | 11.22 ± 4.16* |
| Cd | Contr | 3.97 ± 0.82 | 3.36 ± 0.73* | 2.84 ± 0.51* | 3.24 ± 0.85* | 4.06 ± 1.23 |
|  | RIPC | 4.25 ± 1.10 | 4.00 ± 0.92* | 3.28 ± 0.80* | 4.06 ± 0.92* | 4.27 ± 1.17 |
| RI | Contr | 1.36 ± 0.20 | 2.82 ± 0.38* | 3.71 ± 0.47* | 3.56 ± 0.72* | 2.90 ± 0.87* |
|  | RIPC | 1.42 ± 0.17 | 2.50 ± 0.45* | 3.36 ± 0.60* | 3.31 ± 0.91* | 2.25 ± 1.00* |
| LVEF | Contr | 64.80 ± 6.21 | None |  |  |  |
|  | RIPC | 65.27 ± 5.85 |  |  |  |  |
| LVFS | Contr | 38.43 ± 5.19 | None |  |  |  |
|  | RIPC | 37.53 ± 4.27 |  |  |  |  |
| IS | Contr | None | 15.87 ± 3.10 | 15.87 ± 4.21* | 10.67 ± 2.95* | 6.87 ± 1.74 |
|  | RIPC |  | 18.47 ± 20.00 | 12.10 ± 4.63* | 8.63 ± 3.02* | 6.47 ± 1.68 |
| LDH | Contr | 164.83 ± 43.34* | 509.17 ± 104.81 | 519.49 ± 94.71* | 477.01 ± 110.00* | 418.25 ± 98.38* |
|  | RIPC | 198.19 ± 46.47* | 522.20 ± 118.72 | 447.83 ± 120.98* | 377.27 ± 76.00* | 294.12 ± 79.69* |
| CK | Contr | 41.75 ± 12.47* | 278.84 ± 63.79 | 405.55 ± 84.03 | 473.62 ± 78.94* | 409.65 ± 98.90 |
|  | RIPC | 72.30 ± 11.76* | 286.67 ± 51.81 | 415.74 ± 86.67 | 412.61 ± 75.61* | 388.73 ± 94.46 |
| CK-MB | Contr | 9.46 ± 4.33* | 57.25 ± 17.28* | 75.68 ± 16.04* | 75.12 ± 17.61* | 53.52 ± 11.13* |
|  | RIPC | 15.48 ± 4.32* | 48.84 ± 15.02* | 53.42 ± 18.05* | 56.09 ± 17.67* | 46.13 ± 15.41* |
| TnI | Contr | 0.64 ± 0.30* | 2.35 ± 0.43* | 2.41 ± 0.32* | 2.00 ± 0.29 | 1.65 ± 0.41 |
|  | RIPC | 1.10 ± 0.30* | 2.05 ± 0.41* | 2.10 ± 0.32* | 1.90 ± 0.35 | 1.70 ± 0.39 |
| IL-6 | Contr | 1.44 ± 0.56* | 34.93 ± 14.29 | 138.79 ± 34.30* | 90.05 ± 31.68 | 29.03 ± 6.55 |
|  | RIPC | 2.92 ± 1.54* | 30.81 ± 13.28 | 105.90 ± 35.49* | 87.27 ± 23.54 | 30.28 ± 7.15 |
| IL-8 | Contr | 1.87 ± 0.90* | 18.37 ± 4.50 | 23.46 ± 5.48* | 27.80 ± 7.86* | 12.35 ± 6.64 |
|  | RIPC | 3.96 ± 1.87* | 17.57 ± 6.01 | 19.42 ± 5.87* | 17.58 ± 9.11* | 10.69 ± 6.87 |
| IL-10 | Contr | 3.31 ± 1.50* | 31.21 ± 12.45 | 81.52 ± 21.43 | 35.97 ± 12.70 | 8.12 ± 2.59* |
|  | RIPC | 5.56 ± 1.98* | 32.31 ± 10.61 | 72.03 ± 24.26 | 38.94 ± 15.76 | 10.73 ± 2.97* |
| TNF-α | Contr | 1.51 ± 0.50* | 9.47 ± 2.61 | 10.03 ± 2.42* | 7.04 ± 2.16* | 3.62 ± 0.98* |
|  | RIPC | 2.15 ± 0.56* | 10.07 ± 2.75 | 8.67 ± 2.66* | 5.40 ± 1.72* | 2.57 ± 0.58* |

