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Remote Ischemic Preconditioning Protects the Brain Against Injury After Hypothermic Circulatory Arrest

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Background—Ischemic preconditioning (IPC) is a mechanism protecting tissues from injury during ischemia and reperfusion. Remote IPC (RIPC) can be elicited by applying brief periods of ischemia to tissues with ischemic tolerance, thus protecting vital organs more susceptible to ischemic damage. Using a porcine model, we determined whether RIPC of the limb is protective against brain injury caused by hypothermic circulatory arrest (HCA).

Methods and Results—Twelve piglets were randomized to control and RIPC groups. RIPC was induced in advance of cardiopulmonary bypass by 4 cycles of 5 minutes of ischemia of the hind limb. All animals underwent cardiopulmonary bypass followed by 60 minutes of HCA at 18°C. Brain metabolism and electroencephalographic activity were monitored for 8 hours after HCA. Assessment of neurological status was performed for a week postoperatively. Finally, brain tissue was harvested for histopathological analysis.

Study groups were balanced for baseline and intraoperative parameters. Brain lactate concentration was significantly lower ($P<0.0001$, ANOVA) and recovery of electroencephalographic activity faster ($P<0.05$, ANOVA) in the RIPC group. RIPC had a beneficial effect on neurological function during the 7-day follow-up (behavioral score; $P<0.0001$ versus control, ANOVA). Histopathological analysis demonstrated a significant reduction in cerebral injury in RIPC animals (injury score; mean [interquartile range]: control 5.8 [3.8 to 7.5] versus RIPC 1.5 [0.5 to 2.5], $P<0.001$, t test).

Conclusions—These data demonstrate that RIPC protects the brain against HCA-induced injury, resulting in accelerated recovery of neurological function. RIPC might be neuroprotective in patients undergoing surgery with HCA and improve long-term outcomes. Clinical trials to test this hypothesis are warranted. **(*Circulation*. 2011;123:714-721.)**

Key Words: cardiac surgery ■ cerebral ischemia ■ cardiopulmonary bypass ■ reperfusion injury ■ ischemic preconditioning

In the repair of complex congenital heart defects or in surgery of the aortic arch, normal circulation may be temporarily halted to ensure a clean, bloodless operation field. The brain is the organ most vulnerable to ischemic injury during this period of hypothermic circulatory arrest (HCA), and the mortality and morbidity of these procedures today consists mostly of neurological complications.[1,2] The focus in recent years has shifted from reducing mortality to alleviating long-term functional disability, a major determinant of which is neurocognitive performance.

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Preconditioning, a plausible neuroprotective strategy, describes a concept whereby brief exposure to a harmful stimulus like ischemia, in a dose below the threshold for tissue injury, provides robust protection against or tolerance to the injurious effects of a subsequent more severe insult.[3] In vitro studies have confirmed that hypoxic preconditioning protects cortical neurons from glutamate toxicity.[4] In an

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experimental rat model, hippocampal CA1 cell counts revealed significant neuronal sparing in preconditioned animals observed 6 months after reperfusion with global cerebral ischemia.[5] The cerebral diffusion lesions and infarct volumes following a human stroke have also been shown to be reduced if a patient has a history of previous transient ischemic attacks, thus eliciting a theory of a "natural" preconditioning effect.[6]

As it is not always possible to mimic the exact ischemic insult before the actual intervention, the introduction of remote preconditioning has been eagerly received.[7] In this process, the preconditioning stimulus is applied to nontarget tissue, most commonly to a skeletal muscle in a limb, and the signal is thought to spread systemically by a mechanism that includes activation of the autonomic nervous system and as-yet unidentified humoral mediators. In remote ischemic preconditioning (RIPC), intermittent ischemia is induced in the limb most commonly by use of a blood pressure cuff or a tourniquet, in cycles varying in time and repetitions.

We hypothesized that RIPC would improve brain protection in association with deep HCA. We tested this hypothesis in a chronic porcine model, allowing us to mimic the devices and procedures of human surgery very closely yet giving us the opportunity to explore both acute and chronic parameters and, most importantly, neurological recovery and brain histology after ischemic changes.

Materials and Methods

Animals and Study Design

Twelve Yorkshire piglets of a native stock (25 to 30 kg) were randomized using sealed envelopes to RIPC group (n=6) and control group (n=6). Investigators performing experimental protocols and/or postoperative assessment were blinded to group allocation. All animals received humane care in accordance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals (http://www.nap.edu/catalog/5140.html). The study was approved by the Research Animal Care and Use Committee of the University of Oulu.

Induction of RIPC

RIPC was induced in advance of cardiopulmonary bypass (CPB) by inflating a 9-cm-wide blood pressure cuff placed around the upper part of a hindlimb. The cuff was inflated to 200 mm Hg for 5 minutes (hindlimb ischemia) followed by a 5-minute deflation (reperfusion), and the RIPC stimulus consisted of 4 inflation-deflation cycles (total duration 40 minutes). Interruption of blood supply to the limb was confirmed by vascular Doppler ultrasound. In animals randomized to the control group, the blood pressure cuff was placed in the upper part of the hindlimb, but was inflated to a pressure of 20 mm Hg, and thus did not result in limb ischemia (sham RIPC). Four 5-minute inflations and deflations were applied, as with the RIPC group.

