



Cardiac protective effects of remote ischaemic preconditioning in children undergoing tetralogy of fallot repair surgery: a randomized controlled trial

Qingping Wu^{1†}, Tingting Wang^{1†}, Shiqiang Chen^{1†}, Qianjun Zhou¹, Haobo Li^{2,3}, Na Hu¹, Yinglu Feng¹, Nianguo Dong^{4*}, Shanglong Yao^{1*}, and Zhengyuan Xia^{2,3*}

¹Department of Anaesthesiology, Institute of Anaesthesiology and Critical Care Medicine, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1277 Jiefang Avenue, Wuhan 430022, China; ²State Key Laboratory of Pharmaceutical Biotechnology, University of Hong Kong, 21 Sassoon Road, Hong Kong, China; ³Department of Anaesthesiology, University of Hong Kong, 102 Pokfulam Road, Hong Kong SAR, China; and ⁴Department of Cardiovascular Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1277 Jiefang Avenue, Wuhan 430022, China

Received 19 May 2016; revised 12 October 2016; editorial decision 12 January 2017; accepted 17 January 2017; online publish-ahead-of-print 18 February 2017

Aims

Remote ischaemic preconditioning (RIPC) by inducing brief ischaemia in distant tissues protects the heart against myocardial ischaemia-reperfusion injury (IRI) in children undergoing open-heart surgery, although its effectiveness in adults with comorbidities is controversial. The effectiveness and mechanism of RIPC with respect to myocardial IRI in children with tetralogy of Fallot (ToF), a severe cyanotic congenital cardiac disease, undergoing open heart surgery are unclear. We hypothesized that RIPC can confer cardioprotection in children undergoing ToF repair surgery.

Methods and results

Overall, 112 ToF children undergoing radical open cardiac surgery using cardiopulmonary bypass (CPB) were randomized to either a RIPC group ($n=55$) or a control group ($n=57$). The RIPC protocol consisted of three cycles of 5-min lower limb occlusion and 5-min reperfusion using a cuff-inflator. Serum inflammatory cytokines and cardiac injury markers were measured before surgery and after CPB. Right ventricle outflow tract (RVOT) tissues were collected during the surgery to assess hypoxia-inducible factor (Hif)-1 α and other signalling proteins. Cardiac mitochondrial injury was assessed by electron microscopy. The primary results showed that the length of stay in the intensive care unit (ICU) was longer in the control group than in the RIPC group (52.30 ± 13.43 h vs. 47.55 ± 10.34 h, respectively, $P=0.039$). Patients in the control group needed longer post-operative ventilation time compared to the RIPC group (35.02 ± 6.56 h vs. 31.96 ± 6.60 h, respectively, $P=0.016$). The levels of post-operative serum troponin-T at 12 and 18 h, CK-MB at 24 h, as well as the serum h-FABP levels at 6 h, after CPB were significantly lower, which was coincident with significantly higher protein expression of cardiac Hif-1 α , p-Akt, p-STAT3, p-STAT5, and p-eNOS and less vacuolization of mitochondria in the RIPC group compared to the control group.

Conclusion

In ToF children undergoing open heart surgery, RIPC attenuates myocardial IRI and improves the short-term prognosis.

Keywords

Remote ischaemic preconditioning • Heart protection • Tetralogy of fallot • Paediatric surgery • Cardiac pulmonary bypass

* Corresponding authors. Tel: +852 39179794, Fax: +852 28551654, Email: zyxia@hku.hk; Tel: +86 27 85351633, Fax: +86 27 85351633, Email: ysltian@163.com; and Tel: +86 27 85351610, Fax: +86 27 85351636, Email: dongnianguoxw@163.com

† The first three authors contributed equally to this work.

© The Author 2017. Published by Oxford University Press on behalf of the European Society of Cardiology.

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/4.0/>), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited. For commercial re-use, please contact journals.permissions@oup.com

Introduction

Tetralogy of Fallot (ToF) is a severe congenital cyanotic cardiac disease for which the outcomes are more unfavourable than other types of simple congenital heart diseases, even following surgical repair. Protection of the organs, especially the heart, of children with ToF against ischaemia-reperfusion injury (IRI) during the surgery is necessary to improve their prognosis.

The common goal of cardioprotective techniques is to initiate endogenous mechanisms that can reduce the effects of IRI.¹ Remote ischaemic preconditioning (RIPC) is a noninvasive and clinically relevant strategy of protection whereby acute intermittent ischaemia is induced at a distant site to protect against IRI in remote organs, including the heart.²

The mechanistic pathway linking the remote organ or tissue to the heart is currently unclear, although several mechanisms have been proposed, including neural pathways, humoral pathways, and systemic responses. RIPC involves the regulatory phosphorylation of a number of key intracellular proteins that propagate the signalling for prosurvival metabolic control in the heart. In particular, the inhibition of the opening of mitochondrial permeability transition pore (mPTP) in the heart has been proposed as a major cardioprotective mechanism in the setting of RIPC.³

Protection against IRI is of the utmost importance in children undergoing heart surgery, as prolonged periods of cardioplegic arrest and cardiopulmonary bypass (CPB) are often required. RIPC provides clinically significant protection in children undergoing open heart surgery with CPB for congenital heart defects,⁴ although the effectiveness of RIPC cardioprotection in adult patients undergoing cardiac surgery remains controversial,^{5–8} which may be largely attributable to confounding variables⁹ and/or the choice of anaesthetics,^{7,8} which interfere with the cardioprotection by RIPC.¹⁰ However, ToF is a severe cyanotic congenital heart condition, and an oxygen deficit could remain as a consequence of right ventricle outlet tract stenosis; the impact of RIPC on myocardial protection in the setting of chronic hypoxia in children is largely unknown. Therefore, we aimed to explore the cardioprotective effect of RIPC in children undergoing repair for ToF and to determine the impact of RIPC on the phosphorylation/activation status of the key molecules involved in preconditioning cardioprotection and on the post-operative prognosis.

Methods

Patient recruitment and allocation

This study was approved by the Wuhan Union Hospital Human Ethics Committee and registered in the Chinese Clinical Trial Registry (NO. Chi-CTR-TRC-09000457). Written informed consent was obtained from the patient's guardian before enrolment in the study.

The sample size was determined based on our preliminary study of RIPC in children, which aimed to detect differences in clinical outcomes.¹¹ A prolonged intensive care unit (ICU) stay after open heart surgery has a strong association with poor perioperative cardiac function,¹² and perioperative complications are the strongest predictors of post-operative length of stay (LOS) in the ICU.¹³ Additionally, a prolonged ICU stay is a powerful predictor of adverse outcomes after cardiac operations both immediately and in the long-term.¹⁴ Therefore, the sample size was

determined to be adequate based on the following conditions: RIPC to reduce the ICU stay time by 3 h with a power of 0.85, a two-sided significance level α of 0.05, and a common variance (Sc^2) of 16. Based on the formula for calculating the sample size,¹⁵ the sample size required per group was 32. Thus, we aimed for a sample size of $n = 60$ per group, which would yield ample statistical power.

Patients diagnosed with ToF ($n = 154$), aged between 3 months to 3 years old, undergoing elective radical surgical repair of ToF with standard blood cardioplegia and CPB were recruited, and 120 patients were randomized to the RIPC or control group. Among these 120 patients, the guardians of 39 patients also gave consent to allow the use of right ventricle outflow tract (RVOT) tissue and blood samples for research purposes (20 patients in the control group, 19 patients in the RIPC group). Children with chromosomal defects, airway and parenchymal lung disease, immunodeficiency, severe malnutrition, or blood disorders were excluded. However, 8 patients did not receive allocated treatment because of operation cancellation or a change in the surgical approach (3 cases in the control group and 5 cases in the RIPC group), as shown in *Figure 1*.

Randomization and blinding

After the sample size was determined, 120 patients were allocated into the control group or the RIPC group by block randomization. For this, 10 patients in each block (12 blocks in total) were randomized on a 1:1 basis into the two groups. Codes were computer-generated and kept in sealed envelopes at a central location. For each patient randomized, the next available code was used. On the day of surgery, patients were assigned to undergo either remote ischaemic preconditioning or no ischaemic preconditioning (control group).