*Note*: Cs and Cd measured in ml/cm/H$_2$O; LVEF and LVFS in percent; IS measured in μg/kg/min; LDH, CK, and CK-MB measured in U/l; TnI measured in μg/l; IL-6, IL-8, IL-10, and TNF-α measured in pg/ml

* $p < 0.05$, between groups

between treatment groups might highlight protection from pulmonary ischemia–reperfusion injury. Furthermore, changes in protective effect over time might reflect the natural progression of early-phase and/or late-phase RIPC protection. Shortly after the operation, Cs and Cd values decreased and RI values increased across both treatment groups. Compared to the control group, the RIPC group had significantly higher Cs at 4, 12, and 24 h ($5.83 \pm 1.04$ ml/cm/$H_2O$ vs. $6.57 \pm 1.68$ ml/cm/$H_2O$, $p = 0.044$; $6.34 \pm 1.70$ ml/cm/$H_2O$ vs. $8.55 \pm 2.23$ ml/cm/$H_2O$, $p = 0.000$; and $9.16 \pm 2.95$ ml/cm/$H_2O$ vs. $11.22 \pm 4.16$ ml/cm/$H_2O$, $p = 0.040$, respectively) and Cd at 2, 4, and 12 h ($3.36 \pm 0.73$ ml/cm/$H_2O$ vs. $4.00 \pm 0.92$ ml/cm/$H_2O$, $p = 0.004$; $2.84 \pm 0.51$ ml/cm/$H_2O$ vs. $3.28 \pm 0.80$ ml/cm/$H_2O$, $p = 0.015$; and $4.06 \pm 1.23$ ml/cm/$H_2O$ vs. $4.27 \pm 1.17$ ml/cm/$H_2O$, $p = 0.001$, respectively). The RIPC group also had lower values of RI at all postoperative time points ($2.82 \pm 0.38$ vs. $2.50 \pm 0.45$, $p = 0.005$; $3.71 \pm 0.47$ vs. $3.36 \pm 0.60$, $p = 0.017$; $3.56 \pm 0.72$ vs. $3.31 \pm 0.91$, $p = 0.041$; and $2.90 \pm 0.87$ vs. $2.25 \pm 1.00$, $p = 0.012$, respectively).

Similar to lung function, preoperative heart function was not significantly different between groups as assessed by LVEF and LVFS. However, infants in the control group presented greater inotropic need than those in the RIPC group, at 4 h and 12 h after surgery ($15.87 \pm 4.21$ µg/kg/min vs. $12.10 \pm 4.63$ µg/kg/min, $p = 0.002$; and $10.67 \pm 2.95$ µg/kg/min vs. $8.63 \pm 3.02$ µg/kg/min, $p = 0.011$, respectively). Instead of LVEF and LVFS, heart function was assessed by inotropic score and serum myocardial enzyme concentration at postoperative time points.

Compared with the control group, the RIPC group had higher serum concentrations of myocardial enzymes LDH, CK, CK-MB, and TnI at the baseline measurement ($p = 0.006$, $0.000$, $0.000$, $0.000$, respectively). Postoperatively, the serum levels all of myocardial enzymes increased in both treatment groups. However, in comparison with the control group, the RIPC group had lower LDH at 4, 12, and 24 h ($p = 0.013$, $0.000$, $0.000$, respectively), lower CK at 12 h ($p = 0.003$), lower CK-MB at all time points ($p = 0.049$, $0.000$, $0.000$, $0.000$, respectively), as well as lower TnI at 2 h and 4 h ($p = 0.007$, $0.000$, respectively) (Table 2).