Operative Protocol

Anesthesia

Animals were sedated with intramuscular ketamine hydrochloride (350 mg) and midazolam (45 mg), and a peripheral catheter was inserted into a vein of the right ear for administration of drugs and to maintain fluid balance with Ringer acetate solution. Using thiopental for further sedation as required, the piglets were intubated with a 6.5-mm cuffed endotracheal tube and ventilated with 50% oxygen and a rate of 12 to 15 breaths per minute to achieve an end-tidal carbon dioxide concentration in the expired air (EtCO2) of 4.5% to 5.0%. After induction with fentanyl (50 μg/kg), anesthesia was maintained by a continuous infusion of fentanyl (25 $\mu g \cdot kg^{-1} \cdot h^{-1}$), midazolam (0.25 $mg \cdot kg^{-1} \cdot h^{-1}$) and pancuronium (0.2 $mg \cdot kg^{-1} \cdot h^{-1}$), as well as inhalation anesthesia of 0.5% isoflurane throughout the entire experiment, excluding the period of HCA. Cefuroxime (1.5 g) was administered intravenously at induction of anesthesia. ECG monitoring was carried out throughout the entire operation.

Hemodynamic Monitoring

An arterial line for pressure monitoring and blood sampling was placed on the left femoral artery. A pulmonary artery thermodilution catheter (CritiCath, 7 Fr, Ohmeda GmbH & Co, Erlangen, Germany) was introduced through the left femoral vein for measurement of pulmonary pressure, pulmonary capillary wedge pressure, central venous pressure, cardiac output, and temperature, as well as for blood sampling. A 10 Fr catheter was placed in the urinary bladder to monitor urine output. A right anterolateral thoracotomy was performed in the fourth intercostal space to expose the right atrium for CPB cannulation. The right internal thoracic artery and vein were ligated and cut, and the pericardium was opened. After systemic heparinization (500 IU/kg), the ascending aorta was cannulated with a 16 Fr arterial cannula, and the right atrial appendage was cannulated with a single 24 Fr atrial cannula. A 12 Fr intracardiac sump cannula was positioned into the apex of the heart for decompression during CPB.

Cranial Procedures

Three 5-mm windows were drilled over the parietal cortex. A temperature probe (Thermocouple Temperature Catheter-Micro-Probe, Ref C8.B, GMS, Kiel, Germany) was inserted in the first window to monitor intracerebral temperature throughout the experiment. A pressure-monitoring catheter (Codman Micro-Sensor ICP Transducer, Codman ICP Express Monitor, Codman & Shurtleff Inc, Raynham, MA) was placed into the cerebral tissue through the second window for intracranial pressure measurements. Finally, a microdialysis catheter (CMA 70, CMA/Microdialysis, Stockholm, Sweden) was placed into the brain cortex (through the third window) to a depth of 15 mm below the dura mater. The catheter was connected to a 2.5-mL syringe placed into a microinfusion pump (CMA 106, CMA/Microdialysis) and perfused with Ringer acetate solution at a rate of 0.3 μL/min (Perfusion Fluid CNS, CMA/Microdialysis).

CPB and HCA

After baseline recordings of the RIPC or sham-RIPC protocol, a membrane oxygenator (D905 Eos, Dideco, Mirandola, Italy) was primed with 1 L of Ringer acetate solution and heparin (5000 IU). Fresh whole blood from a donor pig, drawn on the operative day, was transfused into the prime as required to maintain the hematocrit of all animals above 25% after the operation ($\approx$50 mL/kg). Nonpulsatile CPB was initiated at a flow rate of 90 to 110 $mL \cdot kg^{-1} \cdot min^{-1}$, and the flow was adjusted to maintain an arterial pressure of 50 to 70 mm Hg.

A 30-minute cooling period was carried out to attain a brain temperature of 18°C. Cooling was managed according to the pH-stat method of CPB for 25 minutes. The hypercarbia related to the pH-stat strategy causes cerebral vasodilatation, resulting in improved cooling and cerebral tissue oxygenation before the critical ischemic period. Five minutes in advance of HCA, the pH strategy was switched to α-stat principles to normalize the biochemical status. A heat exchanger was used for core cooling.

After 30 minutes of cooling, a 60-minute period of HCA at 18°C was initiated and potassium chloride (40 mmol) was injected toward the heart via CPB arterial cannula. Cardiac cooling with topical ice slush was maintained throughout HCA. The intracerebral temperatures were controlled and maintained at 18°C with ice packs placed over the head.

At the beginning of rewarming, furosemide (40 mg), methylprednisolone (80 mg), mannitol (150 g), calcium bioglyconate (2.25 mmol Ca2+) and lidocaine (40 mg) were administered into the pump. During 45 minutes of rewarming, the piglets were warmed to 35°C with a 100 $mL \cdot kg^{-1} \cdot min^{-1}$ flow rate, so that the warming