The randomized patients were anaesthetized by an anaesthesiologist who was not involved as an investigator in the study. Envelopes were opened after the general anaesthesia induction by another anaesthesiologist who would perform remote ischaemic preconditioning or not according to the grouping. The sample collection, clinical follow-ups and laboratory research were carried out by different researchers who were unaware of the grouping. When the research was completed, all the data were sent to a statistician who was aware of the grouping. The patients, cardiac surgeons, and intensive-care physicians were unaware of the treatment assignments.

RIPC and surgical procedures

All preoperative medications were omitted on the day of surgery, and no premedication was given before surgery. The volatile anaesthetic sevoflurane (8% by volume) was given by mask with pure oxygen immediately after the patient entered the operation room. One minimum alveolar concentration (MAC) of sevoflurane was inhaled after the patient lost consciousness. During anaesthesia induction, patients received i.v. midazolam (0.15–0.25 mg/kg), fentanyl (5–10 μ g/kg), and the neuromuscular blocking agent rocuronium (0.6–1.2 mg/kg). Anaesthesia was maintained with midazolam, sufentanil, and the neuromuscular blocking agent by continuous infusion, and sevoflurane was inhaled when needed.

Following the insertion of radial arterial catheters, RIPC was induced by three cycles of 5 min of lower limb ischaemia and 5 min of reperfusion using a blood-pressure cuff inflated to a pressure 30 mm Hg greater than the systolic arterial pressure measured via the radial arterial line. The patients in the control group underwent sham placement of the blood pressure cuff around the leg without inflation. Blood flow interruption and restoration were monitored by pulse-oximetry. Thereafter, a jugular vein central venous catheter was inserted. There were 55 to 65 min intervals (no significant difference between groups) between the completion of the RIPC protocols and the initiation of CPB in both groups.

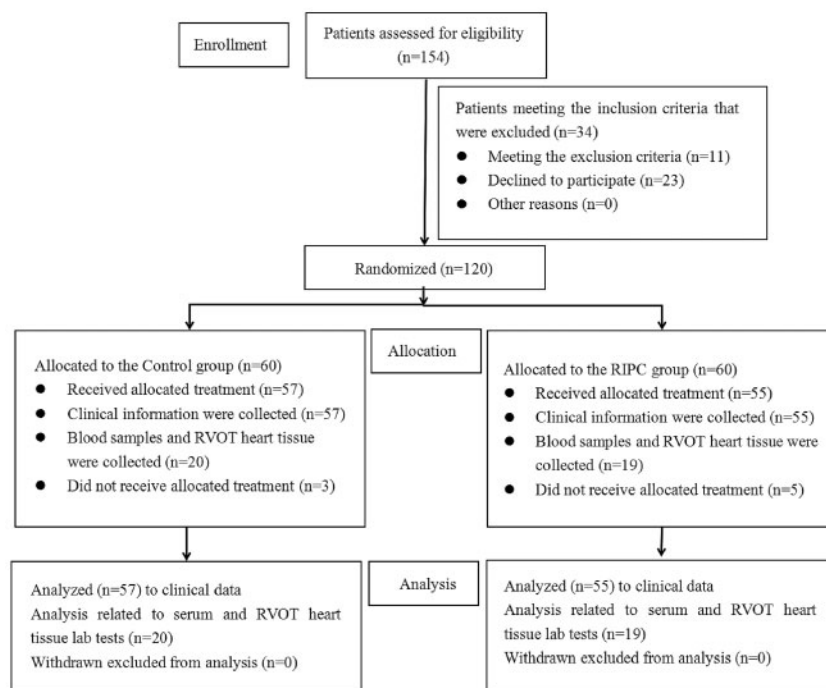


Figure 1 Trial flow diagram.

All children underwent radical surgical repair of ToF using standard CPB techniques with blood cardioplegia. Modified ultrafiltration was carried out in all children. Inotropic therapy was standardized to a treatment algorithm instituted to treat a mean radial arterial blood pressure of 60 mm Hg, despite optimization of the preload, afterload, and heart rate.¹⁶

Blood analysis

The patients who gave consent for heart tissue and blood collection were randomly assigned to control ($n = 20$) or RIPC ($n = 19$) groups. Arterial blood was sampled from the arterial catheter for the measurement of the cytokines interleukin (IL)-8 and IL-10, tumour necrosis factor (TNF)- α , and the levels of serum creatinine, troponin T (TnT), creatine kinase-MB (CK-MB), and human fatty acid-binding protein (hFABP) (Supplementary material online, Method).

Tissue protein extraction and determination by western blot analysis

After CPB was established, right ventricle outflow tract (RVOT) tissues were cut by the surgeon to broaden the right ventricle outflow tract stenosis. The tissue protein was extracted and processed for Western blot analysis of phosphorylated Akt (pAkt) (Ser473), Akt (β), p-STAT3 (Tyr705), STAT3, p-STAT5 (Tyr694), STAT5, p-PTEN, PTEN, p-eNOS (Ser1177), eNOS, and Hif-1 α (Supplementary material online, Method).

Electron microscopy

Each RVOT heart tissue sample was cut into two 1 mm,³ and was processed for mitochondrial morphological analysis (Supplementary material

online, Method). The mitochondrial surface-to-volume ratio (Sv-ratio_{Mi})¹⁷ and morphometric cellular oedema index (CEI)^{18,19} were determined to evaluate the ischaemia-induced swelling of cardiomyocytes. A 48-point grid with angles was projected onto each test field to determine the Sv-ratio_{Mi} by the point and intersection counting method as described.²⁰ The CEI was calculated from the volume densities of the mitochondria, sarcoplasm and myofibrils as described.¹⁸

Statistical analysis

The data are presented as the means \pm SD. The comparison of enumerated data between the treatment groups was conducted using the chi-square test, while an unpaired t -test was applied to compare measurement data where appropriate. Measurements taken across the time course were assessed using repeated measures ANOVA with post hoc comparisons to evaluate any significant effect of group (control vs. RIPC) or time and/or any significant interaction between group and time. The GraphPad version 5.0 statistical program (GraphPad Software Inc., San Diego, CA, USA) and SPSS version 19.0 for Windows (IBM Corporation, Armonk, NY, USA) were used for the statistical analyses. A value of $P < 0.05$ was considered as statistically significantly different.

Results

Patient anthropometric data and perioperative conditions

As shown in Table 1, the data of intra-operative parameters and post-operative parameters, including ventilation time, duration of ICU

Table 1 Patients' perioperative, intraoperative, and postoperative characteristics

