Oiva Arvola

REMOTE ISCHEMIC PRECONDITION BEFORE HYPOTHERMIC CIRCULATORY ARREST IN A PORCINE MODEL

A SPECIAL REFERENCE TO OXIDATIVE STRESS
OIVA ARVOLA

REMOTE ISCHEMIC PRECONDITION BEFORE HYPOTHERMIC CIRCULATORY ARREST IN A PORCINE MODEL
A special reference to oxidative stress

Academic Dissertation to be presented with the assent of the Doctoral Training Committee of Health and Biosciences of the University of Oulu for public defence in Auditorium L1 of Oulu University Hospital (Kajaanintie 5; entrance B1), on 10 February 2017, at 12 noon

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Abstract

In pathologies of the ascending aorta or in congenital heart defects, circulation may be temporarily halted during surgical intervention. This is achieved by operating under deep hypothermic circulatory arrest or under hypothermia combined with isolated perfusion techniques. For deep hypothermic circulatory arrest (DHCA), the patient is cooled below 18°C using an extracorporeal heart-lung machine, and circulation and breathing are stopped. The advantage of hypothermia is that it decreases oxygen and glucose consumption and provides the surgeons the time required to repair complex heart defects. However, there is still a relatively high risk of neurological complications that can affect the quality of life of patients and their families.

One of the methods to mitigate ischaemia-reperfusion injury is remote ischaemic preconditioning. In this work, the neuroprotective mechanisms of remote ischaemic preconditioning (RIPC) were studied in acute and surviving chronic animal models. In study I, we used an acute model, and studied the effects of RIPC in cerebral microcirculation using an intravital microscope and samples analysed by transmission electron microscope. In study II, a chronic model was used to evaluate whether the effects of remote ischaemic preconditioning can be seen in the markers of oxidative stress or in redox-regulating enzymes. Study III was conducted to supplement the findings of study II, considering the markers of oxidative stress. Findings in all studies were consistent with one another.

Study I showed the effect of remote ischaemic preconditioning on leukocyte activation and adhesion to cerebrocortical vessels in piglets after prolonged DHCA. Additionally, cellular preservation of endoplasmic reticulum was present in transmission electron microscope analysis of the central nervous system. In studies II and III, the remote ischaemic preconditioning lowered markers of ischaemia-reperfusion-related oxidative stress. In study III the remote ischemic preconditioning lowered oxidative stress already during cardiopulmonary bypass.

Keywords: brain damage, brain protection, cardiac surgery, oxidative stress, remote ischemic preconditioning
Arvola, Oliva, Esialtistava raajaiskemia sydän- ja aortakirurgiassa porsasmallilla. Oksidatiivisen stressin asema
Oulun yliopiston tutkijakoulu; Oulun yliopisto, Lääketieteellinen tiedekunta; Medical Research Center Oulu
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Tiivistelmä
Ensimmäisessä osatyössä esialtistava raajaiskemia vaikutti leukosyyttiaktivaatioon ja leukosyyttien tarttumiseen aivojen pintaverisuonien hypotermisen verenkierron seisausten jälkeen. Myös hermosolujen sisäisten soluelinten säilyminen näkyi läpäisylelektronimikroskooppilla raajaisksiemia ryhmällä.
Tutkimuksissa II ja III esialtistavan raajaiskemian todettiin alentavan oksidatiivisen reperfusiion aiheuttamaa oksidatiivista stressiä, jonka todettiin tapahtuvan/alkavan jo sydän-keuhkokoneen käytön aikana tutkimuksen III perusteella.

Asiakirjat: aivojen suojaus, aivovaurio, esialtistava ischemia, oksidatiivinen stressi, sydänkirurgia
To my beloved wife Maija
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Lohja, Finland, December 2015

Oiva Arvola
Abbreviations

8-OHdG  8-hydroxydeoxyguanosine  
AAD  Stanford type A acute aortic dissection  
AD  Anoxic repolarisation  
ADP  Adenosine diphosphate  
AIF  Apoptosis inducing factor  
AMPA  $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid  
ARE  Antioxidant response element  
ATP  Adenosine triphosphate  
C  Celsius  
$Ca^{2+}$  Calcium ion  
CBF  Cerebral blood flow  
CK MB  Creatine kinase isoenzyme MB  
CNS  Central nervous system  
CO2  Carbon dioxide molecule  
CPB  Cardiopulmonary Bypass  
CRP  Complement reactive protein  
CXR  Chest radiograph  
e.g.  exempli gratia  
eNOS  Endothelial nitric oxide synthase  
ETC  Electron transport chain  
etc.  et cetera  
H2O2  Hydrogen peroxide  
HCA  Hypothermic circulatory arrest  
HIF-1-$\alpha$  Hypoxia-inducible factor 1, alpha subunit  
HIF-1-$\beta$  Hypoxia-inducible factor 1, beta subunit  
i.e.  id est  
IL  Interleukin  
iNOS  Inducible nitric oxide synthase  
IPC  Ischaemic preconditioning  
K+  Potassium ion  
Keap1  Kelch ECH associating protein 1  
MAPK  Mitogen-activated protein kinase  
Mn  Manganese  
mtDNA  Mitochondrial DNA  
mV  Millivolts
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>Sodium ion</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Transcription factor nuclear factor – κB</td>
</tr>
<tr>
<td>NMIA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>Nrf2</td>
<td>Nuclear factor erythroid 2-related factor 2</td>
</tr>
<tr>
<td>O₂</td>
<td>Oxygen molecule</td>
</tr>
<tr>
<td>OH⁻</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>pCO₂</td>
<td>Partial pressure of carbon dioxide</td>
</tr>
<tr>
<td>pH</td>
<td>Hydrogen ion concentration i.e. acidity</td>
</tr>
<tr>
<td>Pi</td>
<td>Phosphate ion, PO₄³⁻</td>
</tr>
<tr>
<td>PKCε</td>
<td>the protein kinase C ε</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>pO₂</td>
<td>Partial pressure of oxygen</td>
</tr>
<tr>
<td>PTP</td>
<td>Permeability transition pore</td>
</tr>
<tr>
<td>PVC</td>
<td>Polyvinyl Chloride, a synthetic plastic polymer</td>
</tr>
<tr>
<td>RIPC</td>
<td>Remote ischaemic preconditioning</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RER</td>
<td>Rough endoplasmic reticulum</td>
</tr>
<tr>
<td>Se</td>
<td>Selenium</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle cell</td>
</tr>
<tr>
<td>TAA</td>
<td>Thoracic aortic aneurysm</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TGA</td>
<td>Transposition of great arteries</td>
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<tr>
<td>TNFα</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor – f</td>
</tr>
</tbody>
</table>
List of original publications

This thesis is based on the following publications, which are referred throughout the text by their Roman numerals:


* = equal contribution in article
# Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>9</td>
</tr>
<tr>
<td>Tiivistelmä</td>
<td>13</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>15</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>17</td>
</tr>
<tr>
<td>List of original publications</td>
<td>19</td>
</tr>
<tr>
<td>Contents</td>
<td>21</td>
</tr>
<tr>
<td>1 Introduction</td>
<td>23</td>
</tr>
<tr>
<td>2 Review of literature</td>
<td>25</td>
</tr>
<tr>
<td>2.1 Operations under deep hypothermic circulatory arrest in cardiothoracic surgery</td>
<td>27</td>
</tr>
<tr>
<td>2.1.1 Aortic dissection</td>
<td>27</td>
</tr>
<tr>
<td>2.1.2 Aortic aneurysm</td>
<td>28</td>
</tr>
<tr>
<td>2.1.3 Congenital heart defects</td>
<td>30</td>
</tr>
<tr>
<td>2.2 Methods of cerebral protection in aortic arch surgery</td>
<td>32</td>
</tr>
<tr>
<td>2.2.1 Cardiopulmonary bypass</td>
<td>32</td>
</tr>
<tr>
<td>2.2.2 Deep hypothermic circulatory arrest</td>
<td>33</td>
</tr>
<tr>
<td>2.3 Human central nervous system</td>
<td>35</td>
</tr>
<tr>
<td>2.3.1 The brain anatomy</td>
<td>35</td>
</tr>
<tr>
<td>2.3.2 Neurons and glial cells</td>
<td>36</td>
</tr>
<tr>
<td>2.3.3 Cerebral blood flow</td>
<td>37</td>
</tr>
<tr>
<td>2.3.4 Homeostasis of functioning nervous cells</td>
<td>38</td>
</tr>
<tr>
<td>2.4 Nervous cell damage, ischaemia, and infarction after global ischaemia</td>
<td>39</td>
</tr>
<tr>
<td>2.4.1 Loss of function</td>
<td>39</td>
</tr>
<tr>
<td>2.4.2 Glutamate</td>
<td>40</td>
</tr>
<tr>
<td>2.4.3 Mitochondria</td>
<td>41</td>
</tr>
<tr>
<td>2.4.4 The role of accumulating Ca$^{2+}$</td>
<td>42</td>
</tr>
<tr>
<td>2.4.5 Calpains and NO</td>
<td>43</td>
</tr>
<tr>
<td>2.4.6 Reactive oxygen species</td>
<td>44</td>
</tr>
<tr>
<td>2.4.7 DNA damage and 8-OHdG</td>
<td>45</td>
</tr>
<tr>
<td>2.4.8 Neuronal apoptosis and necrosis</td>
<td>46</td>
</tr>
<tr>
<td>2.4.9 Selective vulnerability</td>
<td>47</td>
</tr>
<tr>
<td>2.5 Innate response to neuronal ischaemic injury</td>
<td>48</td>
</tr>
<tr>
<td>2.5.1 Cytokines and ischaemic signalling cascade</td>
<td>49</td>
</tr>
<tr>
<td>2.5.2 White blood cells</td>
<td>50</td>
</tr>
<tr>
<td>Section</td>
<td>Title</td>
</tr>
<tr>
<td>---------</td>
<td>-------</td>
</tr>
<tr>
<td>2.6</td>
<td>Natural countering measures for oxidative stress</td>
</tr>
<tr>
<td>2.6.1</td>
<td>Nrf2</td>
</tr>
<tr>
<td>2.6.2</td>
<td>HIF-1-α</td>
</tr>
<tr>
<td>2.6.3</td>
<td>DJ-1</td>
</tr>
<tr>
<td>2.7</td>
<td>Remote ischaemic preconditioning</td>
</tr>
<tr>
<td>2.7.1</td>
<td>Cerebral and cardial protection</td>
</tr>
<tr>
<td>2.7.2</td>
<td>Molecular mechanisms of remote ischaemic preconditioning</td>
</tr>
<tr>
<td>3</td>
<td>Aims of study</td>
</tr>
<tr>
<td>4</td>
<td>Materials and methods</td>
</tr>
<tr>
<td>4.1</td>
<td>Experimental animals</td>
</tr>
<tr>
<td>4.2</td>
<td>Perioperative management</td>
</tr>
<tr>
<td>4.3</td>
<td>Blood sampling</td>
</tr>
<tr>
<td>4.4</td>
<td>Blood transfusion</td>
</tr>
<tr>
<td>4.5</td>
<td>Remote ischaemic preconditioning</td>
</tr>
<tr>
<td>4.6</td>
<td>Cardiopulmonary bypass</td>
</tr>
<tr>
<td>4.7</td>
<td>Intravital microscopy</td>
</tr>
<tr>
<td>4.8</td>
<td>Transmission electron microscopy (TEM)</td>
</tr>
<tr>
<td>4.9</td>
<td>Sagittal sinus blood sampling</td>
</tr>
<tr>
<td>4.10</td>
<td>Additional cranial procedures</td>
</tr>
<tr>
<td>4.11</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>4.12</td>
<td>Histopathology</td>
</tr>
<tr>
<td>4.13</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>4.14</td>
<td>Cardiac parameters</td>
</tr>
<tr>
<td>4.15</td>
<td>Additional measurements</td>
</tr>
<tr>
<td>4.16</td>
<td>Statistical analysis</td>
</tr>
<tr>
<td>5</td>
<td>Results</td>
</tr>
<tr>
<td>5.1</td>
<td>Study I</td>
</tr>
<tr>
<td>5.1.1</td>
<td>Comparison of study groups</td>
</tr>
<tr>
<td>5.1.2</td>
<td>Intravital microscopy</td>
</tr>
<tr>
<td>5.1.3</td>
<td>Transmission electron microscopy (TEM)</td>
</tr>
<tr>
<td>5.2</td>
<td>Study II</td>
</tr>
<tr>
<td>5.2.1</td>
<td>Comparability of study groups</td>
</tr>
<tr>
<td>5.2.2</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>5.2.3</td>
<td>Histopathology and immunohistochemistry</td>
</tr>
<tr>
<td>5.3</td>
<td>Study III</td>
</tr>
<tr>
<td>5.3.1</td>
<td>Comparability of study groups</td>
</tr>
</tbody>
</table>
5.3.2 Enzyme-linked immunosorbent assay ............................................. 70
5.3.3 Additional measurements .............................................................. 71

6 Discussion .................................................................................. 75
  6.1 Study design ............................................................................... 75
  6.2 Intravital microscopy .................................................................. 75
    6.2.1 Vessel diameters .................................................................. 75
    6.2.2 Leukocytes ......................................................................... 76
    6.2.3 NAD+/NADH redox state ...................................................... 76
  6.3 Transmission electron microscopy (TEM) .................................... 76
  6.4 Reactive oxygen species (ROS) findings ...................................... 77
  6.5 Limitations of the study .............................................................. 78
  6.6 Summary .................................................................................. 79
  6.7 Clinical implications .................................................................. 79

7 Conclusions .............................................................................. 81

References .................................................................................. 83

Original publications ..................................................................... 101
1 Introduction

In aortic arch and congenital heart defect surgery, an interruption of the blood flow is often inevitable in order to make surgical repairing possible. This jeopardises the central nervous system with the associated ischaemic period. The cells of the brain, neurons, rely on a constant supply of oxygen and nutrients. Interruption of the cerebral blood flow causes dysfunction at the cellular level, predisposing the patient to irrepairable brain damage. Hypothermia is used to lower cellular metabolic activity and preserve neurons during the ischaemic period. Cardiopulmonary bypass is used for core cooling prior to hypothermic circulatory arrest.