Baseline serum levels of inflammatory mediators, including IL-6, IL-8, IL-10, and TNF-α, were higher in the RIPC group than in the control group (Fig. 1). After the operation, blood concentrations of cytokines increased markedly in both groups. Yet, compared with patients in the RIPC group, infants in the control group had higher levels of IL-6 at 4 h ($138.79 \pm 34.30$ pg/ml vs. $105.90 \pm 35.49$ pg/ml, $p = 0.001$), higher levels of IL-8 at 4 and 12 h ($23.46 \pm 5.48$ pg/ml vs. $19.42 \pm 5.87$ pg/ml, $p = 0.003$; and $2.41 \pm 0.32$ pg/ml vs. $2.10 \pm 0.32$ pg/ml, $p =$
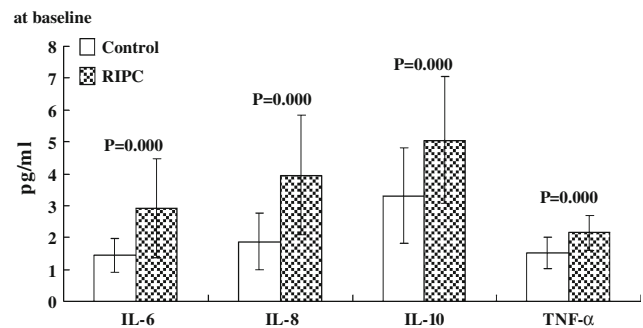


**Fig. 1** At baseline, IL-6, IL-8, IL-10, and TNF-α were higher in the RIPC group

$0.000$, respectively) and lower levels of IL-10 at 24 h ($8.12 \pm 2.59$ pg/ml vs. $10.73 \pm 2.97$ pg/ml, $p = 0.001$); TNF-α was also higher in the control group at 4, 12, and 24 h postoperatively ($10.03 \pm 2.42$ pg/ml vs. $8.67 \pm 2.66$ pg/ml, $p = 0.042$; $7.04 \pm 2.16$ pg/ml vs. $5.40 \pm 1.72$ pg/ml, $p = 0.002$; and $3.62 \pm 0.98$ pg/ml vs. $2.57 \pm 0.58$ pg/ml, $p = 0.000$, respectively).

## MDA and SOD

Before administration of the aortic cross-clamp, the coronary sinus venous blood concentration of MDA and SOD was not significantly different between groups. Five minutes after removal of the aortic cross-clamp, however, MDA was lower ($4.50 \pm 1.68$ nmol/mgpro vs. $3.61 \pm 1.59$ nmol/mgpro, $p = 0.050$) and SOD was higher ($53.16 \pm 11.91$ nmol/mgpro vs. $75.22 \pm 15.22$ nmol/mgpro, $p = 0.000$) in the RIPC group compared with the control group (Table 3).

## HSP 70

Western blot analysis showed that cardiomyocytes from the RIPC group had higher expression of HSP 70 protein compared with the control group ($31.85 \pm 12.20$ vs. $41.41 \pm 13.49$, $p = 0.006$).

**Table 3** Coronary sinus venous blood concentration of MDA and SOD

|  |  | MDA (nmol/mgpro) | SOD (nU/mgpro) |
|---|---|---|---|
| Before clamp | Control | $3.77 \pm 1.54$ | $71.82 \pm 11.66$ |
|  | RIPC | $3.68 \pm 1.52$ | $76.75 \pm 14.23$ |
| After clamp | Control | $4.50 \pm 1.68*$ | $53.16 \pm 11.91*$ |
|  | RIPC | $3.61 \pm 1.59*$ | $75.22 \pm 15.22*$ |

*Note*: In the RIPC group, 5 min after remove the clamp, MDA was lower but SOD was higher, * $p = 0.050$, $0.000$, respectively