	Control (n =57)	RIPC(n =55)	P-value
Preoperative parameters			
Male/female (n, n)	32/25	30/25	0.87
Age, month	11.19±6.22	10.51±6.66	0.58
Weight, kg	9.14±2.81	8.86±2.85	0.59
SpO ₂ , %	96.84±4.26	98.00±3.17	0.11
RVOT, cm	1.07±0.23	1.12±0.26	0.25
Aorta overriding ratio, %	35.09±9.84	32.36±9.81	0.15
Ejection fraction, %	63.86±5.67	64.82 ±4.61	0.33
MAP, mm Hg	56.12±3.50	55.60±3.29	0.42
CVP, cmH ₂ O	12.42±3.64	12.20±2.85	0.72
Intraoperative parameters			
Temperature ^a , °C	30.85±0.86	30.76±0.92	0.60
CPB time, min	109.4±20.2	108.3±20.7	0.79
TACC time, min	46.37±9.42	47.73±9.07	0.44
RVOT sample collect, min	13.05±2.26	12.45±1.76	0.35
MAP during CPB, mm Hg	43.40±3.78	44.15±4.41	0.34
CVP	9.58±1.05	9.26±1.04	0.10
Postoperative parameters			
CVP 0h	12.67±1.52	12.53±1.83	0.66
Ventilation time, h	35.02±6.56	31.96±6.60	0.016*
Oxygenation index 0h	138.2±22.5	139.1±23.9	0.84
Oxygenation index 12h	182.5±21.4	187.9±17.3	0.14
Oxygenation index 24h	224.6±32.1	235.6±30.5	0.10
ICU stay, h	52.30±13.43	47.55±10.34	0.038*
Hospital stay, d	15.56±2.77	15.13±2.18	0.37
RPP 0h	11887±1087	11690±1230	0.37
RPP 6h	11951±1798	12043±1162	0.75
RPP 12h	10282±907	9769±1682	0.046*
RPP 24h	9781±881	9598±999	0.31
0h MAP, mm Hg	55.35±3.73	55.64±3.96	0.70
6h MAP, mm Hg	54.37±5.20	54.11±5.50	0.80
12h MAP, mm Hg	53.81±4.27	54.62±4.54	0.33
24h MAP, mm Hg	55.96±4.69	56.42±3.84	0.58
48h MAP, mm Hg	56.05±4.40	57.22±4.28	0.16
0h IScore, mcg/kg/min	14.17±1.36	13.53±1.44	0.018*
3h IScore, mcg/kg/min	12.25±1.27	11.74±1.50	0.056
6h IScore, mcg/kg/min	11.03±1.37	10.51±1.24	0.035*
12h IScore, mcg/kg/min	10.09 ±1.51	9.63±1.39	0.097
24h IScore, mcg/kg/min	8.84±1.72	8.60±1.45	0.41
24h (ALT), U/L	36.70±8.80	35.58±8.42	0.49
24h Creatinine, umol/L	37.04±15.69	40.87±18.72	0.24
Postoperative complications			
ARF I, n(%)	34(59.65%)	24(43.64%)	0.09
ARF II, n(%)	13(22.81%)	9(16.36%)	0.39
Dialysis therapy, n(%)	6(10.53%)	4(7.27%)	0.74
LCOS, n(%)	12(21.05%)	8(14.55%)	0.46
Reintubation, n(%)	7(12.28%)	5(9.09%)	0.76
Pneumonia, n(%)	18(31.58%)	14(25.45%)	0.53
Hospital death, n(%)	2(3.51%)	1(1.82%)	1.00
30-day mortality, n	0	0	

Data are mean ± SD when appropriate. RVOT, right ventricle outflow tract; MAP, mean arterial pressure; CVP, central venous pressure; CPB, cardiopulmonary bypass; TACC time, Total aortic cross-clamp time; ICU, intensive care unit; RPP, rate pressure product; IScore, Inotropic score; ALT, Alanine aminotransferase; ARF, acute renal failure; LCOS, low cardiac output syndrome.

^aThe temperature refer to minimum core body temperature.

*P < 0.05 vs. control group.

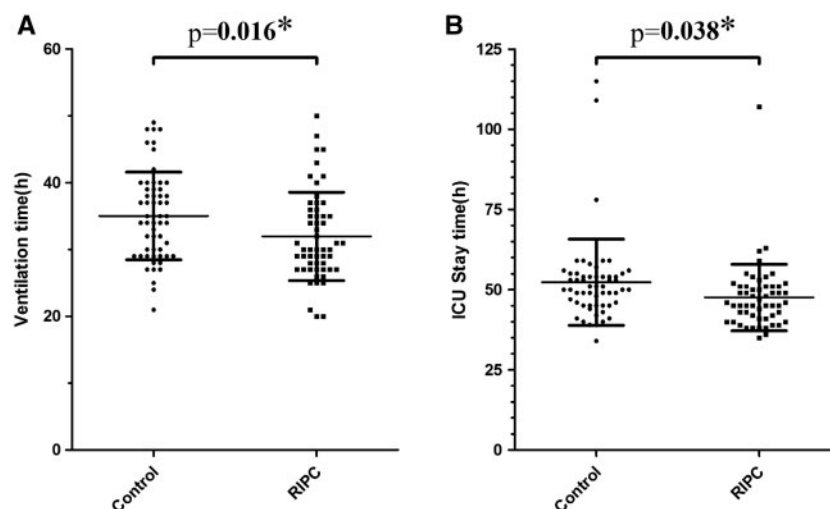


Figure 2 Ventilation time curve (A) and ICU stay time curve (B). $N = 57$ in the control group and $N = 55$ in the RIPC group. * $P < 0.05$ vs. control group.

stay, duration of hospital stay, mean arterial pressure, central venous pressure (CVP), heart rate pressure product (RPP, the product of heart rate and systolic blood pressure), and inotropic score were all collected. Alanine aminotransferase and creatinine levels at 24 h after the operation were used to assess postsurgical liver and kidney function, respectively. No significant differences were observed between the two groups in terms of gender, body weight, age, cardiac colour Doppler flow data or intra-operative monitoring parameters. Patients in the control group needed longer ventilation time than the RIPC group (35.02 ± 6.56 h vs. 31.96 ± 6.60 h, respectively, $P = 0.016$) (Table 1 and Figure 2A). The length of stay in the ICU of the control group was longer than that in the RIPC group (52.30 ± 13.43 h vs. 47.55 ± 10.34 h, $P = 0.039$) (Table 1 and Figure 2B). However, there were no significant differences in the oxygenation index or duration of hospital stay. The mean arterial pressures after operation did not significantly differ between the two groups. However, patients in the control group required moderately but significantly higher doses of inotropes used to maintain haemodynamic stability immediately following surgery (14.17 ± 0.18 mcg/kg/min vs. 13.53 ± 0.19 mcg/kg/min, $P = 0.018$) and at 6 h post-surgically (11.03 ± 0.18 mcg/kg/min vs. 10.51 ± 0.17 mcg/kg/min, $P = 0.035$) (Table 1). RPP, an indirect measure of myocardial oxygen consumption, was also significantly lower in the RIPC group than in the control group at 12 h after the operation. Post-operative complications including hospital death (2 in the control group vs. 1 in the RIPC group) did not significantly differ between the groups during the period of observation.

Systemic inflammatory response and myocardial function and injury

As shown in Supplementary material online, Table S1, the levels of plasma IL-10, IL-8, and TNF- α did not differ between the groups pre-operatively (Preop). However, the secretion of IL-10 was significantly

increased post-operatively in both groups, but the values of post-operative IL-10 in the RIPC group were significantly higher than those in the control group at 12 h ($P < 0.01$). Significant elevations of IL-8 and TNF- α were observed in both groups post-operatively. However, the levels of IL-8 (at 6 and 12 h) and TNF- α (at 6 and 12 h) were significantly lower in the RIPC group than in the control group. There were significant differences in terms of the time effects and interactive effects between time and group for IL-10, IL-8, and TNF- α . In addition, the secretion of IL-10, IL-8, and TNF- α were significantly increased and reached maximal levels at 6 h after CPB (PostCPB6h) and then gradually decreased but remained higher than those at Preop group during the period of observation.

As shown in Table 2, the levels of post-operative TnT, CK-MB and hFABP were significantly lower in the RIPC group than in the control group at 6 h (hFABP), 12 h (TnT), 18 h (TnT), and 24 h (CK-MB), post-operatively. There were significant differences in terms of the time effects and interactive effects between time and group for TnT and hFABP. Meanwhile, there was significant time effect but no significant difference in terms of interaction between time and group for CK-MB. In addition, the value of the area under the plasma concentration time curve (AUC) for TnT in the RIPC group was significantly lower than that in the control group ($P < 0.05$), while the values of AUC for CK-MB and hFABP in the RIPC group were approximately 11.9%, 14.5% lower than those in the control group, but these differences did not reach statistical significance ($P > 0.05$, Table 2).