Due to vulnerability of the central nervous system, new strategies have been studied to mitigate the neurological complications in complex heart surgery. During the last two decades, various cerebral perfusion techniques, in addition to pharmacological agents and stem cell-therapies as well as remote ischaemic preconditioning have been studied. Not only the ischaemia itself, but also the reperfusion to ischaemic tissues, contributes to ischaemic insult.

Remote ischaemic preconditioning is a concept that brief intermittent episodes of non-lethal ischemia, followed by reperfusion at a distant organ protects another (target) organ or tissue from subsequent ischemic insult. The mechanisms behind neuroprotective effects and the outcome of remote ischaemic preconditioning are still unclear, and somewhat controversial.

This study was conducted to explore the cellular mechanisms of remote ischaemic preconditioning in a large animal mode, simulating a prolonged heart operation under deep hypothermic circulatory arrest. In the first study (I), we investigated the effects of remote ischaemic preconditioning in cerebral microcirculation, NAD+/NADH redox state, and ultrastructural chances of single neurons. In studies II and III, markers of oxidative stress were analysed as were histopathological and immunohistochemical changes in preconditioned subjects, after 7 days of prolonged deep hypothermic circulatory arrest.
2 Review of literature

2.1 Operations under deep hypothermic circulatory arrest in cardiothoracic surgery

A large variety of cardiac and pulmonary conditions are treated in the field of cardiothoracic surgery. A minority of those operations are performed under the protection of deep hypothermic circulatory arrest.

2.1.1 Aortic dissection

Stanford type A acute aortic dissection (AAD) (DeBakey type I and II) has high acute phase mortality. For those patients who are not able to be treated in a hospital setting, mortality rate rises 1–2% / hour for the first 48 hours, leading to mortality of almost 90% in 2 days follow-up, untreated. Fully accurate numbers are not available, due to the high mortality rate—not all cases reach the status of knowledge (Erbel et al. 2001). The incidence of AAD varies from 4.7 to 6/100,000/year (Howard et al. 2013a, Pacini et al. 2013, Yeh et al. 2015).

Type A aortic dissection is followed by pathology in the aorta. Usually due to atherosclerosis, the intima thickens, leading to lower nutrient and oxygen supply and degeneration. Vasa vasorum may also be blocked or ruptured, causing necrosis in the aortic arch. These mechanisms can cause rupture in the intima, causing AAD or intramural hematoma via the vasa vasorum rupture pathway. Intramural hematomas often proceed to AAD. AAD may also occur after blunt trauma to the chest, or after iatrogenic trauma. (Erbel et al. 2001, Stefanadis et al. 1993).

When the intima ruptures, blood flows between the intima and the outer layers of the aortic arch. In AAD, the newly formed false lumen is in the ascending aorta, and blood pressure pushes the false lumen to a size that is larger than the actual lumen. The false lumen can tear the dissection in a retrograde and antegrade fashion from the initial breaking point, when pressurised, involving the cranial ascending arteries. Rupture in the dissected aorta and effusion to the pericardium causing tamponade are the causes of death in AAD patients.

The main goal in surgical treatment of AAD is to prevent rupture and pericardial tamponade. The dissected aorta is approached through medial sternotomy, and the patient is cannulated for cardiopulmonary bypass. Depending
on the site of initial tear in the intima and function of the aortic valve, multiple techniques are used for prosthetic replacement of the dissected aorta (Bentall & De Bono 1968, Kouchoukos et al. 1980, Okita et al. 2015). The in-hospital mortality rate after aortic reconstruction of AAD patients varies from 9% to 11% with best practices, and the 5-year-survivability is approximately 69% (Murzi et al. 2014, Rylski et al. 2014). Globally, the in-hospital mortality rate is approximately 18–19% after surgical intervention (Pape et al. 2015, Yeh et al. 2015), with neurological complications ranging from 3 to 20% (Leshnower et al. 2014, Shrestha et al. 2014, Tsiouris et al. 2014).

2.1.2 Aortic aneurysm

Aortic dissection is a devastating complication of thoracic aortic disease. Dissections can occur without aneurysms, but aneurysm is a major risk factor of aortic dissection. The sex- and age-adjusted incidence of thoracic aortic diseases (thoracic aortic aneurysm and dissection) has been increasing, recently reaching 16.3 per 100,000 per year in men and 9.1 per 100,000 per year in women (Howard et al. 2013b, Olsson et al. 2006).

An aneurysm is defined as a permanent arterial dilation to more than 50% of the expected normal diameter (Johnston et al. 1991). Hager et al. suggested that the diaphragmatic level be chosen as the reference level for dilatation, as aneurysms and hypoplasia are rarely found there (Hager et al. 2002). The aortic diameter is influenced by age, body size, sex, and method used for measurement (Hager et al. 2002, Hannuksela et al. 2006). Suggested normal value measurements are listed in Table 1 (Johnston et al. 1991).

Structural changes in the media layer of the thoracic aorta result in development of idiopathic form of thoracic aortic aneurysm (TAA). In healthy aortic sections layers of smooth muscle cells (SMCs) are separated by prominent elastic lamellae, interconnected by a network of small elastin and collagen fibres and proteoglycans (de Figueiredo Borges et al. 2008). In aortic sections from aneurysmal aortas, disorganisation, breakdown of collagen network, and reduction of SMCs are present, as well as cystic medial degeneration of vacuolated basophilic material, causing mechanical dysfunction of the aorta (Borges et al. 2010). Increased elastolytic matrix metalloproteinases (MMPs), MMP-2 and MMP-9, are seen in the media of thoracic aortic aneurysms, explaining part of the biochemical pathways (Ikonomidis et al. 2007, LeMaire et al. 2005).
Other forms of TAA include a variety of different genetic or inherited conditions, and inflammatory conditions. These include Marfan syndrome (Wisler et al. 2015), Loeys-Dietz syndrome (Loeys et al. 2005), Vascular Ehlers-Danlos syndrome (Cury et al. 2013), and Turner syndrome (Matura et al. 2007), as well as familial thoracic aortic aneurysm and dissection syndrome (Regalado et al. 2011) and bicuspid aortic valve (Alegret et al. 2003). Pathologies in the elastic tissue of the medial layer of the aorta via multiple pathways are common to all these conditions (Cury et al. 2013).

The mechanical wall stress in the aortic dilatation zone is increased in accordance to Laplace’s law. In a cylinder, i.e. the aorta, the wall tension (T) equals pressure difference (P) (pressure difference between the inside and outside of the aorta) multiplied by radius (R): \( T = PR \) (Valentinuzzi & Kohen 2011). This enlargement causes further expansion of the aorta due to increased wall tension. As the tensile strength limit is reached, dissection occurs.

<table>
<thead>
<tr>
<th>Table 1. Normal Adult Thoracic Aortic Diameters</th>
</tr>
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<tbody>
<tr>
<td>Thoracic Aorta</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Root (male)</td>
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<tr>
<td>Root (female)</td>
</tr>
<tr>
<td>Ascending</td>
</tr>
<tr>
<td>(male, female)</td>
</tr>
<tr>
<td>Mid-descending (male)</td>
</tr>
<tr>
<td>Mid-descending (female)</td>
</tr>
<tr>
<td>Diaphragmatic (male)</td>
</tr>
<tr>
<td>Diaphragmatic (female)</td>
</tr>
</tbody>
</table>

Modified from Johnston et al. 1991

When the risk for rupture exceeds the known risks of aortic transplant surgery, intervention is needed. Also, patients with symptoms are operated upon, regardless of the size of the aorta. When the diameter of ascending aorta reaches over 6 cm or when the descending aorta’s diameter reaches over 7 cm, the risk of rupture or dissection peaks (Elefteriades 2002). The American Association for Thoracic Surgery jointly updated in 2010 with multiple professional societies the guidelines for surgical intervention for thoracic aortic disease. Repair is
recommended for arch diameter reaching 5.5 cm in low-risk patients without symptoms. The guideline considers each patient’s risk and comorbidity, and the surgical and perioperative management varies accordingly (Hiratzka et al. 2010).

2.1.3 Congenital heart defects

The incidence of moderate or severe congenital heart defects is 3–6/1,000 live births and all forms of defects occur in 75/1,000 live births (Hoffman & Kaplan 2002). A variety of infant heart malformations are operated under hypothermic circulatory arrest; these include transposition of great arteries (TGA), tetralogy of Fallot, ventricular septal defect, and complete common atrioventricular canal defect. These diseases can predispose to stroke and neurodevelopmental disabilities in 10% of patients. Half of the strokes occur prior to operation (Chen et al. 2009). The risk of brain injury in infant heart surgery is a sum of cardiopulmonary bypass-related emboli, duration of total circulation arrest, the depth of hypothermia, pH management during cardiopulmonary bypass, and genetic propensity to inflammatory response and vulnerability to ischaemia reperfusion injury (Newburger & Bellinger 2006).

2.2 Methods of cerebral protection in aortic arch surgery

2.2.1 Cardiopulmonary bypass

In the 1950s, Dr Gibbon successfully used the first heart-lung machine to repair an intracardiac defect of a human patient. Extracorporeal circulation was already being studied, but Gibbon’s team developed the first cardiopulmonary bypass (CPB) pump including oxygenator and tubing with roller pump (GIBBON et al. 1954).

The concept of CPB is to maintain adequate blood pressure and flow via extracorporeal circulation. The CPB machine temporarily replaces the function of the heart and lungs during the operation. A major vein, usually vena cava or vena femoralis, is cannulated and connected to the CPB machine using elastic silicone rubber or PVC heparin-coated tubing, as the venous line. The venous blood flows through to the oxygenator’s reservoir by gravity, as a siphon. From the reservoir, the venous blood is pumped through the oxygenator with a roller pump. This pump gives the CPB circulation a pulsatile effect and maintains its flow rate. The
perfect oxygenator would be the patient’s lungs, but the CPB machine’s oxygenator works as an extracorporeal gaseous exchange device during cardiopulmonary bypass. The blood circulates through the oxygenator’s oxygen (O₂)-rich membranes, and carbon dioxide (CO₂) and O₂ are transferred via diffusion from liquid to the membrane and vice versa. The oxygenated blood then flows through the arterial line back to the patient’s major artery through the arterial cannula. Additional roller pumps and reservoirs are used for certain isolated perfusion depending on the operation performed. A heat exchanger is used to manipulate the returning blood, in order to cool or warm the patient or maintain adequate core temperature.

Cardiopulmonary bypass has its downsides, as 5–10% of patients operated under CPB suffer from coagulopathy as a complication and experience massive blood loss defined as over 2,000 ml chest tube bleeding during the first post-operative day (Thiele & Raphael 2014). CPB also initiates systemic inflammation as the blood contacts air and synthetic tubes. The pump and the oxygenator both function in a sub-physiological manner. Overall, the CPB initiates humoral and cellular inflammatory responses that lead to vasoactive mediator production, cause haemolysis, and alter capillary permeability (Smith et al. 1987).

2.2.2 Deep hypothermic circulatory arrest

Dr Bigelow introduced the medical approach of hypothermia in mitigating ischaemic damage in the 1950s. He conducted multiple animal and human studies in order to determine adequate protocol and temperatures for cooling the patients for surgical operations. His goal was to improve heart surgery, and he realised that the next step was excluding the heart from circulation to achieve a bloodless operating view and open heart surgery. At the same time, heart-lung machines were tested, but Dr Bigelow hypothesised that hypothermic conditions would produce better outcomes in patients with cardiac diseases or abnormal heart, where the cannulae in great vessels may be poorly tolerated (Bigelow et al. 1950). Dr Lewis published just a few years after collecting his results using deep hypothermia in patients to operate under circulatory arrest in open heart surgery, resulting in the concept of deep hypothermic circulatory arrest (DHCA). Both Lewis and Bigelow cooled animals and human patients using surface cooling with ice water, ice packs, and later refrigerating blankets (Lewis 1956, Niazi & Lewis 1957). After the development of cardiopulmonary bypass (CPB) as an extracorporeal form of circulation, Dr Griepp successfully utilised both topical
cooling and cardiopulmonary bypass to achieve total body hypothermia in patients during prosthetic replacement of the aortic arch in the 1970s (Griepp et al. 1975).