Table 1. Experimental and Metabolic Data

			After the Start of Rewarming					
	Baseline	End of Cooling	30 minutes	2 hours	4 hours	8 hours	P_g	$P_{t \cdot g}$
pH							0.050	0.14
RIPC	7.57 (7.55–7.60)	7.26 (7.25–7.29)	7.44 (7.42–7.46)	7.42 (7.36–7.50)	7.56 (7.46–7.61)*	7.61 (7.58–7.63)*		
Control	7.49 (7.41–7.56)	7.20 (7.15–7.32)	7.47 (7.40–7.56)	7.37 (7.27–7.42)	7.42 (7.38–7.51)	7.46 (7.42–7.54)		
$PaCO_2$, kPa							0.11	0.59
RIPC	4.18 (3.82–4.51)	9.36 (8.59–9.63)	4.20 (3.56–4.32)	4.97 (4.38–5.69)	4.4 (3.9–4.9)	3.9 (3.8–4.5)*		
Control	4.53 (3.68–5.40)	11.2 (7.16–12.9)	4.23 (3.31–5.00)	5.55 (5.02–6.26)	5.4 (4.2–6.3)	5.1 (4.3–5.5)		
PaO_2, kPa							0.36	0.29
RIPC	33.0 (29.2–36.8)	106 (106–106)	67.7 (59.1–75.6)	31.2 (26.8–33.7)	31.3 (27.9–32.3)	28.1 (26.5–31.4)		
Control	32.4 (30.1–35.7)	106 (106–106)	68.8 (66.6–73.9)	26.6 (20.5–32.5)	29.8 (24.2–39.8)	31.2 (24.8–39.7)		
SvO_2, %							0.47	0.33
RIPC	77.5 (74.5–85.3)	100 (100–100)	74.5 (70.3–82.8)*	78.0 (73.0–82.0)	77.0 (74.8–79.3)	73.5 (61.0–79.3)		
Control	80.0 (77.8–81.3)	100 (100–100)	89.0 (81.3–90.5)	78.5 (72.8–84.0)	73.5 (60.8–80.3)	75.5 (63.5–80.6)		
Heart rate, bpm							0.64	0.78
RIPC	102 (112–127)	. . .	132 (88–164)	108 (98–130)	124 (111–135)	123 (119–144)		
Control	115 (105–124)	. . .	128 (72–144)	110 (77–131)	127 (105–151)	121 (103–151)		
Mean arterial pressure, mm Hg							0.83	0.41
RIPC	84 (75–87)	65 (62–67)	60 (53–64)	103 (88–112)	104 (96–108)	97 (89–105)		
Control	73 (70–104)	64 (54–72)	60 (59–67)	95 (79–97)	95 (82–105)	93 (89–113)		
Pulmonary artery pressure, mm Hg							0.78/0.78	0.24/0.32
RIPC	18 (16–20)/12 (9–12)	. . .	. . .	29 (26–34)/19 (17–22)	26 (21–30)/17 (16–22)	27 (22–31)/15 (15–19)*		
Control	17 (15–19)/11 (9–14)	. . .	. . .	30 (28–32)/20 (18–21)	30 (25–34)/21 (17–23)	26 (23–26)/13 (12–14)		
Hematocrit, %							0.72	0.57
RIPC	22.0 (18.8–25.3)	24.5 (20.8–26.0)	24.0 (21.0–28.5)	29.0 (25.0–30.1)	30.0 (27.3–30.3)	28.8 (27.9–30.0)		
Control	22.0 (18.4–23.3)	24.0 (20.8–25.8)	24.3 (20.8–24.9)	31.0 (24.5–32.9)	30.5 (29.0–34.8)	28.0 (27.5–33.3)		
Intracerebral temperature, °C							0.75	0.26
RIPC	37.1 (36.7–37.7)	17.0 (16.8–17.3)	31.5 (30.5–33.3)	34.3 (32.2–35.0)	36.0 (34.6–37.2)	37.4 (36.9–38.1)		
Control	37.7 (37.2–38.3)	16.3 (16.0–17.3)	33.2 (30.8–34.6)	33.4 (32.8–34.2)	35.8 (35.5–37.0)	38.0 (37.0–38.4)		
Rectal temperature, °C							0.49	0.83
RIPC	37.6 (37.1–38.2)	16.6 (14.3–17.3)	29.2 (26.5–32.0)	34.1 (32.6–35.0)	36.7 (34.5–37.7)	37.9 (37.2–38.5)		
Control	38.0 (37.8–38.2)	15.3 (14.1–17.5)	27.2 (25.6–30.4)	33.8 (33.0–34.1)	36.0 (35.7–36.3)	37.6 (37.2–38.1)		
Blood temperature, °C							0.70	0.89
RIPC	37.5 (37.1–38.1)	9.6 (7.7–17.7)	36.9 (34.8–37.7)	34.2 (32.8–35.1)	36.8 (34.6–37.6)	37.9 (37.3–38.7)		
Control	37.9 (37.6–38.2)	10.3 (8.0–14.1)	35.0 (33.1–36.4)	33.9 (33.3–34.5)	36.4 (35.9–37.2)	38.1 (37.5–38.4)		

RIPC n=6, Control n=6. Values are shown as medians and 25th and 75th percentiles. $PaCO_2$ indicates arterial CO_2 partial pressure; SvO_2, mixed venous oxygen saturation.

*$P<0.05$ at single time point.

gradient was ≤10°C at all times. During rewarming, the pH was managed according to the α-stat principles. The heart was defibrillated if necessary at 27 to 30°C. The sump cannula was removed after 30 minutes of rewarming. Sufficient ventilation was restored 10 minutes before weaning from CPB, which itself occurred at 45 minutes after the start of rewarming. Throughout rewarming and after weaning from CPB, animal temperature was regulated using heat exchange mattresses, heating lamps, paracetamol infusions (1 to 2g intravenously), and ice packs as required.

Postoperative Management

Noradrenalin was used postoperatively as required to maintain mean arterial pressure over 60 mm Hg. Animals were extubated 8 hours after the start of rewarming and were moved to a recovery room. Each animal was electively euthanized on the seventh postoperative day. Immediately after intravenous injection of pentobarbital (60 mg/kg) and heparin (500 IU/kg), the thoracic cavity was opened and the descending thoracic aorta was clamped. Ringer solution (1 L) was administered through the ascending thoracic aorta to the upper body, and blood was suctioned from the superior vena cava until the perfusate was clear of blood. Then, 10% formalin solution (1 L/15 minutes) was infused through the brain in the same manner to accomplish perfusion fixation. Immediately thereafter, the entire brain was harvested, weighed, and immersed in 10% neutral formalin. No other tissue (including tissue from the heart, kidney and limb) was harvested.