RVOT cardiac hif-1 α and intracellular pro-survival signalling protein expression

As shown in Figure 3A, cardiac Hif-1 α expression was higher in the RIPC group than that in the control group. Total protein amounts of Akt, STAT3, STAT5, eNOS, and PTEN did not significantly differ between the groups (Figure 3B–F). However, the ratios of

Table 2 Myocardial injury factors

	TnT (ng/mL)		CK-MB (ng/mL)		hFABP (ng/mL)	
	Control	RIPC	Control	RIPC	Control	RIPC
Preop	0.12 ± 0.13	0.12 ± 0.10	0.15 ± 0.19	0.11 ± 0.10	3.82 ± 0.97	3.64 ± 1.13
PostCPB6h	3.93 ± 0.75#	3.42 ± 1.08#	0.87 ± 0.38#	0.74 ± 0.39#	183.2 ± 52.5#	143.7 ± 45.9*#
PostCPB12h	7.59 ± 1.42#	6.37 ± 1.93*#	2.17 ± 0.49#	1.85 ± 0.52#	52.2 ± 11.9#	45.6 ± 10.7#
PostCPB18h	6.44 ± 0.74#	4.21 ± 0.80*#	3.12 ± 0.66#	2.98 ± 0.44#	35.05 ± 6.54#	29.58 ± 6.69#
PostCPB24h	3.97 ± 0.85#	3.30 ± 1.07#	4.18 ± 0.86#	3.62 ± 0.87*#	28.7 ± 7.0#	30.3 ± 9.1#
PostCPB48h	1.92 ± 0.48#	1.71 ± 0.62#	0.69 ± 0.37#	0.52 ± 0.22#	14.7 ± 3.5#	15.4 ± 3.4#
AUC	22.9 ± 6.29	18.0 ± 7.90*	10.77 ± 4.05	9.49 ± 3.65	308.2 ± 173.2	263.6 ± 182.2

Values are mean ± SD. $n=20$ in control and $n=19$ in RIPC group. AUC, Area under curve; * $P<0.01$ vs. corresponding control groups; # $P<0.01$ vs. corresponding Preop group. There were significant differences in terms of the time effects and interactive effects between time and group for TnT (Time: $F=312.28$, $P<0.0001$; Interaction: $F=8.68$, $P<0.0001$) and hFABP (Time: $F=307.51$, $P<0.0001$; Interaction: $F=5.57$, $P<0.001$). Meanwhile, there was significant time effect but no significant difference in terms of interaction between time and group for CK-MB (Time: $F=382.02$, $P<0.0001$; Interaction: $F=1.46$, $P=0.2035$).

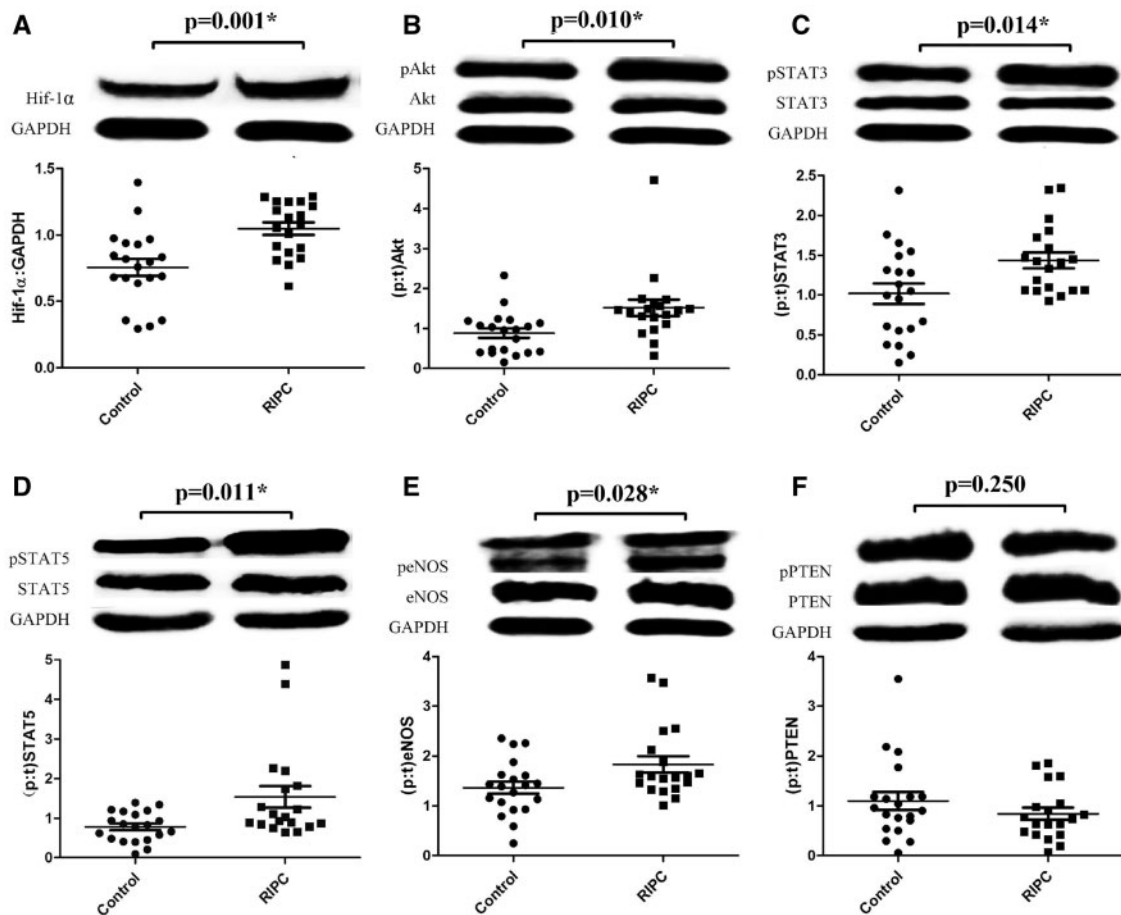


Figure 3 Protein expression of RVOT myocardium. (A): Hif-1 α expression in the RIPC group is higher than that in the control group ($P=0.0008$). (B–E): RIPC group ratio of phosphorylated Akt ($P=0.0096$), STAT3 ($P=0.0144$), STAT5 ($P=0.0156$), and eNOS ($P=0.0279$) to total protein are higher than that in the control group. (F): Phosphorylated PTEN shows no difference between the groups. $n=20$ in the control and $n=19$ in the RIPC group. * $P<0.05$ vs. control group.

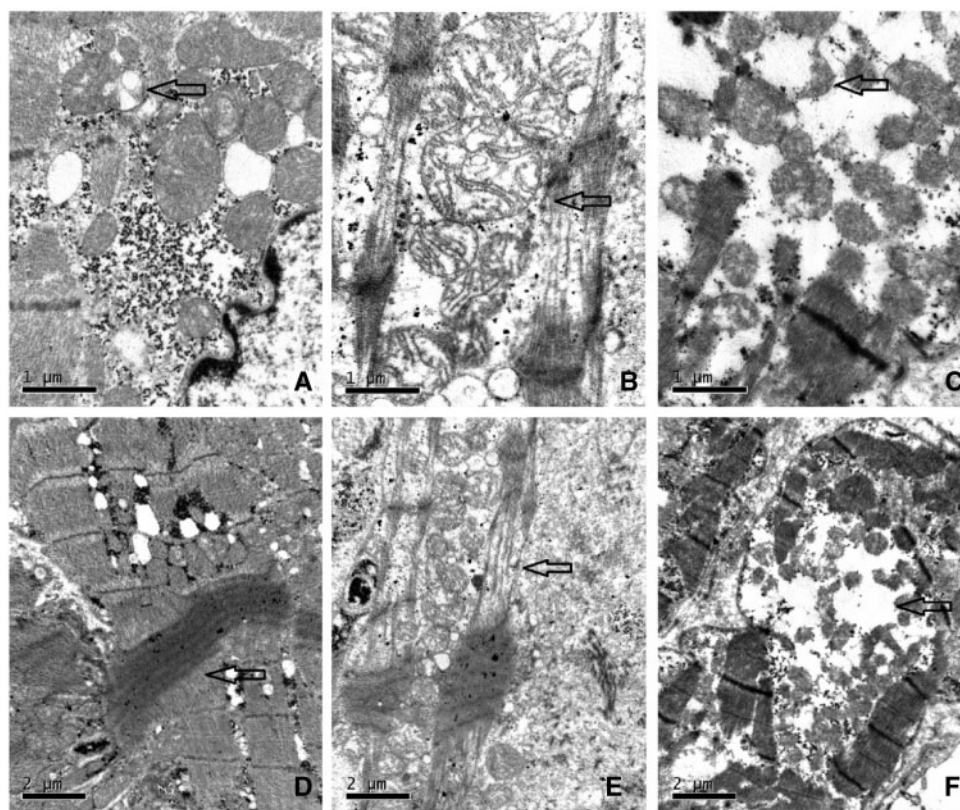


Figure 4 Ultrastructure of RVOT myocardium. (A): All the mitochondria exhibit swelling, with one also showing vacuolization; (B): Mitochondria exhibit marked swelling; (C): A ruptured mitochondrion; (D): Distinct abnormal contraction of myofibrils; (E): Due to abnormal contraction, the myofibrils snapped; (F): The structure of the myofibrils were disrupted, with lysis at the centre. The typical ultrastructural changes of the myocardium are indicated by the arrow.