The DHCA is a method to decrease ischaemic damage in the central nervous system during surgery. The brain uses 20% of circulating oxygen and up to 65% of glucose. Today, the patient is cooled to the target temperature using cardiopulmonary bypass to reduce oxygen consumption in metabolically active tissues, e.g. the brain. After cooling, the circulation is halted to ensure a bloodless operating view. When the heart and extracorporeal circulation is stopped, all circulation in the patient’s cardiovascular system is halted and DHCA is achieved. In animal studies, the cerebral oxygen consumption at 20 degrees Celsius (°C) is only 31% compared with 37 °C body temperature, giving surgeons the time necessary to operate on the heart (Perna et al. 1973). The drop in the cerebral metabolism in the first few degrees is higher, approximately 5% every degree C, indicating that the oxygen consumption correspondence with the whole body temperature drop is not linear (Laptook et al. 1995). In humans, the cerebral metabolic rate is only 24% of the normothermic baseline at 20 degrees C (McCullough et al. 1999).

**Acid-base balance and hypothermia**

When the patient’s blood temperature is lowered, the solubility of gases usually increases and partial pressure of gases decreases. This leads to the possibility of employing different acid-base management strategies during hypothermia. With the pH-stat strategy, the aim is to maintain partial pressure of CO₂ (pCO₂) of 40 mmHg and pH of 7.40 at the patient’s actual temperature, by adding CO₂. Another approach, the alpha-stat strategy, is to maintain at a constant value the degree of ionisation (alpha) of the imidazole of histidine by keeping CO₂ content the same at different body temperatures. The aim of the alpha-stat strategy is to maintain optimal protein function intracellularly and extracellularly, and also reduce tissue acidosis during the DHCA.

The pump flow can be adjusted to maintain high blood flow and arterial pressure. Schwartz _et al._, however, demonstrated that brain autoregulation works even in hypothermia, and that perfusion in the brain is related to arterial pressure and not to pump flow rate (Schwartz _et al._ 1995). The cerebral autoregulation can also be evaded with the pH-stat perfusion strategy. Increased cerebral blood flow (CBF), uniform cooling, reduction of oxygen consumption, and increased tissue
oxygen availability are achieved in infants and young animal models using the pH-stat strategy (Duebener et al. 2002), as temperature-corrected arterial pCO₂ maintained at 40 mmHg (with reduced fresh gas flow or added CO₂ to the CPB circuit) uncouples cerebral blood flow and metabolism, and impairs cerebral autoregulation (Murkin et al. 1987). Additionally, low-flow of 50ml/kg/min after deep hypothermic circulatory arrest is associated with better protection of the brain than the alpha-stat strategy in newborn piglets (Pirzadeh et al. 2011). However, the perfusion strategy during cooling and hypothermia should be chosen according to the patient’s age, as the alpha-stat strategy (pCO₂ is not temperature-corrected / no additional CO₂ is administered) is associated with better neuropsychological outcome in adults (Abdul Aziz & Meduoye 2010).

Cardiopulmonary bypass strategies

For over 5 decades, CPB perfusion rates have been set to approximately 2.4 l/min/m² during normothermic conditions, and 1.8 l/min/m² during hypothermic CPB (Tarhan & Moffitt 1971, Tuble et al. 2009). Although there are guides for standard flow rates, the minimum flow rate varies according to the conditions of bypass (Anttila et al. 2005). The uncoupled, impaired cerebral blood flow by the pH-stat perfusion strategy can be harmful, as higher mean arterial pressure (MAP) and cerebral flow are also associated with microemboli in the brain (Cartwright & Mangano 1998). However, a higher MAP can also be a benefit to areas secondary to emboli, as collateral flow is pressure-dependent (Hartman 1998). Using a standardised flow for each patient may not guarantee adequate tissue perfusion. The information from oxygen consumption, carbon dioxide saturation and intermittent whole blood lactate levels is required for optimal perfusion flow, and the perfusionist should decide the ideal cardiac output for a given condition (De Somer 2007).

Cooling and rewarming

Greissler et al. studied cooling gradients with mongrel dogs in the 1990s. They demonstrated the formation of microemboli with temperature gradients of 10 °C or greater, increasing significantly with higher temperature gradients (Geissler et al. 1997). Temperature gradients between arterial outlet and venous inflow during cardiopulmonary bypass are recommended not to exceed 10 °C (Engelman et al. 2015).
Before rewarming, a 10-minute period of cold perfusion at 20 °C reduces neurological events (Di Mauro et al. 2013). During rewarming, bladder and rectal temperatures are 3.2 °C ±1.9 °C and 2.2 °C ±1.0 °C, respectively, lower than cerebral temperature (Nussmeier et al. 2006). Rewarming must be cautious, to avoid even mild cerebral hyperthermia, which is associated with aggravated neurological injury (Nussmeier 2005). The gradient of no more than 10 °C is also recommended for rewarming, as rapid rewarming of the blood causes dissolved gases to come out of the blood. Additionally, to avoid cerebral hyperthermia, a rewarming rate of only 0.5 °C/min or less is advised when closing separation from bypass, at core temperature over 30 °C (Engelman et al. 2015). Deep hypothermic circulatory arrest itself does not seem to affect the autoregulation of cerebral blood flow during aortic reconstructive surgery (Ono et al. 2013).

**Safe duration of hypothermic circulatory arrest**

The outcome of the cerebral protection is a sum of the depth and duration of the DHCA. McCullough et al. calculated the safe duration of DHCA, based on the oxygen consumption; the safe time is considered 21 minutes at 20 °C. The patient should be cooled towards 10 °C if exceeding 30 minutes of DHCA is anticipated (McCullough et al. 1999). Based on the findings of McCullough et al., the safe duration of hypothermic circulatory arrest was calculated to be 5 minutes at normothermia, 9 minutes at 30 °C, 14 minutes at 25 °C, 21 minutes at 20 °C, 31 minutes at 15 °C and 45 minutes at 10 °C (Luehr et al. 2014). However, a prolonged duration of DHCA is also associated with worse neurodevelopmental outcome in repairs of congenital heart defects, as the outcome steadily worsens after longer durations of DHCA (Bellinger et al. 1995, Wypij et al. 2003). Prolonged duration of DHCA and alpha-stat strategy are also associated with the development of choreoathetosis in children (Levin et al. 2005). The pH-stat strategy as acid-base management in the treatment of congenital heart defects is shown to produce better neurological outcomes (Bellinger et al. 2001).

**Cerebral perfusion strategies**

In addition to HCA, several cerebral perfusion strategies have been used during operation to improve neurological outcome. Using these techniques, the circulation is arrested in the heart and lungs, and most of the body as in HCA, but additional cannulae are used to perfuse the brain.
Selective cerebral perfusion (SCP) can be achieved unilaterally, e.g. via innominate artery with occlusion of the left carotid and subclavian arteries, or bilaterally via innominate and carotid arteries simultaneously. The concept of unilateral SCP is based on total cerebral perfusion through collateral flow via the circle of Willis. However, Papantchev et al. studied the cerebral anatomical variations of 250 dissected patients and 250 individuals with CT angiography, and revealed hypoplasia or aplasia in 58.6% of Willis circles (Papantchev et al. 2013). Olsson and Thelin reported better brain protection with patients operated with bilateral technique compared with unilateral technique (Olsson & Thelin 2006). Zierer et al. could not demonstrate a difference between unilateral and bilateral technique with patients of acute type A aortic dissection operated under mild hypothermia (Zierer et al. 2012).

Retrograde cerebral perfusion (RCP) is a technique to perfuse the brain using the superior vena cava. The potential positive effects are considered to derive from uniform cerebral cooling and flushing of debris, emboli, or toxins and de-airing arch vessels (Griepp et al. 1997, Kouchoukos 1994).

For the past two decades, there has been a debate over the best brain protection strategy during operations of the aortic arch. There are some difficulties in comparing studies with one another, as practices for cerebral protection vary between institutions and may change over time. Misfeld et al. did not demonstrate any difference between groups, when comparing groups of moderate HCA with unilateral antegrade cerebral perfusion (ACP), moderate HCA with bilateral antegrade cerebral perfusion, DHCA alone, and DHCA with RCP (Misfeld et al. 2012). In a randomised, prospective study, DHCA was significantly better compared with ACP and RCP, regarding neurocognitive tests 6 months postoperatively (Svensson et al. 2001). Di Mauro et al. showed ACP to reduce stroke risk more than RCP or DHCA alone, when DHCA takes more than 30 minutes (Di Mauro et al. 2013). Hirsch et al. reviewed the best cardiopulmonary bypass strategies in infants undergoing cardiac surgery, and concluded that with respect to blood gas management, cooling strategy, and perfusion strategy, the data does not demonstrate the superiority of any specific practice relative to others (Hirsch et al. 2012).

2.3 Human central nervous system

In short, the human central nervous system is a network of neurons in the brain and spinal cord gathering data and stimuli from the surrounding environment and
the body, responding accordingly via neurotransmitters. The central nervous system (CNS) covers the brain and the spinal cord. The peripheral nervous system (PNS) consists of nerves and ganglia outside the CNS and is connected to the CNS.

### 2.3.1 The brain anatomy

During significant embryonic development from ectoderm, the brain reaches its macroscopic form prior to birth. The brain is located in the skull, protected by thick surroundings of bone, and it is divided anatomically into cerebrum, cerebellum, midbrain, pons, medulla oblongata, hypothalamus, and thalamus, each of which has its own significant structures and functions. The brain is composed of about 100 billion neurons and many more glial cells. The high demand for oxygen and glucose is nourished by circulation in various small arteries inside the brain tissue (Lechan & Toni 2000).

The brain can be divided into 4 pairs of lobes: the frontal lobes responsible for problem solving, judgement, and motor activity; the parietal lobes responsible for sensation; temporal lobes responsible for hearing and memory; and occipital lobes responsible for the visual data processing. The cerebellum is responsible for coordination and balance (Ackerman 1992).

The outermost part of the brain is called the cortex. It is quite thin, ranging from 1.5 to 4 millimetres deep, and contains 6 layers of cells. From the outer surface inward, these are as follows:

I. **Molecular layer.** Made up mostly of junctions between neurons, dendrites, and axons, for the exchange of signals.

II. **External granular layer.** Mainly interneurons, which serve as communicating nerve bodies within a region, and various axons as well as dendritic connections from deeper layers.

III. **External pyramidal layer.** Large-bodied ‘principal’ cells whose axons extend into other regions.

IV. **Internal granular layer.** Main termination point for fibres from the thalamus, and layer with densely packed stellate cells.

V. **Internal pyramidal layer.** Large pyramidal cells that project their axons mostly to structures below the cortex.
VI Multiform layer. Innermost layer of the cortex, containing small pyramidal cells, cells of Martinotti, stellate cells, and fusiform cells, which in this case project to the thalamus.

2.3.2 Neurons and glial cells

Within the brain, there are two predominant types of cells, the neurons and the glial cells. The neuron is an electrically active unit that responds to stimulus through excitatory or inhibitory chemical signals or via a direct electrical stimulus. The glial cells are mainly composed of astrocytes, oligodendrocytes, and microglia.

The nucleus of the cell is located in the soma, which is the cell’s body. Multiple limbs or branches derive from the soma. Those branches responsible for bringing information to the cell are called dendrites, and those responsible for delivering information are called axons. The axons can be very long processes specialised for the conduction of action potentials.

In the PNS, axons are long and myelinated by supporting Schwann cells for faster information delivery. In the CNS, cells called oligodendrocytes perform the task of myelinating the axons. Myelin sheaths produced by these glial cells reduce ion leakage and decrease capacitance. Between the myelin sheaths are gaps called nodes of Ranvier, where the action potentials hop from one node to another. The glial cells are non-conducting cells, unlike neurons. Oligodendrocytes are mostly present in the white matter, but can also be found in abundance in the grey matter.

Another type of glial cell is the astrocyte, which functions as a supporting cell in the CNS. Astrocytes maintain extracellular ion balance and support endothelial cells that form the blood brain barrier (BBB) with specific tight junctions between the endothelial cells. Astrocytes are highly branched, filling the spaces between neurons, their processes, and other neuroglial cells.

Microglia are the brain macrophages, activated by pathogens, damage, or cytokines (Brown & Vilalta 2015). They transform into large phagocytic cells, and have mainly immunological functions.

2.3.3 Cerebral blood flow

The cerebral activity varies constantly, and the cerebral blood flow (CBF) is altered accordingly, under physiological conditions. Roy and Sherrington
hypothesised over 125 years ago that CBF is regulated first by an intrinsic mechanism, by which the brain’s local requirements determine the blood supply, and secondly via the vasomotor nervous system via alteration of systemic circulation and general arterial blood pressure (Roy & Sherrington 1890). They tested their hypothesis in dogs, cats, and rabbits. Half a century later, Kety and Schmidt published supporting evidence, as they showed elevated CBF values in healthy human subjects by a rise in PCO₂, a lowering of PO₂, or a drop in pH, all of which are the consequences of increased energy metabolism (Kety & Schmidt 1948).

2.3.4 Homeostasis of functioning nervous cells

As in all eukaryotic cells, nervous cells also have a negative membrane potential supported by sodium-potassium pumps (Na⁺/K⁺ pump) and sodium-calcium (Na⁺/Ca²⁺) exchangers. This membrane potential is -60 mV to -70 mV, negative on the inside of the cell. The main function of neuronal cells is the signal transportation beginning with depolarisation. At the peak of the action potential, the membrane potential can be +50 mV and positive on the inside of the cell. This produces a net change of 110–120 mV, and the action potentials move rapidly, at the speed of 100 m/s (Lodish et al. 2000).

