Leg ischaemia before circulatory arrest alters brain leucocyte count and respiratory chain redox state

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Received 29 March 2013; received in revised form 9 August 2013; accepted 19 August 2013

Abstract

OBJECTIVES: Remote ischaemic preconditioning and its neuroprotective abilities are currently under investigation and the method has shown significant effects in several small and large animal studies. In our previous studies, leucocyte filtration during cardiopulmonary bypass reduced cerebrocortical adherent leucocyte count and mitigated cerebral damage after hypothermic circulatory arrest (HCA) in piglets. This study aimed to obtain and assess direct visual data of leucocyte behaviour in cerebral vessels after hypothermic circulatory arrest following remote ischaemic preconditioning.

METHODS: Twelve native stock piglets were randomized into a remote ischaemic preconditioning group (n = 6) and a control group (n = 6). The intervention group underwent hind-leg ischaemia, whereas the control group received a sham-treatment before a 60-min period of hypothermic circulatory arrest. An intravital microscope was used to obtain measurements from the cerebrocortical vessel in vivo. It included three sets of filters: a violet filter to visualize microvascular perfusion and vessel diameter, a green filter for visualization of rhodamine-labelled leucocytes and an ultraviolet filter for reduced nicotinamide adenine dinucleotide (NADH) analysis. The final magnification on the microscope was 400. After the experiment, cerebral and cerebellar biopsies were collected and analysed with transmission electron microscope by a blinded analyst.

RESULTS: In the transmission electron microscopy analysis, the entire intervention group had normal, unaffected rough endoplasmic reticulum’s in their cerebellar tissue, whereas the control group had a mean score of 1.06 (standard deviation 0.41) (P = 0.026). The measured amount of adherent leucocytes was lower in the remote ischaemic preconditioning group. The difference was statistically significant at 5, 15 and 45 min after circulatory arrest. Statistically significant differences were seen also in the recovery phase at 90 and 120 min after reperfusion. Nicotinamide adenine dinucleotide autofluorescence had statistically significant differences at 10 min after cooling and at 120 and 180 min after hypothermic circulatory arrest.

CONCLUSIONS: Remote ischaemic preconditioning seems to provide better mitochondrial respiratory chain function as indicated by the higher NADH content. It simultaneously provides a reduction of adherent leucocytes in cerebral vessels after hypothermic circulatory arrest. Additionally, it might provide some degree of cellular organ preservation as implied by the electron microscopy results.

Keywords: Hypothermia/circulatory arrest • Preconditioning brain • Ischaemia/reperfusion injury brain

INTRODUCTION

In surgery of complex congenital heart defects or in surgery of the aortic arch normal circulation may be temporarily halted to ensure a bloodless operating view. To mitigate ischaemic damage to tissues such as the brain, simultaneous deep hypothermia (<18°C) and isolated cerebral perfusion techniques are used. During the last decade, numerous research groups have attempted to find means of improving neurological outcomes after brain ischaemia or trauma [1–4]. The concept of cerebral ischaemic preconditioning and remote ischaemic preconditioning (RIPC) is still a relatively new method and during the last decade, it has shown promise as a new and efficient neuroprotective method in experimental settings [5, 6].

Cardiopulmonary bypass (CPB) and hypothermic circulatory arrest (HCA) induce systemic inflammatory response through a complex interplay of cellular and humoral mechanisms resulting in capillary leakage, tissue oedema and end-organ dysfunction [7]. These responses include activation of complement, coagulation, cytokine release, endothelial activation and expression of leucocyte adhesion molecules [8]. It has been thought that reduced leucocyte accumulation is associated with reduction of the ischaemic brain injury [9, 10]. In previous studies, this leucocyte adhesion in the wall...
of cerebral microvascular bed caused exacerbated oedema formation [11]. Further, accumulation of leucocytes incites their release of oxygen-free radicals and proteolytic enzymes [12, 13].

In our previous study, our data suggested a change in oxygen consumption or metabolism, indicated by the fact that the HCA group had an altered oxygen tension profile during HCA [14]. Additionally, leucocyte filtration has in our previous works shown to improve brain protection and provide beneficial effects in this setting [15, 16]. With an intravital microscope, we are able to gather data associated with both intracerebral leucocytes and the redox state of the neurones of the cortex. We can assess the redox state of the mitochondria of the cerebral neurones with the intravital microscope. Nicotinamide adenine dinucleotide (NAD+) is a molecule that plays a vital role in the mitochondrial respiratory chain of every cell. Crucially, via a phenomenon called autofluorescence we can measure the amount of its reduced form, NADH. This is important because if NADH fluorescence increases it directly correlates with a worsened metabolic state of the mitochondria [17–19]. It means that citric acid cycle, electron transport chain (i.e. respiratory chain) and the formation of adenosine triphosphate from adenosine diphosphate have slowed down as NADH accumulates.