phosphorylated to total (p:t) protein specifically probed for Akt, STAT3, STAT5, and eNOS were greater in the RIPC group than those in the control group (all $P < 0.05$). There were no significant differences in either total PTEN or the ratio of phosphorylated PTEN to total PTEN between the groups.

Mitochondrial ultrastructure of RVOT myocardium

As shown in *Figure 4* and see Supplementary material online, *Table S2*, the mitochondrial ultrastructure was severely damaged in the control group compared with the RIPC group as observed under transmission electron microscopy. Mitochondrial oedema was present in all of the samples obtained from both groups, whereas the values of the surface-to-volume ratios of mitochondria ($Sv\text{-ratio}_{M_i}$) and the cellular oedema index (CEI) in the RIPC group were significantly lower than those in the control group (all $P < 0.05$). However, 14 samples from the control group were found to be extremely swollen with apparent vacuolization compared to two samples from the RIPC group ($P < 0.001$). No significant difference was observed in terms of broken mitochondria, abnormal contraction bands, myofibrillar fragmentation, or myofibrillar lysis between the two groups.

Discussion

This study is the first to demonstrate the clinical effectiveness of RIPC with a concomitant exploration of its mechanism in ToF children undergoing open-heart surgery. RIPC attenuates myocardial IRI and improves the short-term prognosis in those patients, and the mechanism underlying likely involves the activation of Hif-1 α , Akt, STAT3, STAT5, and eNOS resulting in decreased mitochondrial damage in children undergoing TOF repair surgery as summarized in *Figure 5*.

Although experimental animal studies have demonstrated that RIPC is beneficial, the cardioprotective effects of RIPC in patients undergoing cardiac surgery have yielded contradictory results. The first clinical application of RIPC in patients was reported in a randomized clinical trial (RCT) in children undergoing open-heart surgery and it demonstrated that RIPC is cardioprotective with a decreased inotrope requirement, decreased airway resistance and decreased troponin levels.⁴ Subsequently, other RCTs have demonstrated myocardial protection by RIPC against myocardial IRI in adult patients undergoing coronary artery bypass graft surgery with CPB.^{5,6} However, other recent multicentre trials have failed to demonstrate

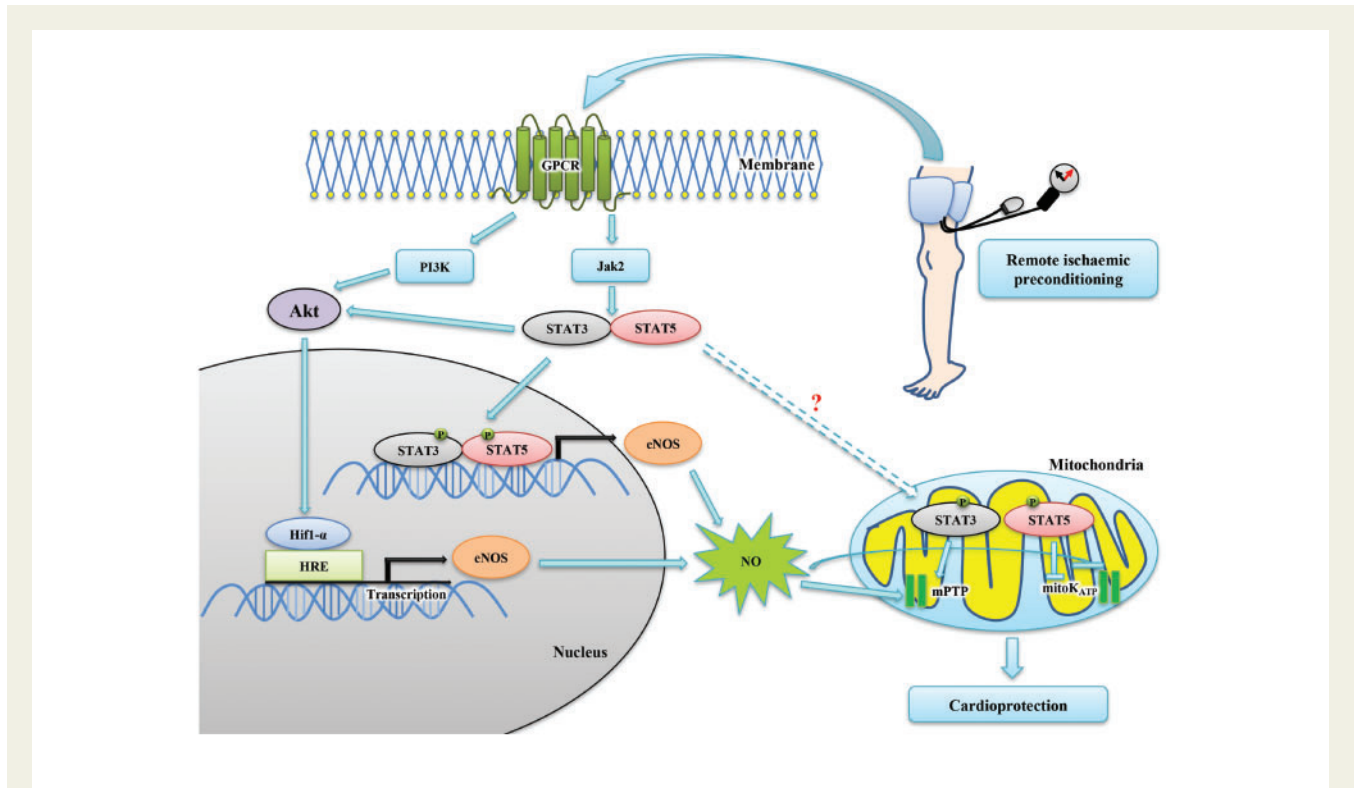


Figure 5 Schematic of proposed signalling involved in remote ischaemic preconditioning cardioprotection (RIPC) in ToF children undergoing elective radical ToF repair surgery. RIPC reduces the postoperative cardiac ischaemic injury through activating the PI3K/Akt and JAK/STAT3/5 signalling pathways, which is associated with nuclear accumulation of Hif-1 α and STAT3/5 and subsequent induction of myocardial eNOS activation and mitochondrial protection.