## Discussion

Ischemia–reperfusion injury seems almost inevitable during the surgical repair of congenital heart defects, which require cardioplegic arrest and a temporary block of cardiomyocyte perfusion. In turn, these procedures result in a measurable degree of left ventricular dysfunction even after the repair of "simple" congenital defects [2]. Direct ischemic preconditioning and RIPC aimed to reduce the adverse effects of IRI after cardiac surgery have been investigated by many researchers, both in the clinical setting [1] and in experimental animal models [7]. The existence of early-phase protection, as well as durable "second-window" protection lasting from the early phase, has been confirmed by landmark studies [4]. We hypothesized that early-phase and second-window RIPC can combine to achieve greater protection from myocardial and pulmonary IRI than a single protocol, in the early postoperative time frame. In this clinical trial, we induced left upper arm RIPC at 24 and 1 h before surgery in infants, to reinforce the protective effects of RIPC. Presumably, the timing of our protocol also ensures that early-phase and second-window protection should overlap. In contrast to direct local IPC, limb RIPC is a noninvasive technique with the advantages of easy application and a lack of ethical concerns. Furthermore, the effects of limb RIPC are relatively benign; no signs of myocardial dysfunction, risk of arrhythmia, low cardiac output, or secondary organ injury caused by RIPC were found during the entire trial. One can confidently infer the safety of RIPC from data on limb function and sensory disability outcomes and from short-term surgical outcomes.

Our data indicate that 5 min before sternotomy (at the baseline measurement), the release of pro-inflammatory cytokines and the leakage of heart enzymes were significantly higher in the RIPC group than in the control group. Most reports using a single treatment of direct IPC [8] or limb RIPC [3, 17] immediately before sustained IRI did not show a significant difference in the level of cytokines or heart enzymes during the early phase of reperfusion. Therefore, we speculate that the increased serum cytokines and enzymes might be primarily associated with late-phase (second-window) protection, induced by the first RIPC treatment given 24 h prior to surgery. Serum levels of pro-inflammatory mediators were lower in the RIPC group, whereas levels of anti-inflammatory mediator IL-10 were higher at various postoperative time points, which suggests that the inflammatory cascade was attenuated by the overlapping effects of early- and late-phase protection from two rounds of preconditioning. Additionally, leakage of LDH, CK, CK-MB, and TnI were also inhibited to varying degrees in the RIPC group at several postoperative time points, compared to the control group. These results also suggest that myocardial IRI was attenuated by the "nonlocal" effect of two rounds of "remote" ischemic preconditioning. Notably, limb RIPC also reduced oxygen free-radical formation in coronary sinus venous blood, as evidenced by increased concentrations of SOD and decreased concentrations of the lipid peroxidation product MDA. Consequently, the control group's higher inotropic score at 4 h and 12 h can be interpreted as reflecting a greater degree of myocardial dysfunction.

During CPB and cardioplegic arrest, the lungs have virtually no circulation except for the limited perfusion provided by the bronchial arteries. As a result, the temperature of the lungs is not substantially reduced during hypothermic CPB; hence, lung tissue undergoes normothermic ischemia and is subjected to IRI. This CPB-induced lung IRI is mainly mediated by neutrophil activation, leading to the release of proteases and oxygen radical species [14]. Therefore, attenuation of the inflammatory reaction cascade and a reduction in oxygen free-radical levels should not only afford the heart protection but also protect the lungs against IRI. In the study that first applied limb RIPC in children, Cheung et al. [3] demonstrated that CPB-induced SIRS was attenuated partially as shown by the increased variance of IL-10 and a reduction in the levels of TNF-α in the RIPC group. Equally important, the authors anticipated that further repression of the inflammatory response to CPB might occur if the RIPC stimulus were applied 1 day before surgery. In our study, we designed our RIPC treatments (24 and 1 h preoperatively) to coincide with the conjecture mentioned here. In addition to the attenuated inflammatory reaction cascade and the inhibited leakage of heart enzymes, we also found that values of Cs and Cd were lower and RI was higher in the control group, suggesting that limb RIPC enhances lung preservation. The current data indicating better cardiopulmonary protection can be explained by the overlap of the two windows of protection derived from the two limb RIPC treatments.