In this study, we aimed to study the effects of RIPC in cerebral microcirculation through direct visualization with intravital microscopy. With the use of three separate filters, we examined vessel diameter changes, leucocyte behaviour and nicotinamide adenine dinucleotide (NAD+/NADH) redox state. We also examined cerebral and cerebellar samples with transmission electron microscopy (TEM) in order to analyse the effect of HCA and RIPC on ultrastructural features of cerebral and cerebellar cells. We hypothesized that some of the beneficial effects reported with RIPC might be due to an altered leucocyte amount or a change in NADH autofluorescence, perhaps even both.

**MATERIALS AND METHODS**

**Experimental setting**

Twelve female (6–7 weeks) piglets from a native stock were randomly assigned to undergo 60 min of HCA at 18°C. Six of the animals were randomized to undergo the 60 min of HCA with right hind leg receiving transient RIPC preoperatively (RIPC group), and six animals were randomized to undergo 60 min of HCA without any preconditioning (control group). For a simplified overview of the study protocol, refer to Fig. 1.

**Preoperative management**

All the 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.

**Anaesthesia protocol and cardiopulmonary bypass**

In our studies, for the sake of comparability, we try to keep the anaesthesia protocol and CPB protocol unchanged. In this study, the anaesthesia protocol and cardiopulmonary procedures are identical to the ones described in our earlier study [14].

**Remote ischaemic preconditioning**

Preconditioning was induced by applying a static pressure of 250 mmHg with a paediatric blood pressure cuff that was wrapped around the right hind leg. The cuff was inflated as proximally as possible, involving most of the thigh and all the tissues distally as to induce a maximum amount of tissue ischaemia in the hind leg. Four cycles of 5-min ischaemia intermittent with three 5-min reperfusion periods were completed. CPB was initiated 60 min after the RIPC, and HCA was achieved in 90 min from the end of the RIPC cycle. The control group had the blood pressure cuff wrapped around the hind leg, but not inflated, for the same period.

**Cranial procedures**

The piglets were positioned on their left side and secured. A cranial window (30 × 30 mm) was created over the right temporal cerebral cortex with a 14/11 mm disposable cranial perforator (200–253 DGR-II, Acra-Cut, Inc., Acton, MA, USA) for intravital microscopy. A probe was inserted medially through the same cranial window to monitor intracerebral temperature (Licox CC1.P1; Integra LifeSciences, Plainsboro, NJ, USA) throughout the experiment. Intracranial temperature readings were recorded at regular intervals during cooling and rewarming perfusion and 10-min interval during HCA.

**Intravital microscopy**

An intravital microscope (Leica Model MZFL III; Leica, Heerbrugg, Switzerland) was placed over the cranial window. Three sets of filters were used: a violet filter, a green filter and an ultraviolet filter for reduced nicotinamide adenine dinucleotide analysis. The image was captured by a video camera. A frame grabber (Kudo Interactive Frame Grabber; Kudo Interactive, Westminster, CO, USA) and a computer-assisted image analysis system (Scion Corporation, Frederick, MD, USA) were used for analysis. The final magnification on the monitor was 400 times. At baseline, the piglet received a 2 ml (4 mg/ml) loading dose of rhodamine 6G chloride MW 479 (Sigma Chemical Co, St. Louis, MO, USA) 5 min before the initial recording. Arterial and venous cerebrocortical...
microvessels with a rough diameter of 0.2–1.0 mm were chosen for the measurements. The vessel chosen were visible without any magnification and the diameters were calculated using the known magnification of the microscope.

**Violet filter.** When a suitable field of vision with both an artery and a venous vessel was discovered, a 1 ml (50 mg/ml) intravenous bolus of fluorescein isothiocyanate (150 kDa; Fluka Chemicals, St. Louis, MO, USA) was administered to label the plasma of the cerebral vessels, and a recording was made with the violet filter of the microscope.

**Green filter.** The piglet received a 1 ml (4 mg/ml) intravenous bolus of rhodamine to stain the activated leucocytes in the circulation. A recording was made with the green filter. The exact number of adherent leucocytes was calculated from an easily determined portion of the vessel, and the number of cells was then related to the surface area of the vessel. The analysis was made by an investigator unaware of the protocol by using both still pictures and videotapes. For rolling leucocytes, a specific point of the vessel was selected, and the number of leucocytes rolling past that point was observed during ~10–15 s in each recording.