beneficial effects of RIPC for patients undergoing cardiac surgery.^{7,21–23} The two recent multicentre clinical trials reported negative effects of RIPC.^{7,8} The most plausible explanation for the lack of cardioprotection of RIPC in these two trials was the concomitant use of propofol anaesthesia^{24,25} in all patients in the study of Meybohm *et al.*⁸ and in approximately 90% of patients in the study of Hausenloy *et al.*,⁷ although the inclusion of many patients who not only underwent CABG but also more traumatic valve surgery that caused direct damage to the hearts was another concern.²⁵ The intravenous anaesthetic propofol possesses antioxidant properties that could obscure the effects of RIPC. Indeed, RIPC, when applied during isoflurane but not during propofol anaesthesia, reduced myocardial damage in patients undergoing CABG surgery.²⁶ RIPC conferred cardioprotection along with cardiac STAT5 activation under isoflurane anaesthesia, but neither RIPC cardioprotection nor STAT5 activation were observed under propofol anaesthesia.¹⁰ In clinical trials reporting protective effects of RIPC, the RIPC procedure was either completed without anaesthetic intervention during the transportation of patients to the hospital (which would allow more time for RIPC to take effect while avoiding the anaesthetic interaction²⁷), or completed during anaesthesia induction with inhalational anaesthetics other than propofol.⁶ Given that adult patients receiving cardiac surgeries generally present with comorbidities such as diabetes and hypertension that may influence the RIPC-induced cardioprotective effects,²⁸ the contradictory effects of RIPC in clinical settings suggest that the cardioprotective effects of RIPC may also be

specific to the patient or disease. Meanwhile, cardioprotective manoeuvres such as ischaemic pre- and postconditioning lose their effectiveness with ageing.²⁹ Ageing affects cardiomyocytes at several subcellular and molecular levels, including alterations at the DNA level, increased oxidative stress [reactive oxygen species (ROS) formation], and age-related damage of mitochondrial function.²⁹ Thus, children may be more sensitive to protection by RIPC than adults. In several clinical trials that showed no beneficial effects of RIPC in children undergoing open-heart surgery, the sample sizes were either too small to detect between-group differences as summarized in a meta-analysis article,²² or RIPC was conducted in the presence of propofol anaesthesia,³⁰ or the RIPC stimulus was inflation of the blood pressure cuff to 15 mm Hg above systolic pressure³¹ rather than 30 mm Hg above systolic pressure, as used in our current study and in other studies.

In our study, we utilized a uniform patient cohort to evaluate the cardiac protective effects of RIPC. The need for longer ventilation times is usually a consequence of cardiopulmonary dysfunction or slower recovery after cardiac surgery, while the need for higher doses of inotropes to maintain haemodynamic stability reflects poorer cardiac function. In addition, both the duration of intubation and the inotrope requirement can be considered as independent predictors of a prolonged ICU stay.¹² Reduction in ventilation time and inotropes requirement suggested that RIPC improved the cardiac function and improved the short-term prognosis. We found that the

concentrations of TnT, CK-MB, and hFABP, indicators of myocardial damage, were significantly lower in the RIPC group than those in the control group. These results suggested that RIPC attenuated myocardial IRI and improved the short-term prognosis and also potentially long-term prognosis as well in ToF children undergoing open-heart surgery, although the underlying mechanism remains unclear.

Endothelial dysfunction and impairment of the mechanical properties of the lungs following CPB have been proposed as the mechanisms for RIPC-mediated shortening of postoperative ventilation duration.^{32,33} RIPC has been shown to confer cardioprotection by reducing neutrophil activation and endothelial dysfunction in patients with valve replacement surgery.³⁴ In our current study, IL-10 levels were increased with a reduction in IL-8 and TNF- α in the RIPC group when compared with the control group. Studies have shown that RIPC induces late protection against myocardial IRI by increasing the expression of IL-10 in the remote muscle, followed by the release of IL-10 into the circulation, and activation of protective signalling pathways in the heart.³⁵ Excessive TNF- α expression induces contractile dysfunction and cell death, while a lower TNF- α concentration is protective.^{36,37} Furthermore, *in vivo* dog experiments have demonstrated time-dependent myocardial dysfunction following intravenous infusion of TNF- α .^{38,39} Importantly, the overall response is modulated by the anti-inflammatory cytokine IL-10 and pro-inflammatory cytokine IL-8⁴⁰ through the inhibition of the expression of TNF- α . The current data are consistent with an acute modification of inflammatory pathways in RIPC.

The transcription factor hypoxia-inducible factor (Hif)-1 has emerged as a central oxygen-sensitive transcription factor involved in the protective response to hypoxia.^{41,42} Hif-1 is composed of Hif-1 α and Hif-1 β subunits. Whereas Hif-1 β is constitutively expressed, Hif-1 α is up-regulated in response to hypoxia. Under normoxic conditions, Hif-1 α stability and transcriptional activity are inhibited.^{43,44} In our study, there was no difference in the aortic overriding ratio, RVOT width, or SpO₂ before surgery between the RIPC group and the control group. However, the RVOT myocardial Hif-1 α protein expression in the RIPC group was higher than that in the control group. This result was consistent with a previous study showing that RIPC increased Hif-1 α levels in the right atrial tissue of patients undergoing heart surgery.⁴⁵ However, whether or not RIPC-induced cardioprotection requires the up-regulation of Hif-1 α remains unknown and merits further study.

Although the mechanistic pathways underlying RIPC cardioprotection remain unclear, studies suggest a neuro-hormonal pathway that may link the RIPC stimulus to the heart. The reperfusion injury signalling kinase pathway including the PI3K/Akt signalling cascade and the protective survivor activating factor enhancement pathway including the JAK2/STAT3 signalling cascade are the most important pathways involved in eNOS activation and ischaemia myocardial protection.^{46–48} A previous study has shown that STAT5, but not STAT3, is activated by RIPC and becomes the unique signalling signature of RIPC in humans.⁴⁹ Our study demonstrated that RIPC could enhance the phosphorylated Akt, STAT3, STAT5, and eNOS expression levels in the RVOT myocardium of patients. These findings suggest that RIPC reduces the postoperative cardiac ischaemic injury through activating the prosurvival signalling pathway. However, Pepe *et al.* performed RIPC in ToF children as young as 1 month who underwent elective open heart surgery, and it was found that there were

no differences in phosphorylated Akt or STAT3 between the control and RIPC-treated groups.⁵⁰ The reasons for this discrepancy may be due to the increased age of the ToF children in this study (the average age was approximately 10 or 11 months in our study vs. an average of approximately 7 months in the study of Pepe *et al.*). Additionally, the timing of the RVOT tissue sampling may affect the detection of changes in signalling proteins after the preconditioning stimuli, with an elevation of pro-survival proteins at a relatively earlier phase of reperfusion.⁵⁰ As such, the timing of RVOT tissue sample collection was set at a relatively early stage of CPB in the current study, while the exact timing of RVOT tissue sample collection was not clear in the study of Pepe *et al.* Therefore, the patterns of changes in the pro-survival signalling proteins as well as how the pro-survival signalling pathway mediated RIPC cardioprotection in ToF children merits further study. Our finding that RIPC can also activate cardiac STAT3 in humans is in line with a recent study that showed that long-term, regular RIPC increased STAT3 activation in human arterial specimens (presumably coronary artery fragments) discarded during CABG surgery and improved endothelial function.⁵¹

The major determinant of cardiomyocyte death following an episode of IRI is mitochondrial dysfunction arising from the formation of the mPTP,^{52–54} while inhibition of the mPTP is the hallmark of cardioprotection.⁵⁵ The opening of the mPTP results in mitochondrial depolarization, swelling, and ultimately apoptotic cell death with the release of mitochondrial cytochrome C. In our study, we found all the RVOT myocardial samples presented mitochondrial swelling, however, more extreme swelling and even vacuolization of the mitochondria was observed in the control group, which suggested that RIPC attenuated the mitochondrial injury and cellular death induced by myocardial IRI in the clinical setting of children undergoing elective radical ToF repair surgery.

Conclusions

Our randomized clinical trial of RIPC in ToF children undergoing elective radical ToF repair surgery has demonstrated that RIPC can reduce ventilation time, the duration of ICU stay, and improve heart function after surgery. The increased cardiac Hif-1 α expression and enhanced phosphorylation of Akt, STAT3, STAT5 and eNOS expression, which can consequently improve mitochondrial function, may be the major mechanisms whereby RIPC confers the cardioprotective effect in ToF children undergoing elective radical ToF repair surgery.

Author contributions

Conceived and designed the experiments: QW, TW, SC, ND, ZX, SY. Performed the experiments: QW, TW, SC, QZ, NH, YF, HL. Analysed the data: QW, TW, SC, ZX, SY. Contributed to the discussion and wrote the paper: QW, TW, SC, HL, ND, ZX, SY.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

Acknowledgements

The authors acknowledge the Shenzhen IVY-Valued Biotechnology Co. Ltd. for technical consulting, and the VanScholar Editors Co., Ltd., Vancouver, Canada for English language editing.