Maintaining normal homeostasis as well as action potentials requires energy, and the energy is provided by the neurons’ mitochondria. Mitochondria generate adenosine triphosphate (ATP) through oxidative phosphorylation in a process called the electron transport chain. The synthesis of ATP from adenosine diphosphate (ADP) and phosphate ion (Pi) is the primary source of energy in aerobic conditions. Electrons are transferred from reduced coenzymes nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH₂) to an oxygen molecule (O₂), forming H₂O and multiple ATP-molecules. (Lodish et al. 2000, Niizuma et al. 2009). A constant supply of O₂ and glucose is needed to maintain adequate levels of ATP production in the neuron’s mitochondria.

2.4 Nervous cell damage, ischaemia, and infarction after global ischaemia

The outcome of an ischaemic insult on the brain is different in global ischaemia and focal ischaemia. In our model, prolonged deep hypothermic circulatory arrest
causes global ischaemia, as there is no blood flow to any part of the CNS. A plaque-induced thrombus or a thromboembolism from fibrillating atrium causes focal ischaemia.

A pronounced hyperemia up to 2 to 3 times of normal perfusion rates occurs after approximately 10 minutes after cerebrovascular reperfusion of global ischaemia. Subsequently, after 30 to 60 minutes of reperfusion, cerebral blood flow decreases to hypoperfusion (Crumrine & LaManna 1991, Eleff et al. 1991).

### 2.4.1 Loss of function

If cerebral blood flow (CBF) decreases to under 20 ml/min/100g, a significant drop in ATP levels is measured as well as an increase in lactate level and a drop in cerebral pH (Obrenovitch et al. 1988). The cells can no longer maintain oxidative phosphorylation, resulting in a great deal of activity inside the cell. Dysfunction in the electron transfer chain prevents the neuron from maintaining its cellular function and homeostasis. The Na⁺/K⁺ pump and Na⁺/Ca²⁺ exchangers cannot function without constant ATP production of the mitochondria, resulting in the loss of membrane potential and anoxic depolarisation (AD) of the cell. The cells lose K⁺ to the extracellular space, and take up Na⁺ via voltage-gated Na⁺ channels. This causes cellular swelling, as the iso-osmotic gain of H₂O follows anoxic depolarisation.

### 2.4.2 Glutamate

During prolonged depolarisation, which occurs in AD, excessive levels of glutamate spill out of the synapses. This extrasynaptic glutamate overstimulates N-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors, and kainate receptors. NMDA receptors are glutamate-gated ion channels that are highly permeable to Ca²⁺, and glutamate overstimulation in combination with activated voltage-gated Ca²⁺ channels leads to significant Ca²⁺ influx (Ankarcrona et al. 1995, Newell et al. 1995, Simon et al. 1984, Stanika et al. 2009).

Also, the reverse operation of glutamate transporters during prolonged AD is a source of extrasynaptic glutamate (Rossi et al. 2002, Soria et al. 2014). As the neuron’s membrane potential is lost in anoxic depolarisation, extracellular K⁺ rises. The brain cannot withstand concentrations over 60 mM of extracellular K⁺, and with higher concentrations, the astrocyte’s glutamate uptake transporters are
reversed, resulting in an efflux of astrocytic glutamate to extrasynaptic space (Longuemare et al. 1999, Zhang et al. 2015). Although NMDA receptors are mostly responsible for the Ca\textsuperscript{2+} accumulation, NMDA receptor blockade is ineffective in preventing the influx of Ca\textsuperscript{2+}, which is associated with mitochondrial dysfunction following the production of reactive oxygen species (ROS) (Carriedo et al. 1998, Lipton 2006).

2.4.3 Mitochondria

Mitochondria are the power stations of the cells. They generate the majority of cellular ATP, the energy source of the cell. NADH and FADH\textsubscript{2} generated by the citric acid cycle, fatty acid oxidation, and amino acid oxidation are processed in the electron transport chain, where they donate their electrons and are oxidised to NAD\textsuperscript{+} and FAD. Electrons are ultimately transferred to O\textsubscript{2}, producing ATP and H\textsubscript{2}O (Stein & Imai 2012).

Under physiological conditions, a mitochondrial electrochemical gradient is generated and utilised by the oxidative phosphorylation system. It is composed of the electron transport chain (ETC) and the complex V, the ATP synthase. NADH dehydrogenase (complex I) and succinate dehydrogenase (complex II) transfer electrons to complex III. Cytochrome c transfers the electrons to cytochrome c oxidase (complex IV). The energy from electron transportation in complexes I, III, and IV also pumps protons (H\textsuperscript{+}) across the inner mitochondrial membrane. This creates a mitochondrial membrane potential between the mitochondrial membranes, and consists of both the pH difference across the inner mitochondrial membrane and electrical component (Kim et al. 2012). The electrochemical gradient is utilised by complex V, the ATP synthase, and produces ATP.

NAD\textsuperscript{+} is known to prevent apoptosis inducing factor (AIF) translocation from mitochondria to the nucleus after glutamate stimulation (Wang et al. 2014a). During anoxic depolarisation and excess glutamate stimulation, the mitochondria are fragmented. NADH is diffused out to the cytosol due to the opening of voltage-gated channel mitochondrial permeability transition pores (MPTP) (Reichert et al. 2001). The NAD\textsuperscript{+}/NADH ratio suffers, causing further dysfunction. A simplified overview of neuronal ischemic cascade is shown in Figure 1.
2.4.4 The role of accumulating Ca\textsuperscript{2+}

Under physiological conditions, Ca\textsuperscript{2+} enters the mitochondria electrophoretically through a Ca\textsuperscript{2+} uniporter and is cycled between the membranes and matrix of the mitochondria, via Na\textsuperscript{+}/Ca\textsuperscript{2+} exchangers (Nicholls & Crompton 1980). The cycling is a rather slow process, enabling the mitochondria to change cytosolic Ca\textsuperscript{2+} level responding to Ca\textsuperscript{2+}-dependent events in the cytosol. Higher levels of Ca\textsuperscript{2+} consequently activate the tricarboxylic acid cycle and oxidative phosphorylation, to produce more ATP (Crompton & Heid 1978). The rate of Ca\textsuperscript{2+} cycling cannot be greater than the activity of the functioning Ca\textsuperscript{2+} uniporter, under physiological conditions. A normal isolated mitochondrion is known to withstand relatively high concentrations of Ca\textsuperscript{2+}, if there are sufficient O\textsubscript{2}, ATP, ADP, and phosphate ions (PO\textsubscript{4}\textsuperscript{3-},P\textsubscript{i}) (Nicholls & Crompton 1980).

During ischaemic injury, with excessive amounts of Ca\textsuperscript{2+} accumulating in the mitochondria, the permeability transition pore opens. This permeability transition
pore (PTP) is a deformed complex of the voltage-dependent anion channel from the outer membrane, the adenine nucleotide translocase from the inner membrane, and cyclophilin-D from the mitochondrial matrix, and is located between the inner and outer membranes (Marzo et al. 1998). The opening of the PTP leads to lethal aftereffects at a cellular level, including the release of apoptogenic proteins, disruption of oxidative phosphorylation, and generation of further reactive oxygen species (ROS) (Ellerby et al. 1997, Marchetti et al. 1996).

2.4.5 Calpains and NO

Calpains are heterodimer proteins that participate in remodelling cytoskeletal and membrane attachments and apoptosis. Two different calpains have been recognised and named as μ-calpain and m-calpain, according to their Ca$^{2+}$ requirements for activation. The calpain system consists of a Ca$^{2+}$-dependent cysteine protease (calpains) and a polypeptide, calpastatin, whose sole purpose is to inhibit calpains. The calpastatin binds reversibly to calpains depending on Ca$^{2+}$. However, the required Ca$^{2+}$ for the inhibition of calpain is less than the Ca$^{2+}$ needed to activate proteolytic activity (Kapprell & Goll 1989, Volbracht et al. 2005). During anoxic depolarisation, the Ca$^{2+}$ homeostasis is lost and chronic activation of calpain occurs, leading to degradation of the cytoskeleton and neurofilaments, and ultimately to necrotic cell death.

Nitric oxide is a free radical, and it has multiple different effects, depending on its concentration. Under physiological conditions, it is produced in lower concentrations by endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS), and higher levels by inducible nitric oxide synthase (iNOS). Lower concentrations of NO promote cell survival, and higher concentrations can lead to apoptosis and necrosis via nitrosative stress. (Kim et al. 1997, Thomas et al. 2008). The nNOS, however, is activated by Ca$^{2+}$, and the influx of Ca$^{2+}$ during anoxic depolarisation and glutamate excitotoxicity also significantly promotes NO formation. NO is also known to deplete Ca$^{2+}$ from endoplasmic reticulum (ER), causing ER stress, and leads to apoptosis through the ER stress pathway (Oyadomari et al. 2001). When the NO reacts with ROS, such as the superoxide anion (O$_2^-$), neurotoxic peroxynitrate (ONOO-) is formed (Lipton et al. 1993). The high concentration of NO also inhibits mitochondrial respiration and causes glutamate release, and even further promotes excitotoxicity. NO concentrations of 100–300 nM are also known to stabilise hypoxia-inducible factor 1, alpha subunit (HIF-1-α) (Brown 2010).
2.4.6 Reactive oxygen species

Free radicals are atoms and molecules with one or more unpaired electrons. The free radicals and hydrogen peroxide (H₂O₂), which is a major source of the hydroxyl radical (OH⁻), are referred to as reactive oxygen species (ROS). Under physiological conditions, small numbers of electrons in the mitochondria escape the ETC complexes I and III, and react with O₂ to form superoxide (O₂⁻). The O₂⁻ accelerates the metal ion-dependent hydroxyl radical (OH⁻), the most unstable of the ROS, resulting in formation from H₂O₂ (Halliwell 1987). This is countered by Mn-containing superoxide dismutase and Se-containing glutathione peroxidase (LeDoux et al. 1999), as well as fat-soluble vitamins A and E, to prevent damage to the cellular and mitochondrial DNA, proteins, or lipids (Banala & Karnati 2015).

During ischaemia, the accumulated Ca²⁺ also leads to dephosphorylation of mitochondrial proteins and increases in mitochondrial H₂O₂ generation (Hopper et al. 2006). The cellular mechanisms to scavenge ROS are insufficient in relation to this massive production of free radicals under ischaemia. ROS are also known to contribute to the decrease of mitochondrial membrane potential during ischaemia, and also initiate lipid peroxidation cascade, causing the loss of integrity of the mitochondrial inner membrane. This process is independent of previously mentioned MPTP opening (Levraut et al. 2003).

2.4.7 DNA damage and 8-OHdG

ROS result in oxidation of mitochondrial DNA (mtDNA) and the DNA of the nucleus. An oxidised form of guanine in the DNA, 8-Oxoguanine, can cause transversion mutations, as it can pair up with both adenine and cytosine. The cells endeavour to maintain their genomic integrity, and the 8-Oxoguanine in the nuclei and in the mitochondria is recognised by OGG1 gene-encoded 8-oxoguanine DNA glycosylase, and removed (Iida et al. 2004). The end-product, 8-hydroxydeoxyguanosine (8-OHdG), is secreted out of the cell and ultimately to the urine, and the DNA is repaired (Ba et al. 2014, Mazurek et al. 2002). The 8-OHdG can be measured and is considered as a reliable marker for oxidative stress (Chiou et al. 2003).
2.4.8 Neuronal apoptosis and necrosis

Neurons exposed to neurotoxic concentrations of glutamate undergo two different fates. A subset of neurons gives up to acute necrosis immediately after the glutamate exposure. The remaining neurons undergo delayed-onset apoptosis, which requires a relatively intact mitochondrial activity. The probability of necrosis increases with higher glutamate exposure (Ankarcrorna et al. 1995). Sustained opening of the mitochondrial PTP leads to necrosis, as the ATP levels drop rapidly. If the opening is transient and sufficient ATP is maintained, the cell can undergo apoptosis.

In apoptosis, the cell death is programmed and happens despite the presence of adequate levels of ATP. It occurs within a few hours of reperfusion and continues for several days, after deep hypothermic circulatory arrest (Ditsworth et al. 2003). It is hypothesised that excitotoxic neuronal death is a mix of necrosis and apoptosis, where cell swelling, cytoplasm vacuolisation, and disruption of cell membranes are present, as are chromosome condensation and fragmentation, and activation of caspases, which are characteristics of apoptosis (Wang & Qin 2010). The principal difference between these two pathways, apoptosis and necrosis, considering ischaemic brain damage, is the stronger inflammatory response induced by necrosis (Stoll et al. 1998).

2.4.9 Selective vulnerability

Some regions of the brain are more sensitive to ischaemic insult than others. The CA1 pyramidal cells of the hippocampus are known to be the most vulnerable to ischaemia, and CA3 neurons are the most resistant to hypoxia (Ordy et al. 1993). The neuronal cell death is delayed and only observable days after the onset of ischaemic insult (Petito et al. 1987). CA1 neuronal damage is associated with difficulties of spatial learning and memory (Bendel et al. 2005). The next vulnerable areas are the neocortex and striatum, as well as the lateral septum, amygdala, substantia nigra, and cerebellar Purkinje cells (Schmidt-Kastner 2015).