**Ultraviolet filter.** The monitoring of NADH is based on the absorption difference of NADH and the oxidized form of nicotinamide adenine dinucleotide (NAD+). The NAD+ does not absorb light at 320–380 nm, but NADH does. The fluorescence emission of NADH is peaked near 450 nm, as described in previous studies [20]. The epi-illumination was limited to <1 min to avoid thermal injury, and the epi-illumination was always stopped between video recordings.

**Biochemical data**

Blood gas values, pH, electrolytes, complete blood cell counts, plasma lactate levels, serum ionized calcium, glucose, haematocrit and haemoglobin levels were measured at baseline, at the end of cooling (immediately before institution of HCA), and at 30 min, 1 h, 2 h and 3 h after the start of rewarming (i-STAT Analyzer; i-STAT Corporation, East Windsor, NJ, USA). Baseline measurements were taken after anaesthesia, cranial procedures, invasive haemodynamic monitoring had been started and the thoracotomy had been performed, but no cardiac manipulation or cannulation had taken place. Additionally, central temperature readings, urine amount, amount of fluids infused and amount of donor blood were recorded.

**Transmission electron microscopy**

Specimens for TEM were collected from the right cerebral cortex of temporal lobe and right cerebellar cortex 3 h postoperatively. The specimen from cerebrum was taken from the same spot the intravital microscopy had taken place. After collecting the specimens, the animals were euthanized using pentobarbitral (60 mg/kg) while anaesthetized. Also specimens were collected from one animal, which did not undergo HCA (zero-control), in order to compare the TEM results with a tissue unaffected by HCA. All specimens were immediately fixed in 1% glutaraldehyde, 4% formaldehyde mixture in 0.1 M phosphate buffer. They were post-fixed in 1% osmiumtetroxide, dehydrated in acetone and embedded in Epon LX 112 (Ladd Research Industries, Williston, VT, USA). Thin sections were cut with Leica Ultracut UCT ultramicrotome, stained in uranyl acetate and lead citrate and examined in Tecnai G2 Spirit transmission electron microscope (FEI Europe, Eindhoven, Netherlands). Images were captured by Veleta CCD camera (Olympus Soft Imaging Solutions GmbH, Munster, Germany).

**Electron microscope score for ischaemia-related ultrastructural changes in cerebral and cerebellar cells**

A blinded experienced analyst assessed three factors in cerebral and cerebellar TEM samples. The three factors analysed were: ultrastructure, rough endoplasmic reticulum (RER) and mitochondria. Ultrastructural changes included neuroglial cells and neurons, and any structural changes in astrocytic perivascular endfeet. The observed mitochondrial changes were swelling, disrupted crystal integrity and loss of matrix density, the observed changes in RER were dilatation and the presence of vacuoles and the observed changes in the ultrastructural features of astrocytes were swelling of astrocytic soma and perivascular endfeet, ruptured cytoplasmic and mitochondrial membranes and electron lucent cytoplasm. Structural alterations were scored as follows: normal = 0, mild = 1, moderate = 2 and severe = 3. The total score was summed of these three scores for both cerebrum and cerebellum, yielding a maximum score of 18.

**Statistical analysis**

Statistical analysis was performed with SPSS (version 20.0; SPSS, Inc., Chicago, IL, USA) and SAS (version 9.2; SAS Institute, Cary, NC, USA) statistical software packages. Continuous and ordinal variables are expressed as mean values with standard deviation (SD) in parenthesis or median values with 25th–75th percentile. Either the Student t-test or Mann–Whitney U-test was used to assess P-values for continuous variables between the groups. The repeatedly measured data were analysed using a linear mixed model with patients fitted as random, and the best covariance pattern was chosen according to Akaike’s information criteria. Two-tailed significance levels are reported. Reported P-values are as follows: P between groups (P × g) indicates a level of difference between the groups; P for time (P × t) indicating difference and changes during time; P for time by group (P × t × g), indicates behaviour difference between the groups within time.

**RESULTS**

**Comparison of study groups**

The median weight of the animals was 19.0 kg (17.4–20.5 kg), with no statistically significant differences between groups. Baseline haemoglobin in the RIPC group was 88.5 g/l (82.0–92.0 g/l) and in the control group 76.5 g/l (71.0–78.0 g/l). The pigs were given 61.9 ml/kg (54.9–70.1 ml/kg) donor blood, with no significant difference between groups. Experimental and metabolic data are given in Table 1.