The protection from RIPC in the early phase of reperfusion is triggered by the release of several mediators (including adenosine [10] and bradykinin [16]) and is dependent on the activation of complex second-messenger systems. Early-phase protection is protein synthesis independent and continues into a later phase of protection, which is protein synthesis dependent. Recently, an early and late phase of remote protection was demonstrated in human endothelium, with early-phase protection being activated immediately after RIPC and lasting for 4 h. A delayed phase of protection begins at 24 h and lasts for 48 h; notably, this phase is associated with protein expression in the vascular endothelium [12]. HSP's are members of highly conserved protein families and function as intracellular molecular chaperones that assist other proteins in the folding, transport, and assembly into complexes [6]. In our study, approximately 5 min before the

aortic cross-clamp was placed, a small mass of right atrial appendage was harvested and then analyzed by Western blot to check the expression of HSP 70. It was noted that even before the heart sustained IRI, the expression of HSP 70 had already increased in the RIPC group. It should be pointed out that HSP 70 upregulation is associated with the synthesis of mechanistic proteins, including inducible nitric oxide synthase and mitochondrial $K_{ATP}$ channels, which mainly occurs after translocation of cytoplasmic nuclear factors to the nucleus [9, 13]. Increased HSP 70 in the myocardial samples from the RIPC group suggests that HSP expression has an important role in the mechanism of action for late-phase RIPC protection.

Despite significantly greater heart and lung injury in the control group shown in our data, there were no differences in indexes of surgical complications and outcomes such as morbidity and mortality. Because we purposely chose infants undergoing surgical repair of simple congenital heart disease, the aortic cross-clamp times and the operating times were relatively short. Accordingly, the morbidity and mortality were expected to be extremely low [5], even in infants weighing less than 7 kg, who are treated with routine CPB and convenient postoperative management protocols. Hence, the lack of a difference in surgical outcomes is not surprising. Limb RIPC might provide more crucial and quantifiable protection against multiorgan IRI in CPB patients with complex heart disease or in patients with concomitant systemic disease. However, additional studies are clearly required to assess this hypothesis.

Another shortcoming of this study is that we did not enroll infants who underwent limb RIPC only one time either 24 or 1 h preoperatively. Therefore, we did not compare the protective effects of double RICP to single RIPC. Additionally, it is impossible to determine the optimal timing for administration of ischemic preconditioning stimuli from our current study. In the future, it will be important to characterize the time course of protection from RIPC in greater detail and, in particular, to establish whether there are two separate phases of protection.

Overall, we have demonstrated the myocardial and pulmonary protective effects of limb RIPC using a simple and noninvasive technique, which can be performed safely and easily in infants without any clear ethical concerns. The risk-to-benefit ratio of this therapy is so exceptional that a large-scale clinical trial should be established at multiple research centers, in order to optimize the time course and magnitude of RICP stimuli as well as to investigate additional benefits that might last longer than 24 h.