Sample Collection and Biochemical Data

Sample collection from microdialysis catheters was performed at set time points intraoperatively (baseline, 30 minutes cooling, 30 minutes HCA, 60 minutes HCA, 30 minutes rewarm, 1 hour rewarm, 1 hour 30 minutes rewarm, 2 hours rewarm, 2 hours 30 minutes rewarm, 3 hours rewarm, 4 hours rewarm, 5 hours rewarm, 6 hours

Table 2. Experimental and Metabolic Data

			After the Start of Rewarming					
	Baseline	End of Cooling	30 minutes	2 hours	4 hours	8 hours	P_g	$P_{t \cdot g}$
Central venous pressure, mm Hg							0.31	0.79
RIPC	6 (3–6)	3 (1–6)	2 (1–4)	9 (7–9)	9 (8–9)	9 (8–9)		
Control	5 (4–5)	2 (1–3)	2 (2–2)	9 (7–11)	8 (7–9)	7 (5–10)		
Intracranial pressure, mm Hg							0.18	0.86
RIPC	8 (5–13)	9 (8–15)	9 (6–11)	14 (10–17)	12 (10–19)*	15 (12–19)†		
Control	3 (1–8)	8 (4–10)	5 (4–8)	9 (8–13)	9 (8–10)	11 (9–12)		
Cerebral perfusion pressure, mm Hg							0.55	0.66
RIPC	71 (56–79)	57 (47–59)	50 (47–53)*	80 (67–92)	91 (70–92)	81 (70–86)		
Control	76 (62–94)	62 (43–69)	55 (54–56)	80 (71–84)	78 (67–90)	79 (76–92)		
Oxygen consumption, $mL \cdot min^{-1} \cdot m^{-2}$)							0.037	0.37
RIPC	7.4 (6.5–8.1)	0.0 (0.0–0.1)	5.5 (4.2–7.2)*	7.4 (6.3–8.8)	8.7 (6.7–10.3)	10.8 (7.9–14.3)		
Control	5.4 (4.5–6.8)	0.0 (0.0–0.2)	2.7 (2.3–3.9)	5.1 (3.3–8.6)	7.7 (4.7–8.7)	7.4 (3.1–10.6)		
Oxygen delivery, $mL \cdot min^{-1} \cdot m^{-2}$)							0.69	0.72
RIPC	4.1 (3.9–5.3)	3.2 (2.8–3.8)	3.0 (2.0–4.1)	4.2 (3.6–5.4)	5.7 (5.5–6.8)	5.7 (5.5–6.2)		
Control	3.9 (3.3–4.6)	3.1 (2.5–4.3)	3.0 (2.7–4.0)	3.9 (3.5–4.5)	6.0 (5.2–6.2)	5.5 (5.0–6.0)		
Oxygen extraction, mL/dL							0.71	0.25
RIPC	2.0 (1.7–2.4)	0.0 (0.0–0.0)	2.9 (1.9–3.8)*	2.7 (2.5–3.4)	3.0 (2.9–3.3)	3.4 (2.6–5.0)		
Control	2.0 (1.–2.1)	0.0 (0.0–0.1)	1.3 (1.1–1.9)	2.7 (1.7–3.9)	3.8 (2.6–6.0)	3.5 (2.4–4.8)		
Venous glucose, mmol/L							0.90	0.013
RIPC	4.6 (3.9–4.9)	4.1 (3.8–4.6)	10.8 (9.4–11.5)	5.9 (4.9–6.7)	5.2 (4.7–5.7)	5.3 (4.6–5.5)		
Control	5.2 (3.2–7.4)	4.9 (3.2–5.8)	8.3 (7.1–11.4)	5.9 (5.0–7.4)	4.7 (3.8–5.4)	4.2 (4.0–5.5)		
Osmolality, mmol/kg							0.82	0.67
RIPC	274 (269–277)	276 (272–279)	278 (273–280)	277 (272–279)	275 (274–278)	275 (275–279)		
Control	274 (272–280)	278 (276–283)	273 (272–284)	275 (272–281)	273 (269–278)	274 (269–282)		

RIPC n=6, Control n=6. Values are shown as medians and 25th and 75th percentiles.
*$P<0.05$ at single time point; †$P>0.05$ and <0.06.

rewarm, 7 hours rewarm, 8 hours rewarm). The concentrations of cerebral tissue glucose, lactate, pyruvate, glutamate, and glycerol were measured immediately after collection with a microdialysis analyzer (CMA 600, CMA/Microdialysis), using established enzymatic methods. Arterial blood gases, pH, electrolytes, serum ionized calcium, glucose and hemoglobin levels (i-STAT Analyzer, i-STAT Corp, East Windsor, NJ) were measured at baseline, at the end of cooling (immediately before institution of HCA), as well as 30 minutes, 2 hours, 4 hours, and 8 hours after the start of rewarming. At these time points, samples for full blood cell count, cardiac troponin I, creatine kinase isoenzyme MB, and plasma analysis were collected. In order to control arterial CO_2 gas tension precisely, sampling was performed at least every 15 minutes during CPB.