Blood gas analysis and electrolyte content were comparable preoperatively, with only significant difference at PCO₂, which was slightly higher in the control group than in the RIPC group: 5.53 kPa (5.46–5.60) and 5.33 kPa (4.86–5.36), respectively (P < 0.05).
There were no significant differences in mean arterial pressure, central venous pressure and pulmonary capillary wedge pressure between the groups at the baseline. Troponin I levels were at the baseline similar between groups.

**Temperatures**

Blood, rectal and intracerebral temperatures had no statistically significant differences preoperatively and during HCA. The only significant difference at 2 h after HCA, when blood temperature was lower in the control group compared with the RIPC group: 35.8°C vs 35.7°C. However, that difference was rectified by the last time point.

**Systemic blood cell count**

Total leucocyte count in blood was in the RIPC group 24.4 × 10⁹/l (16.1 × 10⁹/l–25.7 × 10⁹/l) and in the control group 18.0 × 10⁹/l (16.9 × 10⁹/l–21.4 × 10⁹/l) at the baseline, with no statistically significant differences between groups (Table 1).

**Adherent leucocytes and rolling leucocytes**

There were no statistically significant differences in arterial vessel calibres at any time points. The diameters of selected venous vessels did not differ significantly at any time point during the experiment.

**Vessel diameter**

The calculated vessel diameters of selected arteries were 0.36 mm (0.33 mm–0.42 mm) in the RIPC group and 0.38 mm (0.26 mm–0.51 mm) in the control group, at baseline with no statistically significant difference between groups. There were no statistically significant differences in arterial vessel calibres at any time points. The diameters of venous vessels were 0.57 mm (0.46 mm–0.66 mm) at baseline in the RIPC group and 0.77 mm (0.73 mm–0.78 mm) in the control group. (P = 0.12) at baseline. The diameter of selected venous vessels did not differ significantly at any time point during the experiment.

**Nicotinamide adenine dinucleotide autofluorescence**

The total score of both cerebral and cerebellar changes in the control group was 9.66 (SD 1.71) and in the RIPC group 7.66 (SD 1.40) (P = 0.54). Statistically significant differences were seen in the

**Transmission electron microscopy**

There were no statistically significant differences in mean arterial pressure, central venous pressure and pulmonary capillary wedge pressure between the groups at the baseline. Troponin I levels were at the baseline similar between groups.

### Table 1: Metabolic data

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Cooling, 30 min</th>
<th>After the end of HCA</th>
<th>P&lt;sub&gt;G&lt;/sub&gt;</th>
<th>P&lt;sub&gt;tg&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>RIPC</td>
<td>7.43(7.42–7.48)</td>
<td>7.36(7.35–7.46)</td>
<td>7.42(7.30–7.52)</td>
<td>7.27(7.24–7.32)</td>
<td>7.33(7.28–7.37)</td>
</tr>
<tr>
<td>CTRL</td>
<td>7.43(7.41–7.45)</td>
<td>7.32(7.27–7.36)</td>
<td>7.52(7.45–7.56)</td>
<td>7.24(7.14–7.29)</td>
<td>7.24(7.20–7.30)</td>
</tr>
<tr>
<td>PaO&lt;sub&gt;2&lt;/sub&gt; (kPa)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIPC</td>
<td>5(4.7–5.1)</td>
<td>41.65(34.9–52.9)</td>
<td>6(5.6–6.2)</td>
<td>3.9(3.4–4.5)</td>
<td>4.45(4.4–4.8)</td>
</tr>
<tr>
<td>CTRL</td>
<td>5(5–5.5)</td>
<td>40.15(38.1–42)</td>
<td>5.15(5–5.4)</td>
<td>5(4.7–5.2)</td>
<td>5.3(4.5–6)</td>
</tr>
<tr>
<td>Systemic white blood cell count ×10⁹/l</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>RIPC</td>
<td>20.2(16.1–24.4)</td>
<td>7.4(4.7–10.1)</td>
<td>12(9.3–14.6)</td>
<td>17(9.2–24.8)</td>
<td>32.3(32.1–32.5)</td>
</tr>
<tr>
<td>CTRL</td>
<td>18(16.6–20.7)</td>
<td>7.7(6.7–9.6)</td>
<td>13.1(11.9–18.4)</td>
<td>18.6(14.5–20.5)</td>
<td>27(22.8–35)</td>
</tr>
<tr>
<td>Cardiac index (cardiac output/pump flow)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>RIPC</td>
<td>2.72(2.41–3.03)</td>
<td>1.89(1.27–2.20)</td>
<td>1.78(1.36–1.92)</td>
<td>2.4(2.21–2.61)</td>
<td>2.27(1.83–3.30)</td>
</tr>
<tr>
<td>CTRL</td>
<td>2.87(2.50–3.38)</td>
<td>1.82(1.30–2.05)</td>
<td>1.36(1.20–1.64)</td>
<td>2.01(1.98–2.76)</td>
<td>2.68(2.51–2.69)</td>
</tr>
<tr>
<td>Haemoglobin (g/l)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIPC</td>
<td>89(82–92)*</td>
<td>65(58–71)</td>
<td>64(58–71)</td>
<td>66(58–71)</td>
<td>75(65–85)</td>
</tr>
<tr>
<td>CTRL</td>
<td>75(71–78)*</td>
<td>61(58–68)</td>
<td>68(58–79)</td>
<td>78(75–88)</td>
<td>75(65–78)</td>
</tr>
</tbody>
</table>