## References

1. Ali ZA, Callaghan CJ, Lim E, Ali AA, Nouraei SA, Akthar AM, Boyle JR, Varty K, Kharbanda RK, Dutka DP, Gaunt ME (2007) Remote ischemic preconditioning reduces myocardial and renal injury after elective abdominal aortic aneurysm repair: a randomized controlled trial. Circulation 116(11S):I98–I105
2. Chaturvedi RR, Lincoln C, Gothard JW, Scallan MH, White PA, Redington AN, Shore DF (1998) Left ventricular dysfunction after open repair of simple congenital heart defects in infants and children: quantitation with the use of a conductance catheter immediately after bypass. J Thorac Cardiovasc Surg 115(1):77–83
3. Cheung MMH, Kharbanda RK, Konstantinov IE, Shimizu M, Frndova H, Li J, Holtby HM, Cox PN, Smallhorn JF, Van Arsdell GS, Redington AN (2006) Randomized controlled trial of the effects of remote ischemic preconditioning on children undergoing cardiac surgery: first clinical application in humans. J Am Coll Cardiol 47(11):2277–2282
4. Kanoria S, Jalan R, Seifalian AM, Williams R, Davidson BR (2007) Protocols and mechanisms for remote ischemic preconditioning: a novel method for reducing ischemia reperfusion injury. Transplantation 84(4):445–458
5. Kanter KR (2007) Management of infants with coarctation and ventricular septal defect. Semin Thorac Cardiovasc Surg 19(3):264–268
6. Kodiha M, Chu A, Lazrak O, Stochaj U (2005) Stress inhibits the nucleocytoplsmic shuttling of heat shock protein hsc70. Am J Physiol Cell Physiol 289(4):1034–1041
7. Konstantinov IE, Li J, Cheung MMH, Shimizu M, Kharbanda RK, Redington AN (2005) Remote ischemic preconditioning of the recipient reduces myocardial ischemia–reperfusion injury of the donor heart via a Katpchannel-dependent mechanism. Transplantation 79(12):1691–1695
8. Leesar MA, Stoddard MF, Xuan YT, Tang XL, Bolli R (2003) Nonelectro-cardiographic evidence that both ischemic preconditioning and adenosine preconditioning exist in humans. J Am Coll Cardiol 42(3):437–445
9. Li G, Labruto F, Sirsjö A, Chen F, Vaage J, Valen G (2004) Myocardial protection by remote preconditioning: the role of nuclear factor kappa-B p105 and inducible nitric oxide synthase. Eur J Cardiothorac Surg 26(5):968–973
10. Liem DA, te Lintel Hekkert M, Manintveld OC, Boomsma F, Verdouw PD, Duncker DJ (2005) Myocardium tolerant to an adenosine-dependent ischemic preconditioning stimulus can still be protected by stimuli that employ alternative signaling pathways. Am J Physiol Heart Circ Physiol 288(3):H1165–H1172
11. Liu XJ, Yi SY, Liao B, Deng MB, Chen SJ, Wang F (2007) Clinical study of limb ischemic preconditioning on myocardial ischemia–reperfusion injury during open heart surgery. Chin J Cardiovasc Rehabil Med 16(4):347–349 (in Chinese)
12. Loukogeorgakis SP, Panagiotidou AT, Broadhead MW, Donald A, Deanfield JE, MacAllister RJ (2005) Remote ischemic preconditioning provides early and late protection against endothelial ischemia–reperfusion injury in humans: role of the autonomic nervous system. J Am Coll Cardiol 46(3):450–456
13. Mabanta L, Valane P, Bourne J, Frame MD (2006) Initiation of remote microvascular preconditioning requires K(ATP) channel activity. Am J Physiol Heart Circ Physiol 290(1):H264–H271
14. Naidu BV, Woolley SM, Farivar AS, Thomas R, Fraga CH, Goss CH, Mulligan MS (2004) Early tumor necrosis factor-alpha

release from the pulmonary macrophage in lung ischemia–reperfusion injury. J Thorac Cardiovasc Surg 127(5):1502–1508

15. Przyklenk K, Bauer B, Ovize M, Kloner RA, Whittaker P (1993) Regional ischemic 'preconditioning' protects remote virgin myocardium from subsequent sustained coronary occlusion. Circulation 87(3):893–899

16. Schoemaker RG, van Heijningen CL (2000) Bradykinin mediates cardiac preconditioning at a distance. Am J Physiol Heart Circ Physiol 278(5):H1571–H1576

17. Sun ZD, Chi YF, Yang TN, Hou WM, Niu ZZ, Tang ZL (2004) Experimental study of immature cardioprotection and mechanism with double limbs ischemic preconditioning in neonatal rabbits. Chin J Exp Surg 21(3):334–335 (in Chinese)

18. Zhou WW, Chen WJ, Gao JP, Liu PB, Li XY (2008) The effects of remote ischemic preconditioning of endothelia function in infants. J Clin Pediatr Surg 7(5):18–20 (in Chinese)