2.5 Innate response to neuronal ischaemic injury

When the cerebral blood flow decreases below 20 ml/100min/100g, the neurons lose their ability to function. It is shown in stroke patients that cerebral blood flow between 10 to 20 ml/min/100g results in reversible neurological deficiency, and in
focal ischaemic insults, it is referred to as the penumbra (Olsen et al. 1983, Symon et al. 1974).

The innate response to transient total cerebral ischaemia differs with stroke, as there is no penumbra. After ischaemic insult, immune and inflammatory responses occur. Resident cells, such as microglial cells, astrocytes, and endothelial cells, in addition to infiltrated white blood cells from the circulation, take part in the brain inflammation. These cells express pro-inflammatory cytokines and acute-phase proteins.

After 3 months after global ischaemia, 40% of CA1 neurons are repopulated via neurogenesis and contribute to neurological recovery (Bendel et al. 2005). However, not all of those repopulated cells function properly. This may be due to long-lasting neuroinflammation that occurs after global ischaemia (Langdon et al. 2008).

2.5.1 Cytokines and ischaemic signalling cascade

The first response to global brain ischaemia is an innate immune response. During the first moments of I/R injury, cerebral concentrations of tumor necrosis factor-α (TNFα), Interleukin-1 (IL-1), and Interleukin-6 (IL-6) rise (Saito et al. 1996). IL-1 is considered a family of early response pro-inflammatory cytokines that induce a cascade of effects leading to inflammation (Huising et al. 2004), and affect nearly every cell type (Dinarello 1997). IL-1 signalling after ischaemic insult, from microglial and immune cells, leads to the activation of the transcription factor nuclear factor - κB (NF-κB) pathway (Osborn et al. 1989). The IL-1 receptor antagonist is known to attenuate plasma complement reactive protein (CRP) and lower IL-6 concentrations, leading to a reduction in total white cell count and neutrophil count compared with placebo, in acute stroke patients (Emsley et al. 2005).

TNFα is a proinflammatory cytokine secreted in response to tissue trauma. Microglial cells (Liesz et al. 2009) and invading macrophages secrete TNFα briefly after ischaemia (Buttini et al. 1996). The released TNFα and IL-1 also stimulate astrocytes to secrete IL-6 (Aloisi et al. 1992). TNFα increases cerebrovascular permeability via NF-κB activation, resulting in alteration in the blood brain barrier (BBB) integrity (Trickler et al. 2005), leading to the accumulation of proteins and water in the brain, and causing oedema.

NF-κB is a transcription factor that regulates inflammatory and apoptotic pathways (Li et al. 2013). Interestingly, there seems to be a dual role for NF-κB,
as the early activity from 0 to 3 hours after ischaemic insult contributes to neuronal damage, but late activity is regarded as neuroprotective and antiapoptotic (Nijboer et al. 2008).

### 2.5.2 White blood cells

The migration of leukocytes into the brain is a key event in the pathogenesis of ischaemia reperfusion (I/R) injury. In response to cytokines and transcription factors, white blood cells migrate and adhere to the neurovascular endothelium (Ritter et al. 2000). The inflammatory agonists stimulate cerebral endothelial cells as well as leukocytes, which then express adhesion molecules, resulting in transient bonding between the two. TNFα and IL-1 induce endothelial E-selectin and P-selectins, which promote leukocyte adhesion (Zhang et al. 1998). This results in leukocytes moving along the blood vessel (Zimmerman et al. 1996).

**T** lymphocytes are part of the adaptive immune system and critical mediators of inflammation. They are formed in the bone marrow, but mature in the thymus. The main function of T lymphocytes is to recognise pathogens and generate immune memory. Antigen-specific CD4+ T lymphocytes differentiate into two major populations, T helper 1 (Th1) and T helper 2 (Th2) cells (Mosmann et al. 2005). Th1 cells activate macrophages and cytotoxic T cells, thus mediating a cellular immune response. Th2 cells secrete multiple cytokines and promote the maturation of B cells, inducing humoral response (Santana & Rosenstein 2003). Specific regulatory T cells (CD4+CD25+ T_{reg}) are also known to attenuate the harmful inflammatory response to cerebral ischaemia, via Interleukin 10 secretion (Liesz et al. 2009).

Antigen-specific CD8+ T lymphocytes are considered cytotoxic T cells (Tc), and they play a role in viral infections and tumours, in addition to destroying damaged cells. CD8+ cells also worsen ischaemic brain damage, contrary to natural killer T cells (NK) (Mracsko et al. 2014). Both the CD4+ and CD8+ T cells contribute to neuronal injury following global cerebral ischaemia (Deng et al. 2014).

### 2.6 Natural countering measures for oxidative stress

The levels of oxidised DNA, phospholipids, and proteins are elevated during ischaemia-derived oxidative stress. The cells have developed multiple ways to counter ROS and repair its end products.
2.6.1 Nrf2

Nuclear factor erythroid 2-related factor 2 (Nrf2) is one of basic leucine zipper proteins. In order to function, it forms heterodimers with the Maf family proteins (named after the *musculoaponeurotic fibrosarcoma oncogene homolog*) in the nucleus (Toki *et al.* 1997) and binds to antioxidant response elements (ARE), which promote the expression of protective proteins to respond to oxidative stress (Rushmore *et al.* 1991). Under physiological conditions, Nrf2 is bound to Kelch ECH associating protein 1 (Keap1), and bound to cytoskeleton (Itoh *et al.* 1999). If the oxidative stress is insignificant, the Nrf2-keap1 complex is isolated in the cytoplasm. Nrf2 is constantly expressed, and is degraded by the proteasome through ubiquitination (Nguyen *et al.* 2003). Under oxidative stress, the Nrf2-keap1 interaction is weakened, and Nrf2 translocates to the nucleus and begins its induction of phase II detoxifying and oxidative stress enzyme genes (Itoh *et al.* 2003). Huang *et al.* also demonstrated that the pathway mediated by protein kinase C, apart from the direct influence of oxidative stress to Keap1, also acts via Nrf2 phosphorylation and activation (Huang *et al.* 2000).

2.6.2 HIF-1-α

Hypoxia-inducible factor 1 is a heterodimer complex composed of two subunits: hypoxia-inducing factor -1-α (HIF-1-α), a 120-kDa-sized subunit and hypoxia-inducing factor -1-β (HIF-1-β), a 91–94kDa subunit (Wang & Semenza 1995). HIF-1-α subunits are constantly produced and rapidly destroyed by the ubiquitin-proteasome pathway. Under hypoxic conditions, HIF-1-α ubiquitination is significantly decreased, and the HIF-1-α accumulates (Kallio *et al.* 1999). Oxidative stress, which is notably present in cerebral ischaemic insult, also contributes to HIF-1-α stabilisation under hypoxic conditions (Brunelle *et al.* 2005). Stabilised HIF-1-α binds to other subunits and regulates multiple hypoxia-induced target genes, such as erythropoietin, glucose transporters, and vascular endothelial growth factor (VEGF) to counter cellular and systemic hypoxia and oxidative stress (Bernaudin *et al.* 2002, Ebert *et al.* 1995, Semenza *et al.* 1994). HIF-1-α contributes to mitigating ROS-related damage in the brain, after cerebral ischaemic insult, as HIF-1-α deficiency has been shown to increase brain damage (Sheldon *et al.* 2009), and experimental drug therapy employing HIF-1-α seems to increase the cerebral ischaemic tolerance (Li *et al.* 2014). Interestingly, HIF-1-
α also down-regulates the Nrf2 pathway, indicating the complexity of cellular responses to oxidative damage and hypoxia (Loboda et al. 2009).

2.6.3 DJ-1

Another ROS-countering protein is the ubiquitous DJ-1. It is a H$_2$O$_2$-responsive, redox-sensitive protein, and functions as an antioxidant (Taira et al. 2004); it is associated with the cell death cascade (Canet-Aviles et al. 2004). During ischaemic insult to the neurons, DJ-1 translocates to the inner membrane of the mitochondria and protects complex I of the electron transport chain against ROS (Pantcheva et al. 2014). The neuroprotection of DJ-1 is evident, as mutations in the DJ-1 gene are shown to cause neurodegeneration and are associated with parkinsonism (Bonifati et al. 2003).

2.7 Remote ischaemic preconditioning

Murray et al. first published reports of a classic phenomenon they termed ischaemic preconditioning (IPC) (Murry et al. 1986). It was the same year in which Schurr et al. demonstrated adaptation to anoxia in rat hippocampal CA1 cells, following 5-minute anoxic episodes (Schurr et al. 1986). A few years later, evidence was published that by applying ischaemia to a remote organ or tissue, another organ was protected from subsequent ischaemic insult (Przyklenk et al. 1993), and the concept of remote ischaemic preconditioning (RIPC) was formed. In experimental models, myocardial protection (Birnbaum et al. 1997), renal protection (Yoon et al. 2015), and liver protection (Kanoria et al. 2006) have been shown with RIPC, in addition to cerebral protection (Jensen et al. 2011, Meng et al. 2012).

2.7.1 Cerebral and cardial protection

Kitagawa et al. demonstrated that brief carotid occlusion in a gerbil model protects hippocampal pyramidal CA1 neurons from prolonged global ischaemia, and first described this phenomenon as ‘ischemic tolerance’ (Kitagawa et al. 1991). Ischaemic tolerance can be described as a protection against a normally injurious duration of cerebral ischaemia, if the brain is first exposed to a non-injurious level of ischaemic stress. There are two preconditioning time windows that are protective after ischaemic preconditioning. Rapid preconditioning exists
between 1 hour to 3 hours, and a second window reappears between 24 hours to 72 hours (Kuzuya et al. 1993). The second window is mediated via new gene expression and protein synthesis. Barone et al. demonstrated that preconditioning induces the expression of IL-1 receptor antagonist (IL-1ra), which inhibits IL-1-mediated immune and inflammatory response (Barone et al. 1998). In their model, however, the ischaemic tolerance only took place in the area made ischaemic, leaving other brain regions vulnerable to subsequent ischaemic insult. Ren et al. published a conflicting study considering the time windows, using RIPC in a rat model, and demonstrated an additional neuroprotective effect after 12 hours of preconditioning (Ren et al. 2008). A longer-lasting effect was also shown, as RIPC 48 hours prior to ischaemic insult reduced hippocampal CA1 neuron degeneration in a normothermic rat model of asphyxia cardiac arrest (Dave et al. 2006).

In experimental models of myocardial infarction, RIPC has been demonstrated to improve myocardial protection (Gho et al. 1996). Jensen et al. reported better cardiac index and decreased cardiac enzymes creatine kinase MB and troponin I in RIPC-treated piglets prior to DCHA (Jensen et al. 2011). Pavione et al. also published differences compared with control group in plasma cardiac troponin I levels in RIPC-treated children undergoing surgery for congenital heart disease with cardiopulmonary bypass. However, RIPC did not provide clinically relevant cardioprotection between the groups (Pavione et al. 2012). Despite the promising results in animal models, the effects of RIPC are controversial. Meybohm et al. recently published a clinical multicenter, double-blinded, randomised, prospective, controlled human trial with patients undergoing coronary artery bypass grafting (CABG) using cardiopulmonary bypass, and could not demonstrate significant differences between the treatment groups (Meybohm et al. 2015). Similar results were published by Hausenloy et al. with patients undergoing CABG with or without valve surgery (Hausenloy et al. 2015). Zaugg et al. discussed these findings and proposed some explanations, including pharmacological agents masking the effects of RIPC (Zaugg & Lucchinetti 2015).

### 2.7.2 Molecular mechanisms of remote ischaemic preconditioning

The mechanisms of RIPC-triggered neuroprotection are not yet completely understood, but the mechanisms of RIPC in all tissues have been extensively studied.
Adenosine A₁ receptor activation and K\textsubscript{ATP} channels

One key element of ischaemic tolerance is the adenosine triphosphate-sensitive potassium channel (K\textsubscript{ATP} channel), located in the sarcolemma and mitochondria. K\textsubscript{ATP} channels are mainly activated via A₁ adenosine receptors. Under physiological conditions, K\textsubscript{ATP} channels are activated when the intracellular ATP concentration decreases and ADP increases (de Weille & Lazdunski 1990). K\textsubscript{ATP} channel opening leads to the decrease of both synaptic transmission and K\textsuperscript{+}-stimulated glutamate release, and inhibition of neuronal presynaptic Ca\textsuperscript{2+} influx, all of which contribute to the neuronal ischemic cascade (Rudolphi \textit{et al.} 1992). Mitochondrial K\textsubscript{ATP} channels also regulate the pH gradient and membrane potential, mitochondrial volume and ionic homeostasis (Bajgar \textit{et al.} 2001).

The opening of K\textsubscript{ATP} channels is considered as one of the main mechanisms of remote ischemic preconditioning. In animal studies, the adenosine A₁ receptor antagonist weakened the protective effect of RIPC (Nakamura \textit{et al.} 2002, Yoshida \textit{et al.} 2004). Hu \textit{et al.} also combined the findings of the A₁ receptor antagonist and RIPC, as well as subsequent cellular oxidative state, as they showed a decrease in cerebral superoxide dismutase activity following RIPC (Hu \textit{et al.} 2012). The K\textsubscript{ATP} channel agonist diazoxide mimicked the protective effect of preconditioning (Kristiansen \textit{et al.} 2005), also implying the K\textsubscript{ATP} channel as the mediator of protective effect.