Protein Biomarkers for Brain Damage

All samples were analyzed, with the analyst being blinded to all other data. Serum neurofilament heavy chain (NfH) levels were measured using an in-house developed enzyme-linked immunosorbent assay (ELISA). Adhering to a previously proposed nomenclature, the soluble fraction of NfH measured is indicated with the capture of antibody in the superscript (NfH^{SMI35}). We concentrated on NfH^{SMI35} instead of the neurofilament light chain because of its relative resistance to proteolysis, an important feature for reliable measurement in body fluids.[8] Serum S100-B levels were measured using a modified in-house developed ELISA, as described previously.[9]

Multimodal Neurophysiological Monitoring

Cortical electric activity was registered by four 5-mm stainless-steel screw electrodes implanted into the skull over the parietal and frontal areas using a digital electroencephalography (EEG) recorder (Nervus, Reykjavik, Iceland) and amplifier (Magnus EEG 32/8, Reykjavik, Iceland). Sampling frequency was 256 Hz and bandwidth was

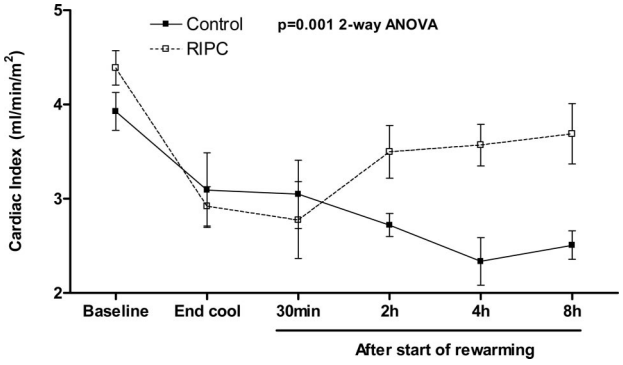

Figure 1. Cardiac index. Mean±1 SE.

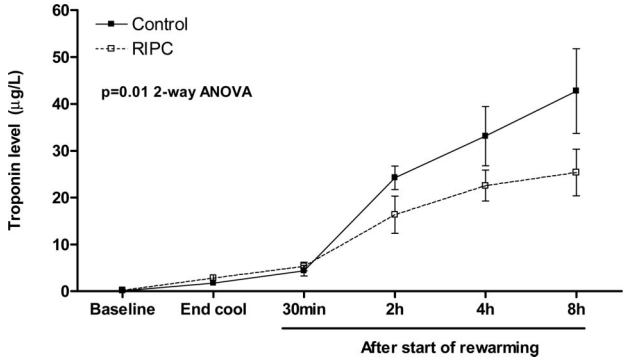

Figure 2. Cardiac troponin I. Mean±1 SE.

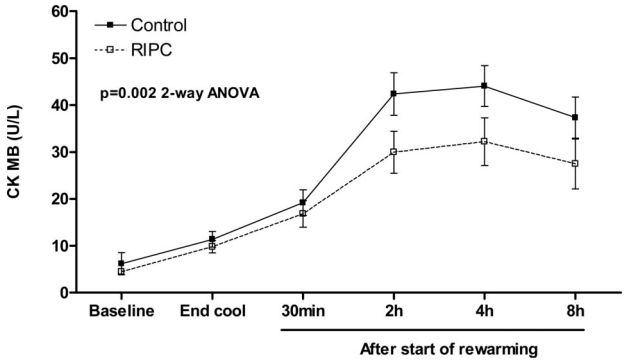

Figure 3. Creatine kinase isoenzyme MB. Mean±1 SE.

0.03 to 100 Hz. All EEG recordings were referenced to a frontal screw electrode that, together with a ground screw electrode, was implanted over the frontal sinuses. A 10-minute baseline EEG recording was performed in advance of the cooling period. EEG recording was reinstituted after HCA and continued until extubation. Artifact periods were excluded from each 5-minute sample. The EEG energy recovery was evaluated by an algorithm based on a nonlinear energy operator.

Regional cerebral blood oxygen saturation was measured noninvasively by near-infrared spectroscopy (INVOS Cerebral Oxymeter, Somanetics, Troy, MI). Two adhesive near-infrared light sensors (SomaSensors, Somanetics, Troy, MI) were positioned over the frontal cortex (one on each side to measure right and left hemisphere regional cerebral blood oxygen saturation), and regional cerebral blood oxygen saturation monitoring was performed continuously from CPB induction up to extubation and recovery (total of 24 hours).

Behavioral Evaluation

Postoperatively, all animals were evaluated daily by an experienced observer using a species-specific quantitative behavioral score. The quantified assessments of mental status (0=comatose, 1=stuporous, 2=depressed, and 3=normal), appetite (0=refuses liquids, 1=refuses solids, 2=decreased, and 3=normal), and motor function (0=unable to stand, 1=unable to walk, 2=unsteady gait, and 3=normal) were summed to obtain a final score, with a maximum score of 9 reflecting apparently normal neurological function and with lower values indicating substantial brain damage.