*Values are shown as medians and 25th and 75th percentiles; P between groups: level of difference between groups.
RIPC: remote ischaemic preconditioning group; CTRL: control group; *P < 0.05, P<sub>G</sub>: P-value between groups (level of difference between groups); P<sub>tg</sub>: P-value time × group (behaviour between groups over time).
RIPC could possibly provide cerebral blood vessels of the brain. Additionally, RIPC has been documented to decrease vascular resistance and endothelial dysfunction in the early phases of ischaemia, which may partially explain this finding [23]. Interestingly, the total systemic white blood cell counts were actually higher in the RIPC group at 1-3 h postoperatively, while the cerebral leucocyte count was lower than in the control group. Obviously, a higher temperature increases the number of rolling and adherent leucocytes in itself [24] but at 2 h postoperatively the control group was cooler than the RIPC group and nonetheless the leucocytes counts in cerebral vessels were higher at that time. There were no statistically significant differences between the groups in intracerebral, rectal or blood temperatures, which add to the credibility of the result. This observation could indicate that RIPC does not have beneficial effects on total leucocyte count but could possibly reduce accumulation of leucocytes to the brain. It also implies that systemic white blood cell count may not directly correlate with the numbers of leucocytes in the brain.

As expected after long HCA and reperfusion, alterations in cerebral and cerebellar ultrastructura were evident. However, only one statistically significant difference between groups was measured. The control group had a higher cerebellar RER score than RIPC. The clinical relevance of this finding, however, remains unclear as it is an isolated result in our data. TEM score showed considerable variability in the severity of alterations, as analysed changes varied from non-detectable to severe in individual animals within both experimental groups.

NADH autofluorescence measurements can be used to measure in vivo the redox state of the mitochondria [17]. The increase of reduced nicotinamide adenine dinucleotide (NADH) autofluorescence implicates worse tissue oxygenation [18] and maximal NADH autofluorescence readings are measured in anoxic conditions [19].
Although, haemoglobin had a statistically significant difference at baseline (Table 1); the difference was nullified in subsequent time points. This is important because haemoglobin differences can cause artefacts in NAD+–NADH autofluorescence measurements [17]. A higher NADH autofluorescence directly correlates with a worsened metabolic state of the mitochondria and worsened respiratory chain function [17–19]. A worsened respiratory chain function in turn correlates with an increase in reactive oxygen species production and cellular protein and membrane peroxidation [25].

RIPC has been studied more in other organs besides the brain, although it has shown some advantageous effects in brain protection in studies performed with small rodents. Our previous results [5, 6, 14] combined with the current study result are, to our knowledge, the first studies utilizing large animals that have shown beneficial results with RIPC conferring resistance against global ischaemia of the brain. Some studies with RIPC and the protection of other organs, such as the heart, have even reached small-scale clinical human trials. However, RIPC has not yet been studied in humans with cases of global brain ischaemia.

In conclusion, our study demonstrates that RIPC reduces the number of adherent leucocytes in the cerebral topical circulation after HCA. Additionally, the NADH autofluorescence result indicates that the RIPC group had a better respiratory chain function during the recovery phase. It is important to keep in mind that our study has limitations; secondly, this study involved a small number of animals and due to this a few outlier results can sway the data either way.

Nonetheless, we feel that our study warrants long-term studies that include detailed immunological and white blood cell analysis in conjunction with RIPC. We hope that large animal studies with RIPC and brain protection could form a steppingstone between the animal studies performed with the use of rodents and possible future human clinical trials with RIPC and global ischaemia.

**FUNDING**

This work was supported by the Finnish Foundation for Cardiovascular Research and the Sigrid Juselius Foundation.

**Conflict of interest:** none declared.

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