Protein kinase C ε and MAPK

Protein kinase C (PKC) is a family of enzymes that regulate other intracellular proteins through phosphorylation.

In studies of myocardial protection as well as neuroprotection, the protein kinase C ε (PKCε) pathway is identified as an early mediator of the protective effect (Perez-Pinzon & Born 1999, Wolfrum \textit{et al.} 2002). The PKCε translocation to mitochondria accelerates the activity of mitochondrial K\textsubscript{ATP} channels (Wang \textit{et al.} 2001). Additionally, the PKCε-mediated pathway includes activation of mitogen-activated protein kinase (MAPK) and is considered part of the pathway (Baines \textit{et al.} 2002). MAPK is thought to open mitochondrial K\textsubscript{ATP} channels (Baines \textit{et al.} 1999), and also induce transcription factors including NF-κB. The protective effect of mitochondrial K\textsubscript{ATP} channel opening and PKCε is thus interdependent.
PKCε also acts via Nrf2 phosphorylation and activation (Huang et al. 2000). \( K_{ATP} \) channel opener diazoxide-treated mice showed increased neuronal Nrf2 translocation compared with control mice (Virgili et al. 2013). This demonstrates an activation of the endogenous Nrf2 anti-oxidative pathway as a part of the neuroprotective mechanism.

**HIF-1-α**

RIPC is shown to increase HIF-1-α protein levels in the atrial tissue of cardiosurgical patients, prior cardiopulmonary bypass (Albrecht et al. 2013). Furthermore, heme oxygenase-1, up-regulated by HIF-1-α (Ockaili et al. 2005), was induced in the liver in mice after RIPC (Wang et al. 2014b), implying that RIPC stabilises HIF-1-α in another tissue.

Additionally, the RIPC-induced \( \text{H}_2\text{O}_2 \) production, leading to HIF-1-α stabilisation and HIF-1 pathway activation, is thought to play a role in the mechanism of RIPC (Chang et al. 2008). HIF-1-α knockout mice did not receive brain protection by hypoxia preconditioning, supporting the theory that RIPC is mediated by the HIF-1-α pathway (Cai et al. 2008, Sheldon et al. 2014). However, Kalakech et al. demonstrated RIPC-induced cardioprotection in partially HIF-1-α deficient and pharmacologically HIF-1-α inhibited mice and rats (Kalakech et al. 2013), indicating the complexity of mechanisms behind the protective effects of RIPC.

**Mediators and effectors**

The mediators and effectors of remote ischemic preconditioning consider a complex intracellular and humoral signal transduction. The ischemic preconditioning activates an intracellular signalling, including \( K_{ATP} \) channel opening and PKCε activation. The PKCε-mediated pathway in neurons induces cyclooxygenase-2 (COX-2) expression via NF-κB induction. The NF-κB activation depends on PKCε and extracellular signal-regulated kinase (ERK 1/2) signalling pathway, and promotes COX-2 up-regulation (Kim et al. 2010). COX-2 is a proinflammatory mediator of ischemic tolerance in the brain (Gendron et al. 2005). This results in activation of transcription factors HIF-1-α and Nrf2, in addition to NF-κB, and promotion of *de novo* proteins, including iNOS, COX-2 and HO-1.
Humoral versus neuronal pathway

There are studies of both humoral and neuronal pathways explaining the protective effect of ischaemic preconditioning. Dickson et al. demonstrated that the protective effect of preconditioning can be transferred from one canine to another via coronary effluent transfer (Dickson et al. 1999). In another study design, the protective effect of RIPC was diminished when systemic trimetaphan was administered, implicating a neurogenic pathway (Loukogeorgakis et al. 2005).
3 Aims of study

I To study the effects of remote ischaemic preconditioning (RIPC) in cerebral microcirculation, vessel diameter, leukocyte behaviour, and NAD⁺/NADH redox state, after prolonged deep hypothermic circulatory arrest

II To investigate whether the effects of remote ischaemic preconditioning can be observed in the markers of oxidative stress or in redox-regulating enzymes in a chronic porcine model

III To study whether the protective effect of oxidative stress could be seen by measuring serum 8-hydroxydeoxyguanosine (8-OHdG) or cardiac enzymes
4 Materials and methods

This section covers the materials and methods of the three different studies. Study I was performed in an acute model, and studies II and III were chronic in design. The summary of methods used for this thesis are presented in Table 2.

<table>
<thead>
<tr>
<th>Experimental setup</th>
<th>Study I</th>
<th>Study II</th>
<th>Study III</th>
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<tr>
<td>Piglets (n)</td>
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<td>Intravital microscopy</td>
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<td>Transmission Electron Microscope</td>
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<td>Immunohistochemical stainings</td>
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<td>Blood 8-OHdG concentrations</td>
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<td>Troponin I and CK-MB measurements</td>
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4.1 Experimental animals

The experiments on which this doctoral thesis is based could not have been carried out with human patients, due to the nature of histopathological data collection. The most suitable model was the porcine model, developed by Professor Randall Grieff and his group at the Mount Sinai School of Medicine in the 1990s. Professor Tatu Juvonen continued developing the model, and the first experiments were carried out in the Department of Experimental Surgery at Oulu University Hospital, Oulu, Finland, in 1997. The model was further improved by Docent Vesa Anttila in the 2000s with the intravital microscopy technique, and improved perioperative management was introduced with knowledge adopted from porcine studies conducted at Children's Hospital, Boston, Harvard Medical School. The basic porcine model is seen in Figure 2.

Our experiments were conducted with young native stock female domestic piglets weighting from 16 kg to 28 kg. All animals received humane care in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Research Council (published by the National Academy Press, revised in 1996) and with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research. The studies were approved by the Research Animal Care and Use Committee of the University of Oulu, Oulu, Finland. The animals arrived 1–2 weeks prior to experiments to adjust to the new surroundings of the laboratory.
facilities before the elective operation. Their well-being and behaviour were monitored daily, before and after the experiments, by the researchers and the staff of the Laboratory Animal Centre.

**Fig. 2.** The basic porcine model used. RIPC = Remote ischemic preconditioning; CPB = Cardiopulmonary Bypass.

### 4.2 Perioperative management

Preoperatively, the piglets were premedicated with a sedative intramuscular injection of midazolam (2 mg/kg), ketamine hydrochloride (15 mg/kg), and medetomidine (0.075 mg/kg), and a peripheral catheter was inserted into a vein in the left ear for intravenous administration of drugs and Ringer’s acetate (15 ml/kg/h). Electrocardiographic monitoring was initiated prior to anaesthesia induction. Anaesthesia was induced with intravenous injection of thiopental (6 mg/kg) and fentanyl (50 µg/kg) prior to endotracheal intubation with 6.5 mm cuffed tube. Cefuroxime 750 mg was administered intravenously after anaesthesia induction. Anaesthesia was maintained by a continuous infusion of fentanyl (25 µg/kg/h), midazolam (0.25 mg/kg/h), and pancuronium (0.2 mg/kg/h) (Study I) or rocuronium (1.5 mg/kg/h) (Studies II and III), in addition to inhalation anaesthesia of 1.5% sevoflurane throughout the surgical protocol, excluding DHCA. The piglets were ventilated with positive pressure of 5 cmH₂O, and end-tidal oxygen (EtO₂) was ensured at 50% in expired air with End-Tidal Control of the GE Aisys Carestation™ (GE Healthcare, Madison, WI, USA). A rectal
thermometer was used for body temperature measurements. A catheter inserted into the bladder was used for continuous monitoring of diuresis.

4.3 Blood sampling

An arterial catheter was positioned in the left femoral artery for arterial blood pressure monitoring and arterial blood sampling. Venous blood samples were collected, and hemodynamic monitoring was performed using a 7F pulmonary artery thermodilution catheter (CritiCath; Ohmeda GmbH & Co, Erlangen, Germany) inserted through the left femoral vein into the pulmonary artery. Cardiac output, pulmonary capillary wedge pressure (PCWP), central venous pressure, systemic blood temperature, and pulmonary pressure were monitored. Arterial blood gas values, electrolytes, plasma lactate levels, pH, serum ionised calcium, venous glucose, hematocrit, and hemoglobin levels (i-STAT Analyzer; i-STAT Corporation, East Windsor, NJ, USA), and blood count, as well as differential cell count (Cell-Dyn Analyser, Abbot, Santa Clara, CA) were measured. Summary of the experimental protocol is shown in Figure 3, and time points for hemodynamic monitoring and measurements are shown in table 3.

Fig. 3. The experimental protocol. After sedation, all piglets received either RIPC treatment or sham treatment. The piglets were then cannulated for cardiopulmonary bypass and cooled to 18°C for 60 minutes of hypothermic circulatory arrest. After HCA, the piglets were rewarmed to normothermic temperature for the follow-up period. The arrows represent the time points for hemodynamic measurements and blood sampling.
Table 3. Summary of time points in each study (I–III)

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<th>Study</th>
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BL = Baseline; C = Cooling phase; W = Warming phase; 20 = 20 minutes, 10 = 10 minutes etc.

4.4 Blood transfusion

Hemodilution during extracorporeal circulation was mitigated using blood transfusions from donor pigs prior to the experiment. Preoperatively, a membrane oxygenator (D905 Eos, Dideco, Mirandola, Italy) was primed with 800 ml of Ringer acetate solution and heparin (15,000 IU) and 1,200 ml of donor blood. The donor blood and receiver blood were tested in a separate test tube for cross-reactions. The donor piglets were sedated and anaesthetised as previously described and euthanised after the collection using pentobarbital (60 mg/kg) while still under anaesthesia.

4.5 Remote ischaemic preconditioning

The intervention of remote ischaemic preconditioning was applied to the right hind leg identically in all studies. A paediatric blood pressure cuff was used for preconditioning in the intervention group. A static pressure of 250 mmHg was applied for 4 cycles of 5-minute ischaemia followed by 5-minute reperfusion. Reactive hypotension and hypertension were observed and confirmed from systemic arterial pressure readings during the intervention. Cardiopulmonary bypass was initiated 60 minutes after the intervention in studies I and III, and 40 minutes after the intervention in study II. The effects of RIPC were studied in the brain using intravital microscope and transmission electron microscopy techniques in study I. In study II, the returning blood from the brain was analysed from sagittal sinus blood samples, and also histological and immunohistochemical samples were harvested and examined from the brain, heart, kidney, and ovary. Cardiac acute phase ischemic markers, as well as 8-OHdG, were analysed from blood samples in study III. The sampling protocol of this study is presented in Figure 4.
4.6 Cardiopulmonary bypass

A right anterolateral thoracotomy was performed through the 4th intercostal space in studies I and II, and for a few study subjects from the 3rd intercostal space in study III. The intercostal vessels were ligated and cut, and the heart was exposed. After the pericardium was opened, a 16F straight tip arterial cannula was placed in the aorta for an arterial line. A venous line was introduced to the right atrial appendage using a 24F venous cannula. Cardiopulmonary bypass was initiated and flow was adjusted to maintain mean arterial pressure of 60 mmHg.

Cooling started immediately, and the core temperature was lowered to 18°C in 30 minutes using a heat exchanger. In all studies, the pH-stat strategy was employed as acid-base management, and blood sampling was performed at least every 15 minutes to allow corrections in PaCO₂ levels. This was achieved by adding CO₂ to the inflowing gas of cardiopulmonary bypass and correcting the PaCO₂ for the actual core temperature. Potassium chloride (40 mmol) was mixed with 40 ml of blood and used for cardioplegia to stop the heart at the end of the cooling phase.

After 30 minutes of cooling, the extracorporeal circulation was stopped and deep hypothermic circulatory arrest was initiated. The core temperature was monitored continuously and maintained at 18°C with topical ice packs surrounding the body and the head.
After 60 minutes of deep hypothermic circulatory arrest, extracorporeal circulation was initiated and the piglets were rewarmed to a normothermic state in 45 minutes, using the pH-stat strategy. During the first 5 minutes of reperfusion, furosemide (40 mg), lidocaine (40 mg), methylprednisolone (40 mg), and calcium gluconate (90 mg) were administered to the CPB circulation, and mannitol 100 ml (15 mg/100 ml) was administered after the blood samples at 10 minutes of rewarming, to avoid dilution of the samples. The heart was defibrillated, if necessary, after reaching 30°C core temperature. Ventilation was started approximately 10 minutes prior to weaning from CPB. During the cooling phase and rewarming phase, hemodynamic measurements were recorded, blood samples were collected, and perfusion line temperature and rectal temperature were measured and recorded. Additionally, arterial gases were measured at regular intervals.

4.7 Intravital microscopy

In study I, the right temporal cerebral cortex was drilled with a 14/11 mm disposable cranial perforator, and a cranial window of 30mm · 30mm was created. The right ear was resected to ensure a clear view for an intravital microscope (Leica Model MZFL III; Leica, Heerbrugg, Switzerland). The dura was removed and the right cerebral cortex revealed. The microscope was placed over the cranial window and mounted to stabilise the image. The microscope included 3 sets of filters and lighting: a green filter (536–556 nm excitation > 590-nm emission wavelength) for visualisation of rhodamine-labelled leukocytes, a violet filter (450–490 nm excitation > 515-nm emission wavelength) to visualise microvascular perfusion, and an ultraviolet filter (340–380 nm excitation >420 nm emission wavelength) for NAD+/NADH redox state analysis. Samples of green and violet filter data are shown in Figures 5 and 6.