Histopathological Analysis

During autopsy, the brain was excised immediately and the hemispheres were separated. One half was immersed in 10% neutral formalin and allowed to fix for 2 weeks en bloc. Coronal samples 3-mm thick were sliced from the frontal lobe, thalamus (including the adjacent cortex), and hippocampus (including the adjacent brain stem and temporal cortex), and sagittal samples were sliced from the posterior brain stem (medulla oblongata and pons) and cerebellum. The pieces were fixed in fresh formalin for another week. After fixation, the samples were processed as follows: rinsing in water for 20 minutes and immersion in 70% ethanol for 2 hours, in 94% ethanol for 4 hours, and in absolute ethanol for 9 hours. Thereafter, the pieces were kept 1 hour in an absolute ethanol-xylene mixture, 4 hours in xylene, and embedded in warm paraffin wax for 6 hours. The samples were sectioned at 6 μm and stained with hematoxylin-eosin. The sections of the brain samples from each animal were screened by a single experienced senior pathologist unaware of the experimental design and the identity and fate of individual animals. Each section was carefully investigated for the presence or absence of any ischemic or other damage.

Sections of brain specimens (cortex, thalamus, hippocampus, posterior brain stem, cerebellum) from each animal were screened by a single blinded neuropathologist. The signs of injury were scored as follows: 1 (slight edema, dark or eosinophilic neurons, or cerebellar Purkinje cells), 2 (moderate edema, at least 2 hemorrhagic foci in the section), and 3 (severe edema, several hemorrhagic foci, infarct foci [local necrosis]). The total regional score was the sum of the scores in each specific brain area (cortex, thalamus, hippocampus, posterior brain stem, and cerebellum). A total histopathological score was calculated by summing all regional scores to allow semiquantitative comparison between the animals.

Statistical Analysis

Statistical analysis was performed using SPSS (SPSS, version 12.0, SPSS Inc, Chicago, IL) and SAS (version 9.1.3, SAS Institute Inc., Cary, NC) statistical software. Continuous and ordinal variables are expressed as the median and 25th and 75th percentiles in the tables. SAS procedure Mixed was used for these repeated measurements. Because the measurement intervals were uneven, spatial exponential covariance structure was chosen in repeated statement, assuming that the further apart the measurements are in time, the weaker the correlation between them. Complete independence was assumed across animals (by random statement). Reported P values are as follows: P between groups (P_g) indicates a level of difference between the groups, P time · group ($P_{t \cdot g}$) indicates behavior between the groups over time. Student t test, 2-way ANOVA, or Mann-Whitney U test was used as appropriate to assess the distribution of variables between the study groups. Two-tailed significance levels are reported. $P<0.05$ was considered statistically significant. In the figures, mean and error bar of 1 SE was used.

Results

Comparability of the Study Groups

The mean weight of the pigs was 29.6 kg in the RIPC group and 26.8 kg in the control group ($P=0.076$). The amount of

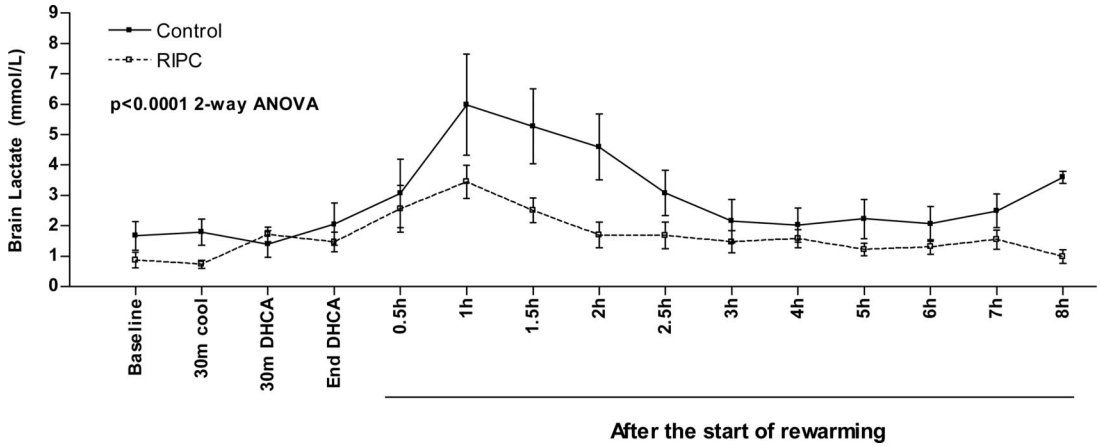

Figure 4. Cerebral concentration of lactate. Mean±1 SE.

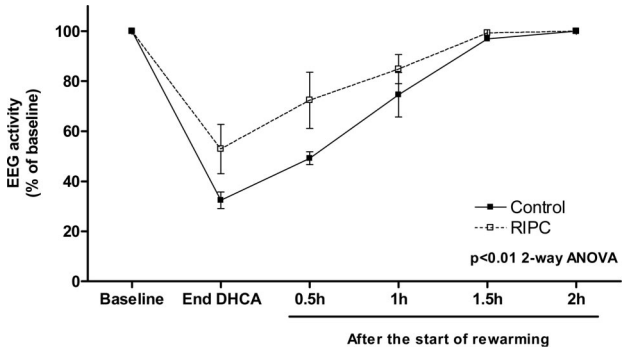

Figure 5. EEG activity. Mean±1 SE.

transfused blood was 52.2 mL/kg (48.4 to 54.8) in the RIPC group and 50.9 mL/kg (44.9 to 53.7) in the control group (P=0.66). The 2 study groups did not differ in duration of cooling (P=0.140) or rewarming (P=1.0), number of defibrillations (P=0.857), or the need for inotropes after the operation (P=0.195). The temperatures during HCA were similar in both groups both relative to the cerebral temperature (P_g=0.67) and the rectal temperature (P_g=0.13). Experimental and metabolic data are summarized in Tables 1 and 2. No clinically significant differences were observed between the groups.