Funding

This study was supported by grants from the National Natural Science Foundation of China (NO. 81370112 and 81200609) and by a General Research Fund (GRF) from the Research Grants Council of Hong Kong (17123915M).

Conflict of interest: none declared.

References

- Heusch G. Cardioprotection: chances and challenges of its translation to the clinic. *Lancet* 2013;**381**:166–175.
- Heusch G, Botker HE, Przyklenk K, Redington A, Yellon D. Remote ischaemic conditioning. *J Am Coll Cardiol* 2015;**65**:177–195.
- Heusch G. Molecular basis of cardioprotection: signal transduction in ischaemic pre-, post-, and remote conditioning. *Circ Res* 2015;**116**:674–699.
- Cheung MM, Kharbada RK, Konstantinov IE, Shimizu M, Frndova H, Li J, Holtby HM, Cox PN, Smallhorn JF, Van Arsdel GS, Redington AN. Randomized controlled trial of the effects of remote ischemic preconditioning on children undergoing cardiac surgery: first clinical application in humans. *J Am Coll Cardiol* 2006;**47**:2277–2282.
- Hausenloy DJ, Mwamure PK, Venugopal V, Harris J, Barnard M, Grundy E, Ashley E, Vichare S, Di Salvo C, Kolvekar S, Hayward M, Keogh B, MacAllister RJ, Yellon DM. Effect of remote ischaemic preconditioning on myocardial injury in patients undergoing coronary artery bypass graft surgery: a randomised controlled trial. *Lancet* 2007;**370**:575–579.
- Thielmann M, Kottenberg E, Kleinbongard P, Wendt D, Gedik N, Pasa S, Price V, Tsagakis K, Neuhauser M, Peters J, Jakob H, Heusch G. Cardioprotective and prognostic effects of remote ischaemic preconditioning in patients undergoing coronary artery bypass surgery: a single-centre randomised, double-blind, controlled trial. *Lancet* 2013;**382**:597–604.
- Hausenloy DJ, Candilio L, Evans R, Ariti C, Jenkins DP, Kolvekar S, Knight R, Kunst G, Laing C, Nicholas J, Pepper J, Robertson S, Xenou M, Clayton T, Yellon DM, Investigators ET. Remote ischemic preconditioning and outcomes of cardiac surgery. *N Engl J Med* 2015;**373**:1408–1417.
- Meybohm P, Bein B, Brosteau O, Cremer J, Gruenewald M, Stoppe C, Coburn M, Schaelte G, Boning A, Niemann B, Roesner J, Kletzlin F, Strouhal U, Reyher C, Laufenberg-Feldmann R, Ferner M, Brandes IF, Bauer M, Stehr SN, Kortgen A, Wittmann M, Baumgarten G, Meyer-Treschan T, Kienbaum P, Heringlake M, Schon J, Sander M, Treskatsch S, Smul T, Wolwender E, Schilling T, Fuernau G, Hasenclever D, Zacharowski K, Collaborators RIS. A multicenter trial of remote ischemic preconditioning for heart surgery. *N Engl J Med* 2015;**373**:1397–1407.
- Kleinbongard P, Neuhauser M, Thielmann M, Kottenberg E, Peters J, Jakob H, Heusch G. Confounders of cardioprotection by remote ischaemic preconditioning in patients undergoing coronary artery bypass grafting. *Cardiology* 2016;**133**:128–133.
- Kottenberg E, Musiolik J, Thielmann M, Jakob H, Peters J, Heusch G. Interference of propofol with signal transducer and activator of transcription 5 activation and cardioprotection by remote ischaemic preconditioning during coronary artery bypass grafting. *J Thorac Cardiovasc Surg* 2014;**147**:376–382.
- Hepponstall M, Ignjatovic V, Binos S, Attard C, Karlaftis V, D'udekem Y, Monagle P, Konstantinov IE. Remote ischemic preconditioning (RIPC) modifies the plasma proteome in children undergoing repair of tetralogy of fallot: a randomized controlled trial. *PLoS One* 2015;**10**:e0122778.
- Azarfarin R, Ashouri N, Totonchi Z, Bakhshandeh H, Yaghoubi A. Factors influencing prolonged ICU stay after open heart surgery. *Res Cardiovasc Med* 2014;**3**:e20159.
- Almashrafi A, Alsabti H, Mukaddirov M, Balan B, Aylin P. Factors associated with prolonged length of stay following cardiac surgery in a major referral hospital in Oman: a retrospective observational study. *BMJ Open* 2016;**6**:e010764.
- Mahesh B, Choong CK, Goldsmith K, Gerrard C, Nashef SA, Vuylsteke A. Prolonged stay in intensive care unit is a powerful predictor of adverse outcomes after cardiac operations. *Ann Thorac Surg* 2012;**94**:109–116.
- Sakpal TV. Sample size estimation in clinical trial. *Perspect Clin Res* 2010;**1**:67–69.
- Xia Z, Huang Z, Ansley DM. Large-dose propofol during cardiopulmonary bypass decreases biochemical markers of myocardial injury in coronary surgery patients: a comparison with isoflurane. *Anesth Analg* 2006;**103**:527–532.
- Schmiedl A, Schnabel PA, Mall G, Gebhard MM, Hunneman DH, Richter J, Bretschneider HJ. The surface to volume ratio of mitochondria, a suitable parameter for evaluating mitochondrial swelling. Correlations during the course of myocardial global ischaemia. *Virchows Arch a Pathol Anat Histopathol* 1990;**416**:305–315.
- DiBona DR, Powell WJ, Jr., Quantitative correlation between cell swelling and necrosis in myocardial ischemia in dogs. *Circ Res* 1980;**47**:653–665.
- Muhlfeld C, Richter J. High-pressure freezing and freeze substitution of rat myocardium for immunogold labeling of connexin 43. *Anat Rec a Discov Mol Cell Evol Biol* 2006;**288**:1059–1067.
- Muhlfeld C, Coulibaly M, Dorge H, Sellin C, Liakopoulos O, Ballat C, Richter J, Schoendube F. Ultrastructure of right ventricular myocardium subjected to acute pressure load. *Thorac Cardiovasc Surg* 2004;**52**:328–333.
- Healy DA, Boyle E, McCartan D, Bourke M, Medani M, Ferguson J, Yagoub H, Bashar K, O'donnell M, Newell J, Canning C, McMonagle M, Dowdall J, Cross S, O'daly S, Manning B, Fulton G, Kavanagh EG, Burke P, Grace PA, Moloney MC, Walsh SR. A multicenter pilot randomized controlled trial of remote ischemic preconditioning in major vascular surgery. *Vasc Endovascular Surg* 2015;**49**:220–227.
- Tie HT, Luo MZ, Li ZH, Wang Q, Wu QC, Li Q, Zhang M. Remote ischemic preconditioning fails to benefit pediatric patients undergoing congenital cardiac surgery: a meta-analysis of randomized controlled trials. *Medicine (Baltimore)* 2015;**94**:e1895.
- Walsh M, Whitlock R, Garg AX, Legare JF, Duncan AE, Zimmerman R, Miller S, Fremes S, Kieser T, Karthikeyan G, Chan M, Ho A, Nasr V, Vincent J, Ali I, Lavi R, Sessler DI, Kramer R, Gardner J, Syed S, VanHelder T, Guyatt G, Rao-Melacini P, Thabane L, Devereaux PJ. Effects of remote ischemic preconditioning in high-risk patients undergoing cardiac surgery (Remote IMPACT): a randomized controlled trial. *CMAJ* 2016;**188**:329–336.
- Heusch G, Gersh BJ, ERICCA and RIPHeart: two nails in the coffin for cardioprotection by remote ischemic conditioning? Probably not! *Eur Heart J* 2016;**37**:200–202.
- Heusch G, Rassaf T. Time to give up on cardioprotection? A critical appraisal of clinical studies on ischemic pre-, post-, and remote conditioning. *Circ Res* 2016;**119**:676–695.
- Kottenberg E, Thielmann M, Bergmann L, Heine T, Jakob H, Heusch G, Peters J. Protection by remote ischemic preconditioning during coronary artery bypass graft surgery with isoflurane but not propofol—a clinical trial. *Acta Anaesthesiol Scand* 2012;**56**:30–38.
- Botker HE, Kharbada R, Schmidt MR, Bottcher M, Kaltoft AK, Terkelsen CJ, Munk K, Andersen NH, Hansen TM, Trautner S, Lassen JF, Christiansen EH, Krusell LR, Kristensen SD, Thuesen L, Nielsen SS, Rehling M, Sorensen HT, Redington AN, Nielsen TT. Remote ischaemic conditioning before hospital admission, as a complement to angioplasty, and effect on myocardial salvage in patients with acute myocardial infarction: a randomised trial. *Lancet* 2010;**375**:727–734.
- Ferdinandy P, Hausenloy DJ, Heusch G, Baxter GF, Schulz R. Interaction of risk factors, comorbidities, and comedications with ischemia/reperfusion injury and cardioprotection by preconditioning, postconditioning, and remote conditioning. *Pharmacol Rev* 2014;**66**:1142–1174.
- Boengler K, Schulz R, Heusch G. Loss of cardioprotection with ageing. *Cardiovasc Res* 2009;**83**:247–261.
- Pedersen KR, Ravn HB, Povlsen JV, Schmidt MR, Erlandsen EJ, Hjortdal VE. Failure of remote ischemic preconditioning to reduce the risk of postoperative acute kidney injury in children undergoing operation for complex congenital heart disease: a randomized single-center study. *J Thorac Cardiovasc Surg* 2012;**143**:576–583.
- McCordle BW, Clarizia NA, Khaikin S, Holtby HM, Manlihot C, Schwartz SM, Caldaroni CA, Coles JG, Van Arsdel GS, Scherer SW, Redington AN. Remote ischemic preconditioning in children undergoing cardiac surgery with cardiopulmonary bypass: a single-center double-blinded randomized trial. *J Am Heart Assoc* 2014;**3**:e000964.
- Pearl JM, Nelson DP, Wellmann SA, Raake JL, Wagner CJ, McNamara JL, Duffy JY. Acute hypoxia and reoxygenation impairs exhaled nitric oxide release and pulmonary mechanics. *J Thorac Cardiovasc Surg* 2000;**119**:931–938.
- Schulze-Neick I, Li J, Penny DJ, Redington AN. Pulmonary vascular resistance after cardiopulmonary bypass in infants: effect on postoperative recovery. *J Thorac Cardiovasc Surg* 2001;**121**:1033–1039.
- Kharbada RK, Peters M, Walton B, Kattenhorn M, Mullen M, Klein N, Vallance P, Deanfield J, MacAllister R. Ischemic preconditioning prevents endothelial injury and systemic neutrophil activation during ischemia-reperfusion in humans in vivo. *Circulation* 2001;**103**:1624–1630.
- Cai ZP, Parajuli N, Zheng X, Becker L. Remote ischemic preconditioning confers late protection against myocardial ischemia-reperfusion injury in mice by upregulating interleukin-10. *Basic Res Cardiol* 2012;**107**:277.