Video recordings were collected at baseline, at 5 minutes before CBP cannulation; at 10 and 20 minutes of cooling phase of CPB; at 50 minutes after the beginning of DHCA; at 5, 15, and 45 minutes of rewarming; and at 90, 120, and 180 minutes after HCA. The epi-illumination did not exceed 1 minute, to avoid thermal damage.

The image was captured by the charge-coupled device video camera (Dage-MTI CCD 300-ETRCX), transferred to a monitor (Samsung LCD SyncMaster 710 mp), and recorded. A frame grabber (Kudo Interactive Frame Grabber) and a computer-assisted image analysis system (Scion Corporation, Frederick, MD)
were used for offline data analysis. The final magnification on the monitor was 400–800 times.

**Fig. 5.** Sample image of violet filter used in combination with fluorescein isothiocyanate administration, for later vessel diameter assessment. The uniformly bright vessel in the left is a cerebrocortical artery, branching into the brain tissue. The fluorescein has not completely reached the veins in this image, showing darker colour, as in the centre and top of the image.

**Violet Filter**

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, to ensure vessel diameter assessment.
Fig. 6. Sample image of green filter used for leukocyte counting. The S-shaped vessel is a cerebrocortical vein. The white dots are adherent leukocytes in the vessel endothelium.

**Green filter**

When a suitable field of vision that included both an artery and a venous vessel was discovered, the piglet received an intravenous 2 ml (4 mg/ml) loading dose of rhodamine 6G chloride MW 479 (Sigma Chemical Co., St. Louis, MO, USA) 5 minutes before the initial recording at baseline, and thereafter 1 ml (4 mg/ml) of rhodamine before each imaging to stain the activated leukocytes in circulation, for observation of leukocyte-endothelial cell interactions. A postcapillary venule was used for calculation of adherent and rolling leukocytes. An easily determined portion of the vessel was chosen, and the exact number of adherent leukocytes was calculated from still images, and related to the venule’s surface area for which the calculation was performed.
NADH is a natural intracellular fluorophore, and its concentration increases during ischaemia (Vollmar et al. 1998). The ultraviolet filter measurements are based on differences on NAD+/NADH redox state, as the 330-nm light excitation results in a fluorescence peak at 440–450 of NADH, but NAD⁺ does not emit such a peak (Chance et al. 1979).

4.8 Transmission electron microscopy (TEM)

In study I, we also analysed intracellular morphological changes using transmission electron microscopy (TEM). TEM samples were collected after the last intravital microscopy imaging, hemodynamic data measurements, and blood sample collection. A 10mm·10mm·5mm biopsy was collected from the right cerebral cortex of the temporal lobe and right cerebellar cortex 3 h postoperatively, while the subject was still under anaesthesia. After collection, the subjects were euthanised using 60 mg/kg pentobarbital intravenously.

All samples were immediately fixed in 1% glutaraldehyde, 4% formaldehyde mixture in 0.1 M phosphate buffer and post-fixed in 1% osmiumtetroxide, dehydrated in acetone, and embedded in Epon LX 112 (Ladd Research Industries, Williston, VT, USA). Representative sectional planes were chosen from cerebral cortex and cerebellar samples using a microscope, and thin sections were cut with a Leica Ultracut UCT ultramicrotome, stained in uranyl acetate and lead citrate, and examined with a Tecnai G2 Spirit transmission electron microscope (FEI Europe, Eindhoven, Netherlands). Images were captured by Veleta CCD camera (Olympus Soft Imaging Solutions GmbH, Munster, Germany).

Three factors of the TEM samples were analysed: ultrastructure, rough endoplasmic reticulum (RER), and mitochondria. Ultrastructural changes included neuroglial cells and neurones, and any structural changes in astrocytic perivascular endfeet. The easiest cell to compare was the cerebellar Purkinje’s cell, as it is notably larger, compared with other cells. The observed mitochondrial changes were swelling, disrupted crystal integrity, and loss of its 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 (low affinity) cytoplasm. Structural alterations were scored as follows: normal = 0, mild = 1, moderate = 2, and severe = 3. The
total score was a sum of these three scores for both cerebrum and cerebellum, summing to a maximum score of 18.

4.9 Sagittal sinus blood sampling

In study II, a 5cm · 5cm U-shaped flap of skin was transversally cut and bent open in the middle of the skull. A cranial window (35 mm · 40 mm) was created in the middle of the skull at a coronal suture with a 14 mm/11 mm disposable cranial perforator (200–253 DGR-II, Acra Cut Inc., Acton MA, USA). Sagittal sinus was then cannulated through dura mater with a 22G 0.8 · 25 mm (22 G x 25 mm BD Venflon™ Peripheral IV Catheter with Injection port) cannula, and secured. Blood samples of the returning blood of the brain were collected through the cannula. After the experiment, the sagittal sinus was decannulated, the cannula entry wound was sutured with 7-0 non-absorbable polypropylene sutures to avoid subdural hematoma, and the flap closed and sutured.

4.10 Additional cranial procedures

In study III, blood samples of a consecutive series of 52 piglets from multiple studies were analysed. In all of the studies, a 7-cm longitudinal incision was made on top of the scalp and the skull was revealed. Two to 3 holes of 5 mm diameter were drilled through the skull over the parietal cortex. A microdialysis catheter (CMA 70, CMA/Microdialysis, Stockholm, Sweden), an intracranial pressure-monitoring catheter (Codman Micro-Sensor ICP transducer, Codman ICP Express Monitor, Codman & Shurtleff Inc., Raynham, MA), a temperature probe (Thermocouple Temperature Catheter-Micro-Probe, Ref C8.B, GMS, Kiel Germany), or a combination of these instruments was placed into the cerebral tissue. In one study, electroencephalography (EEG) data were also gathered from 4 leads placed in the periosteum. The data gathered from cranial measurements during the individual studies were excluded from study III and also from this thesis, due to divergent methodology.

4.11 Enzyme-linked immunosorbent assay

In studies II and III, serum values of 8-OHdG were analysed. After collection, the serum samples were stored in polypropylene tubes at -80 to -88°C until analysis. Highly Sensitive 8-OHdG Check ELISA kits were used to analyse the samples
(from the Japan Institute for the Control of Aging, Fukuroi, Japan), following the manufacturer’s instructions.

In study II, we measured 8-OHdG from both vena cava serum samples and sagittal sinus serum samples at baseline, post-intervention, and post-sham treatment, and 1 h and 6 h after the start of rewarming. In study III, frozen serum samples were thawed and analysed. The samples were collected at baseline, end of cooling, and 2 hours and 8 hours after the beginning of rewarming.

4.12 Histopathology

Brains from study II, as well as right ovaries, and 2 cm × 2 cm biopsies from the right ventricle of the heart and right kidney were obtained after euthanasia and were fixed in buffered 10% formalin solution for 2 weeks prior to paraffin-embedding and staining with hematoxylin-eosin (HE). 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 (pons) and cerebellum. Similar slices were cut from the kidney samples, heart samples, and ovaries. The pieces were fixed in fresh formalin for another week. After fixation, the samples were further processed by 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 incubated for 1 hour in an absolute ethanol-xylene mixture, 4 hours in xylene, and embedded in warm paraffin wax for 6 hours. The samples were then sectioned at 6 μm and stained with hematoxylin-eosin.

After staining, an experienced blinded pathologist analysed the samples. Samples from the right ventricle, ovary, kidney, and 3 regions of the CNS were scored. Scoring of the HE samples was based on the presence of oedema (0 to 3), neuron degeneration (0 to 1), haemorrhages (0 or 2), and presence of infarcted tissue (0 or 3). The CNS total regional score was the sum of the scores in each specific brain area (cortex, thalamus, hippocampus, brainstem, and cerebellum). A total histopathological score comprises these 5 regional scores, to allow semiquantitative comparison between the animals.
4.13 Immunohistochemistry

In study II, the immunohistochemical stainings were scored for DJ-1, 8-OHdG, HIF-1-α, Nrf2, and Ogg1 using the following semiquantitative protocol: 0 = negative, 1 = positive, 2 = strongly positive, and 3 = very strongly positive. All neurons from the cortex and hippocampus in addition to Purkinje neurons from the cerebellum were chosen for regions of the CNS. The heart, kidneys, and ovaries were also chosen for immunohistochemical stainings. Immunohistochemical methods are presented in Table 4.

Table 4. Immunohistochemical methods used in study II

<table>
<thead>
<tr>
<th>Antibody (clone/product code)</th>
<th>Dilution</th>
<th>Immunostaining Method</th>
<th>Source of Primary Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nrf-2 (C-20)</td>
<td>1:300</td>
<td>Dako Envision Kit</td>
<td>Santa Cruz Biotechnology, Santa Cruz, USA</td>
</tr>
<tr>
<td>Ogg-1 (pAb anti Ogg-1 Antibody)</td>
<td>1:500</td>
<td>Dako Envision Kit</td>
<td>Novus Biologicals</td>
</tr>
<tr>
<td>HIF-1-α (Ab-4 H1alpha67)</td>
<td>1:1000</td>
<td>Novo Link kit</td>
<td>Neo Markers</td>
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<tr>
<td>8-OHdG</td>
<td>1:50</td>
<td>Invitrogen kit</td>
<td>Japan Institute For the Control of Aging</td>
</tr>
<tr>
<td>DJ-1/ PARK 7 (ab 18257)</td>
<td>1:20000</td>
<td>Envision kit</td>
<td>Abcam Inc., Cambridge, UK</td>
</tr>
</tbody>
</table>

4.14 Cardiac parameters

In study III, frozen blood samples collected at baseline, end of cooling, and 2 hours and 8 hours after the beginning of rewarming were thawed and analysed. The levels of cardiac troponin I, and creatine kinase isoenzyme MB, and cardiac index were measured.

4.15 Additional measurements

In addition to regular blood sampling of blood gas values, electrolytes, ionised calcium, plasma lactate levels, pH, glucose, and hematocrit, we also recorded rectal and systemic temperature, cardiac output, fluid balance, and heart rate and ECG.
4.16 Statistical analysis

Analyses were performed by an experienced biostatistician using SPSS (IBM Corp., Released 2012, IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.) and SAS (version 9.3, SAS Institute Inc., Gary, NC., USA). Summary measurements are expressed as mean and standard deviation (SD) or as median and 25th – 75th percentiles. Either the Student t-test or Mann-Whitney U test was used to assess the distribution of continuous and ordinal variables between the study groups. The repeatedly measured data were analysed using a linear mixed model (LMM) with patients fitted as random, and the covariance pattern was chosen according to Akaike’s Information Criterion. P-values reported for the LMM are as follows: pt indicates change over time, pg indicates a level of difference between groups, and pt*g indicates interaction between groups and time. Univariate comparison between groups for categorical variables was made using the $\chi^2$-test and Fisher’s exact test, when appropriate. Two-sided p-values are reported.
5 Results

5.1 Study I

5.1.1 Comparison of study groups

No differences in weight of the porcine mean arterial pressures (MAP), central venous pressures (CVP), pulmonary capillary wedge pressures (PCWP), leukocyte counts, or serum calcium or glucose measurements were detected between the RIPC and control groups during the protocol. However, at baseline, haemoglobin levels differed as the 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) (P = 0.004). Also, arterial blood gas values differed at baseline, as the PCO₂ was 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). These differences were nullified at the subsequent time points.

5.1.2 Intravital microscopy

There were no statistically significant differences in the number of rolling leukocytes or in venous vessel diameters between the groups, throughout the experiment. The number of adherent leukocytes referred to baseline was higher in the control group at multiple time points after reperfusion, with statistical power (pₐ=0.003), as seen in Figure 7. The difference between groups in cerebral leukocytes equalised at 3 hours.

The reduced nicotinamide adenine dinucleotide (NADH) autofluorescence differed statistically significantly between the groups (pₐ=0.029). The differences were significant also at individual time points 2 h postoperatively (P = 0.023) and 3 h postoperatively (P = 0.002). At these time points, the RIPC group exhibited lower autofluorescence values, indicating lower concentration of NADH.
5.1.3 Transmission electron microscopy (TEM)

Both groups demonstrated ultrastructural changes in cerebral and cerebellar samples. For comparative purposes, a sham piglet was operated that did not undergo HCA but only similar anaesthesia with similar surgical procedures. A sample picture of scored ultrastructural changes is shown in Figure 8.

The total score of both cerebral and cerebellar changes in the RIPC group was 7.66 (SD 1.40) and in the control group was 9.66 (SD 1.71) (P = 0.54). All RIPC animals had ultra-structurally normal RERs in their cerebellar tissue, whereas the control group’s animals had a mean score of 1.06 (SD 0.41) (P = 0.026). The cerebral score of the sham-control animal was 3, and the cerebellar score was 0.5, summing the total score to 3.5.
Fig. 8. Representative images from study I of normal (left) and affected (right) mitochondria and RER of glial cells. Affected RER is prominent and moderately dilated, whereas affected mitochondria show swelling, disrupted cristae, and loss of matrix density N, nucleus, M, mitochondria, * RER. Scores: left image: Mitochondria = 0, RER = 0, right image: Mitochondria = 3, RER = 2.