Cardiac Function

After weaning from CPB, the animals in the RIPC group demonstrated a tendency for a better cardiac index (Figure 1). Overall, the difference between group pattern of this variable was significant ($P_{2\text{-way ANOVA}}$=0.001), with the cardiac index showing higher mean levels at 4 and 8 hours after the start of rewarming. The levels of cardiac enzymes also differed between the groups, reflected in the increased release of Troponin I ($P_{2\text{-way ANOVA}}$=0.01) and creatine kinase isoenzyme MB ($P_{2\text{-way ANOVA}}$=0.002) in the control group with mean differences emerging at 2, 4, and 8 hours after the start of rewarming (Figure 2 and 3).

Intracranial Measurements

Microdialysis Data

We did not observe any differences in the concentrations of cerebral glucose (P_g=0.24), glutamate (P_g=0.40), pyruvate (P_g=0.16) or glycerol (P_g=0.16). However, the concentration of cerebral lactate tended to be higher in the control group ($P_{2\text{-way ANOVA}}$=0.0001) and also demonstrated more variance than in the RIPC-group (Figure 4). In spite of this, no differences between the groups were displayed in the glucose-lactate ratio (P_g=0.31) or the lactate-pyruvate ratio (P_g=0.73).

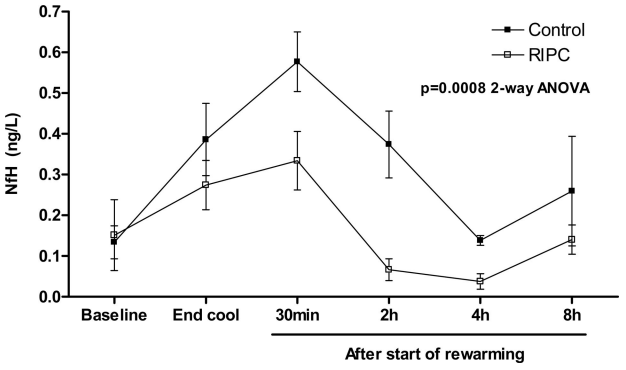

Figure 7. Concentration of NfH. Mean±1 SE.

EEG Monitoring

The EEG activity returned to baseline levels during the 8-hour follow-up period in all animals in both groups. However, the pattern of EEG recovery in the RIPC group seemed to be faster than that of the control group ($P_{2\text{-way ANOVA}}$ <0.01, Figure 5).

Intracranial Pressure

Intracranial pressure tended to be slightly higher in the RIPC group toward the end of the follow-up period (Table 2), however it remained within normal range, and no overall difference between the groups were detected (P_g=0.18).

Neurological Markers

The concentration of S100-B behaved rather similarly in both groups, beginning to rise as rewarming started, peaking at 2 hours after HCA, and falling back to almost baseline levels at the end of the 8-hour follow-up (Figure 6). However, peak concentrations seemed to be higher in the control group ($P_{2\text{-way ANOVA}}$=0.0014).

The level of NfH was observed to be similar in both groups at baseline, but after that the concentration was persistently higher in the control group ($P_{2\text{-way ANOVA}}$=0.0008). In both groups, peak levels were observed right at the beginning of rewarming after HCA (Figure 7).

Behavioral Recovery

All 12 animals survived the experiment and lived for the entire follow-up period. All but 1 animal in the control group

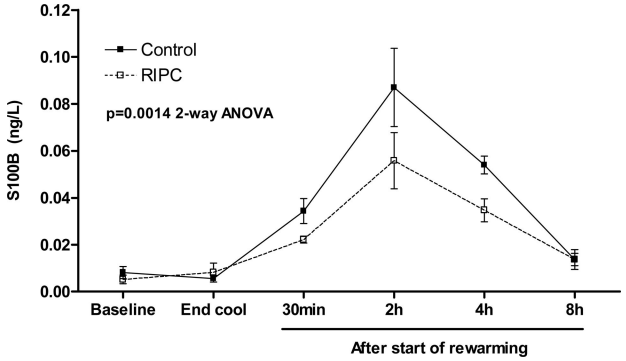

Figure 6. Concentration of S100-B. Mean±1 SE.

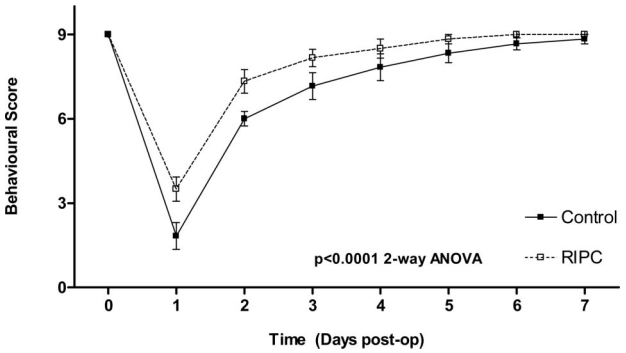

Figure 8. Behavioral score in the postoperative period. Mean±1 SE.