36. Kleinbongard P, Schulz R, Heusch G. TNF α in myocardial ischemia/reperfusion, remodeling and heart failure. *Heart Fail Rev* 2011;**16**:49–69.
37. Cain BS, Meldrum DR, Dinarello CA, Meng X, Joo KS, Banerjee A, Harken AH. Tumor necrosis factor- α and interleukin-1 β synergistically depress human myocardial function. *Crit Care Med* 1999;**27**:1309–1318.
38. Natanson C, Eichenholz PW, Danner RL, Eichacker PQ, Hoffman WD, Kuo GC, Banks SM, MacVittie TJ, Parrillo JE. Endotoxin and tumor necrosis factor challenges in dogs simulate the cardiovascular profile of human septic shock. *J Exp Med* 1989;**169**:823–832.
39. Walley KR, Hebert PC, Wakai Y, Wilcox PG, Road JD, Cooper DJ. Decrease in left ventricular contractility after tumor necrosis factor- α infusion in dogs. *J Appl Physiol* 1985;**76**:1060–1067.
40. Donnelly RP, Freeman SL, Hayes MP. Inhibition of IL-10 expression by IFN- γ up-regulates transcription of TNF- α in human monocytes. *J Immunol* 1995;**155**:1420–1427.
41. Ong SG, Hausenloy DJ. Hypoxia-inducible factor as a therapeutic target for cardioprotection. *Pharmacol Ther* 2012;**136**:69–81.
42. Prabhakar NR, Semenza GL. Adaptive and maladaptive cardiorespiratory responses to continuous and intermittent hypoxia mediated by hypoxia-inducible factors 1 and 2. *Physiol Rev* 2012;**92**:967–1003.
43. Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O₂ tension. *Proc Natl Acad Sci USA* 1995;**92**:5510–5514.
44. Lee SH, Wolf PL, Escudero R, Deutsch R, Jamieson SW, Thistlethwaite PA. Early expression of angiogenesis factors in acute myocardial ischemia and infarction. *N Engl J Med* 2000;**342**:626–633.
45. Albrecht M, Zitta K, Bein B, Wennemuth G, Broch O, Renner J, Schuett T, Lauer F, Maahs D, Hummitzsch L, Cremer J, Zacharowski K, Meybohm P. Remote ischemic preconditioning regulates HIF-1 α levels, apoptosis and inflammation in heart tissue of cardiosurgical patients: a pilot experimental study. *Basic Res Cardiol* 2013;**108**:314.
46. Amour J, Brzezinska AK, Weihrach D, Billstrom AR, Zielonka J, Krolkowski JG, Bienengraeber MW, Wartier DC, Pratt PF Jr, Kersten JR. Role of heat shock protein 90 and endothelial nitric oxide synthase during early anesthetic and ischemic preconditioning. *Anesthesiology* 2009;**110**:317–325.
47. Lecour S. Activation of the protective Survivor Activating Factor Enhancement (SAFE) pathway against reperfusion injury: does it go beyond the RISK pathway? *J Mol Cell Cardiol* 2009;**47**:32–40.
48. Hausenloy DJ, Iliodromitis EK, Andreadou I, Papalois A, Gritsopoulos G, Anastasiou-Nana M, Kremastinos DT, Yellon DM. Investigating the signal transduction pathways underlying remote ischemic conditioning in the porcine heart. *Cardiovasc Drugs Ther* 2012;**26**:87–93.
49. Heusch G, Musiolik J, Kottenberg E, Peters J, Jakob H, Thielmann M. STAT5 activation and cardioprotection by remote ischemic preconditioning in humans: short communication. *Circ Res* 2012;**110**:111–115.
50. Pepe S, Liaw NY, Hepponstall M, Sheeran FL, Yong MS, D'udekem Y, Cheung MM, Konstantinov IE. Effect of remote ischemic preconditioning on phosphorylated protein signaling in children undergoing tetralogy of Fallot repair: a randomized controlled trial. *J Am Heart Assoc* 2013;**2**:e000095.
51. Liang Y, Li YP, He F, Liu XQ, Zhang JY. Long-term, regular remote ischemic preconditioning improves endothelial function in patients with coronary heart disease. *Braz J Med Biol Res* 2015;**48**:568–576.
52. Di Lisa F, Bernardi P. Mitochondria and ischemia-reperfusion injury of the heart: fixing a hole. *Cardiovasc Res* 2006;**70**:191–199.
53. Griffiths EJ, Halestrap AP. Mitochondrial non-specific pores remain closed during cardiac ischaemia, but open upon reperfusion. *Biochem J* 1995;**307**: 93–98.
54. Yellon DM, Hausenloy DJ. Myocardial reperfusion injury. *N Engl J Med* 2007;**357**:1121–1135.
55. Heusch G, Boengler K, Schulz R. Inhibition of mitochondrial permeability transition pore opening: the Holy Grail of cardioprotection. *Basic Res Cardiol* 2010;**105**:151–154.