Table 3. Histopathology

Protocol	Survival	Cortex Score	Thalamus Score	Hippocampus Score	Brainstem Score	Cerebellum Score	Total Score	Edema Score	Hemorrhage Score	Neuronal Damage Score	Infarction Score
RIPC											
Mean	7	0.5 (0.0–1.0)	0.7 (0.0–1.5)	0.2 (0.0–0.3)	0.0 (0.0–0.0)	0.2 (0.0–0.3)	1.5 (0.5–2.5)	1.0 (0.0–2.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.5 (0.0–0.8)
Control											
Mean	7	2.0 (1.0–3.3)	1.8 (0.8–3.5)	1.0 (0.8–1.3)	0.3 (0.0–0.5)	0.7 (0.0–1.0)	5.8 (3.8–7.5)	3.5 (2.8–5.0)	0.3 (0.0–1.0)	1.0 (0.0–2.5)	1.0 (0.0–2.5)
P-value		0.02	0.1	0.026	0.317	0.093	0.001	0.002	0.138	0.140	0.523

RIPC n=6, Control n=6. Survival: All animals in both groups survived until the end of the experiment (ie, the seventh postoperative day).

recovered fully in terms of their neurological status, scoring a full 9 points by the seventh postoperative day. The animals of the RIPC group recovered faster ($P_{2\text{-way ANOVA}}=0.0001$), a difference most notable during the first 2 postoperative days (Figure 8). The cumulative behavioral recovery score was 54 (52 to 56) in the RIPC group and 50 (44 to 52) in the control group ($P=0.078$).

Histological Injury

The total histopathological score of ischemic injury was 1.5 (0.5 to 2.5) in the RIPC group and 5.8 (3.8 to 7.5) in the control group ($P=0.001$). The most defining difference between the groups was observed in the cortex and hippocampus (Table 3). Hemorrhages and neuronal damage were observed only in the control group, and the rate of edema was clearly higher compared to the RIPC group ($P=0.002$).

Discussion

The present study demonstrates that RIPC of the limb protects the brain against ischemia–reperfusion injury in a porcine model of HCA. In this surviving animal trial we found that the brain lactate concentration sequentially measured after surgery was lower, and the recovery of EEG activity was faster in the RIPC group compared to the control group. RIPC had a beneficial effect on neurological function during the 7-day follow-up period in terms of behavioral score. Histopathological analysis demonstrated a significant reduction in cerebral injury in RIPC animals compared to controls. Study groups were balanced for baseline and intraoperative parameters.

The overall mortality for complex congenital heart surgery has come a long way. Attention has been directed to neurological dysfunction and developmental sequelae after neonatal cardiac surgery with its support techniques and deep HCA. Some surgical and CPB strategies have been developed to offer cerebral protection at the time of aortic arch reconstruction, such as the pH-stat strategy,[10] high hematocrit,[11] continuous low-flow CPB,[12] and selective regional perfusion.[13]

In experimental myocardial infarction models, RIPC has been shown to reduce infarction size and improve myocardial protection.[14,15,16] RIPC has also been shown to be protective against ischemia–reperfusion damage in the kidney,[17] liver,[18] skeletal muscle,[19] pancreas,[20] small intestine,[21] and brain in association with stroke.[22] In humans, RIPC has been shown to protect against experimental endothelial dysfunction,[16,23] to modify circulating neutrophil gene expression,[24] to reduce myocardial damage, to improve lung function, and to decrease inflammation markers in children undergoing cardiac surgery.[25] Its potential has been strengthened by our recent findings in an acute porcine model of HCA, where RIPC provided a faster recovery of cortical neuronal activity, protection against potential cerebral oxygen radical–mediated ischemia damage, and a decrease in late-phase lactate and pyruvate burst in the brain, mitigating possible damage from an anaerobic metabolism phase.[26]

The present study demonstrates that the neuroprotective effect after a series of blood pressure cuff inflations before HCA in a surviving-animal model was associated with a significant reduction in some important markers of neurological injury, accelerating neurological recovery and mitigating histopathological findings. RIPC might be neuroprotective in patients undergoing surgery with HCA and improve long-term outcomes. If intermittent limb occlusions are shown consistently to reduce neurological injury during complex cardiac surgery with HCA, clinical implications can be very important. The intervention is practical and noninvasive, and clinical trials to further investigate its applications are warranted.

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Disclosures

Dr Tsang is a principal investigator in a study that applies a mortality risk model for case mix in pediatric cardiac surgery for the UK using the Central Audit Database, with funding from the UK National Institute of Health Research. Dr Juvonen is principal investigator in a study on brain protection in cardiac and aortic surgery with grant support from the Finnish Foundation of Cardiovascular Research and the Sigrid Juselius Foundation. The other authors report no conflicts.

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CLINICAL PERSPECTIVE

In the repair of complex congenital heart defects or in surgery of the aortic arch, normal circulation may be temporarily halted to ensure a clean bloodless operation field. The brain is the organ most vulnerable to ischemic injury during this period of hypothermic circulatory arrest (HCA), and the mortality and morbidity of these procedures today consists largely of neurological complications. Ischemic preconditioning, a plausible neuroprotective strategy, describes a concept whereby brief exposure to ischemia, in a dose below the threshold for tissue injury, provides robust protection against the injurious effects of a subsequent more severe insult. In remote ischemic preconditioning, intermittent ischemia is induced in a nontarget tissue such as the limb, and the signal is thought to spread systemically by a mechanism that includes activation of the autonomic nervous system and as yet unidentified humoral mediators. Our study demonstrates that the neuroprotective effect after a series of blood pressure cuff inflations of the hind leg before HCA in a surviving porcine model was associated with a significant reduction in some important markers of neurological injury, accelerated neurological recovery and mitigated cerebral histopathological findings. Remote ischemic preconditioning might be neuroprotective in patients undergoing surgery with HCA and improve long-term outcomes. If intermittent limb occlusions are shown consistently to reduce neurological injury during complex cardiac surgery with HCA, clinical implications can be very important. The intervention is practical, cost effective, and noninvasive. Clinical trials to further investigate its applications are warranted.