5.2 Study II

5.2.1 Comparability of study groups

One piglet was excluded due to failed cannulation of sagittal sinus, leading to a control group of 5 out of 11 total subjects. There were no statistically significant differences between the groups, considering the weight, the amount of donor blood, baseline electrolyte content, hemoglobin hemodynamic data, or blood gas values.

5.2.2 Enzyme-linked immunosorbent assay

The measured 8-OHdG level referred to baseline differed significantly in the sagittal sinus samples at 360 minutes. The compared value in the control group at 360 minutes after the reperfusion was 1.08 (1.01–1.10) and was 0.93 (0.89–0.93) in the RIPC group (p = 0.018). There were no significant differences at any other time points, or in the vena cava samples, in this study design.
5.2.3 Histopathology and immunohistochemistry

The total histopathological score was 3.8 (1.8–6.0) in the RIPC group and 4.4 (2.5–6.5) in the control group, with no statistically significant difference (p=0.72). The HIF-1-α differed significantly, as the control group scored 2.8 (2.5–3), whereas the RIPC group scored 1.3 (0.8–2.3) (p = 0.026). With this group size, there were no statistically significant differences in Nrf2 or HIF-1-α. The immunohistochemistry is shown in Table 5.
Table 5. Immunohistochemistry

<table>
<thead>
<tr>
<th>Immunostaining Protocol</th>
<th>Cortex Score</th>
<th>Hippocampus Score</th>
<th>Cerebellum Score</th>
<th>Total Score</th>
<th>Ovary Score</th>
<th>Kidney Score</th>
<th>Heart Score</th>
</tr>
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<tr>
<td>DJ-1 RIPC</td>
<td>0 (0-0.3)</td>
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<td>0.7 (0-1.0)</td>
<td>0.8 (0.75-1.0)</td>
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<td></td>
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<tr>
<td>CRTL</td>
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<td>0</td>
<td>1.0 (1-1.0)</td>
<td>1.0 (1-1.0)</td>
<td>0</td>
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<tr>
<td>P-value</td>
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<td>0.18</td>
<td>0.36</td>
<td>-</td>
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<tr>
<td>8-OHdG</td>
<td>0 (0-0.3)</td>
<td>0.2 (0-0.3)</td>
<td>0.7 (0-1.5)</td>
<td>0.3 (0-0.3)</td>
<td>0.8 (0-2.3)</td>
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<tr>
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<td>0.8 (0-2.0)</td>
<td>0.4 (0-1.0)</td>
<td>0.4 (0-1.0)</td>
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<td>0.36</td>
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<td>HIF-1-α</td>
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<td>5.3 (3.0-9.0)</td>
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<tr>
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<td>2.4 (2.0-3.0)</td>
<td>7.2 (7.0-8.0)</td>
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<td>2.8 (2.5-3.0)</td>
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</tr>
<tr>
<td>P-value</td>
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<td>0.36</td>
<td>0.36</td>
<td>0.19</td>
<td>0.026*</td>
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<tr>
<td>Nrf2</td>
<td>2.3 (1.0-3.0)</td>
<td>1.7 (0-3.0)</td>
<td>6.2 (5.0-7.0)</td>
<td>0.8 (0-1.0)</td>
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</tr>
<tr>
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<tr>
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</tr>
</tbody>
</table>

RIPC n = 6, Control n=5. Immunohistochemical data after the 7th postoperative day. Score; mean (interquartile range), Mann-Whitney U.
5.3 Study III

5.3.1 Comparability of study groups

Baseline hemoglobin and haematocrit differed statistically significantly between the groups. The hemoglobin in the RIPC group was 82 g/l (77–88) and was 78 g/l (73–80) in the control group \((p=0.011)\). The baseline haematocrit was 24 (22–26) in the RIPC group and 23 (21–24) in the control group \((p=0.017)\). There were no statistically significant differences with respect to the weight, the amount of donated blood, blood gas analysis, plasma electrolyte content, cardiac index, 8-OHdG concentration, troponin isoenzyme I levels, or creatine kinase isoenzyme MB levels between the groups, at baseline. Blood and rectal temperatures were comparable and showed no statistically significant differences throughout the experiment.

The data gathered from cranial measurements were excluded from this study due to divergent methodology between piglets. Only 4 time points (baseline, end of cooling, 2 hours and 8 hours after the beginning of rewarming) were identical for all animals, and were included in study III, for all measurements.

5.3.2 Enzyme-linked immunosorbent assay

The level of 8-OHdG was observed to rise in the control group after cardiopulmonary bypass. The difference was most notable at 8 hours after rewarming, when the control group remained at 1.84 (1.62–2.44) ng/ml, whereas the RIPC group, 8-OHdG concentration was lower at 1.48 (1.39–1.69) ng/ml \((p=0.004)\). The 8-OHdG measurements are shown in Figure 9.
5.3.3 Additional measurements

No statistically significant differences were found in cardiac troponin I, creatine kinase isoenzyme MB, or cardiac index in this experimental setup, as seen in Figures 10–12. There were no differences between the groups with respect to white blood cell count and differential white cell count at any time point throughout the follow-up period.
Fig. 10. The measured level of creatine kinase isoenzyme MB; U/l (units per litre) (Arvola et al. 2016, published with permission from the Heart Surgery Forum).
Fig. 11. The measured cardiac index, l/min/m²; There is no difference in cardiac index between the groups (Arvola et al. 2016, published with permission from the Heart Surgery Forum).
Fig. 12. The measured troponin level µg/l (Arvola et al. 2016, published with permission from the Heart Surgery Forum).
6 Discussion

6.1 Study design

The hypothesis in this experimental study design was that remote ischaemic preconditioning (RIPC) alters cerebral microcirculation, vessel diameter, leukocyte behaviour, and NAD⁺/NADH redox state, after prolonged deep hypothermic circulatory arrest (DHCA). Furthermore, we sought to investigate whether the direct markers of oxidative stress are lower with preconditioned subjects and whether there are changes in redox-regulating enzymes in a chronic model.

In this study design, a prolonged 60-minute DHCA was induced, to mimic a challenging operation of the aortic arch or repair of congenital heart defect that exceeds the safe time of DHCA. Brain samples were gathered in acute and chronic phases, and neurological damage was evident in both groups, in the form of TEM images and in histopathological samples.

6.2 Intravital microscopy

We studied leukocyte activation, NAD⁺/NADH redox state, and changes in cerebrocortical vessel diameters directly in vivo using an intravital microscope, during prolonged DHCA-mediated I/R injury.

6.2.1 Vessel diameters

A profound hyperemia, in which the autoregulation of cerebral blood flow is lost and cerebral perfusion exceeds metabolic demand, is known to occur after ischaemic stroke (Bushnell et al. 1987, Nir et al. 1997). This affects the BBB and causes swelling and a rise in intracranial pressure affecting the cerebral perfusion pressure, as well. Contrary to earlier findings with hypoxia preconditioned rodents (Woitzik et al. 2006), we could not demonstrate differences in the diameter of cerebrocortical vessels during, or soon after, the prolonged DHCA, between the groups, in this model. This implies that the possible neuroprotection of RIPC is not caused by preservation of cerebrocortical autoregulation, after I/R injury. The visual data of the intravital microscope are restricted to the cortical capillary bed. The majority of the autoregulation happens in deeper cerebral
vessels (Yoshino et al. 2009), but we could not assess cerebral core autoregulation with this experimental setup.

6.2.2 Leukocytes

Leukocytes activate via multiple mechanisms, e.g. mechanical shear stress, interaction with air, contact with CPB tubing, and ischaemia- and/or infection-mediated inflammatory signals. The endothelia of cerebrocortical vessels’ response to cytokines such as TNFα and IL-1, causing endothelial E-selectin and P-selectins to activate, mediates the leukocyte rolling and adhesion.

The adherent leukocytes were significantly lower in the RIPC group after DHCA (Figure 3). Leukocytes are known to partly mediate I/R injury. A lower number of adherent leukocytes could partly explain the neuroprotective effect of RIPC, as activated adherent leukocytes contribute to ischemia reperfusion injury in the central nervous system (Clark et al. 1991). There were variations in vessel length, width, and form between subjects. Therefore, we compared the measured leukocytes to the baseline values. The same vessels were used for measurements at all time points. Only the numbers of adherent and rolling leukocytes were measured in this experimental setup. We did not investigate the functionality or antigen specificity of the leukocytes.

6.2.3 NAD+/NADH redox state

The control group demonstrated elevated NADH autofluorescence measurements, indicating worse NAD+/NADH redox state. NAD⁺ prevents apoptosis-inducing factor translocation from mitochondria to nucleus, and is also associated with better intracellular ATP management; it also participates in the preservation of mitochondrial membrane potential (Hong et al. 2015). Our findings demonstrate that the neuroprotective effects of RIPC can be seen in an improved NAD⁺/NADH redox state.

6.3 Transmission electron microscopy (TEM)

We analysed ultrastructural changes of several cellular organelles from the cerebrum and cerebellum. The only statistically significant difference between the groups was the difference in cerebellar RER score. The RER score was higher in the control group, and interestingly, all animals in the RIPC group in study I had
ultrastructurally normal RERs. RER serves multiple major functions, including posttranslational modification, folding, and assembly of newly synthesised proteins, and its proper function is essential to cell survival. Oxidative stress leads to functional flaws in RER (ER stress), and impairment in the folding environment of the RER induces CHOP gene-mediated apoptosis (Zinszner et al. 1998). The induced CHOP gene also results in additional creation of oxidative stress, when there is already oxidative stress present (McCullough et al. 2001). During ischaemia, NO also contributes to ER stress, and leads to apoptosis through the ER stress pathway (Oyadomari et al. 2001). The neuroprotective effect of RIPC is partly explained by the preservation of cellular RER function.

6.4 Reactive oxygen species (ROS) findings

In study II, the 8-OHdG showed a slight difference between the groups in sagittal sinus samples. There were no differences between the groups in the samples of systemic blood, with this group size and experimental setup. We analysed 52 piglets’ systemic blood samples in study III for adequate statistical power, and a clear difference is also seen in systemic blood samples.

The 8-OHdG is a reliable and measurable marker for oxidative stress. We demonstrate the measurable difference in oxidative stress between the groups, indicating that RIPC reduces oxidative during open heart surgery. The reduction in oxidative stress attenuates the ischaemic cascade and preserves cellular integrity.

In study II, we also measured the levels of 8-OHdG immunohistochemically, after 7 days of follow-up. Only a few piglets demonstrated minor findings of 8-OHdG, with no difference between the groups. This was expected, as the 8-OHdG is an acute marker of hydroxylated DNA damage, associated with oxidative stress. After 7 days of follow-up, there should not be substantial oxidative stress present. Interestingly, there were close to significant findings in cerebral Nrf2 and HIF-1-α, even after 7 days of follow-up. There are conflicting studies considering the protective time windows of RIPC. Kuzuya et al. suggested a rapid protecting period after preconditioning to exist between 1 hour to 3 hours, and a second window that reappears between 24 hours to 72 hours (Kuzuya et al. 1993). Barone et al. showed a neuroprotective effect to occur after 12 hours of preconditioning (Barone et al. 1998). The immunohistochemical findings in study II could imply that the RIPC might alter cellular oxidative balance, even 7 days after preconditioning and ischaemic insult. The findings were statistically
nonsignificant, however, and additional investigation is needed to determine if this is the case.

The HIF-1-α in the heart samples of study II was lower in the RIPC group, scoring 1.3 (0.8–2.3), whereas the control group scored 2.8 (2.5–3.0) (p=0.26), indicating higher HIF-1-α activity, after 7 days of follow-up. This is a single finding, and the clinical relevance remains unclear. In study III, there were no differences in cardiac troponin I, creatine kinase isoenzyme MB, or cardiac index between the groups, indicating no relevant heart protection that could explain the HIF-1-α findings of study II.

In study III, the significant reduction in ROS activity was found prior to the major ischemia, and moreover, the difference became more obvious during the follow-up period. During hypoxia-reperfusion phases, ROS formation and oxidative stress are notably increased (Raedschelders et al. 2012). A significant inflammatory response and subsequent I/R injury are also known to be triggered by surgery and the CPB per se (Franke et al. 2005). The findings of study III indicate that the effects of RIPC take place rapidly after the precondition stimulus, and most conspicuously, the attenuation of oxidative stress can already be seen during CPB.

6.5 Limitations of the study

The clinical relevance of this study is limited due to the experimental model used. The number of animal subjects is small because of ethical reasons. Study I is also an acute model, and the animals were euthanised at the end of follow-up. We do not know what the eventual outcome would have been for those individual subjects, had they been studied in a surviving model. The histopathological chances develop fully in 4–7 days. The correlation of our TEM findings and eventual histopathological score were not studied in this experimental set-up.

The animal models were also conducted on healthy individuals. In contrast, human patients with diseases of the aortic arch are usually over 50 years of age, with possible multiple pathologies including atherosclerosis and diabetes, that also contribute to systemic inflammation and endothelial pathologies. In this experimental model, the pigs used were aged around 10 weeks (roughly equivalent to human child aged 3–4 years. The aorta size is more or less analogous with an aorta of a 10-year-old child.), weighing 18 to 28 kg. The healing effect is probably better in healthy piglets than in human patients due to the lack of other systemic pathologies. The model is, however, more suitable for
profiling operations of congenital heart defects than aortic pathologies of aged people.

6.6 Summary

In study I, remote ischemic preconditioning (RIPC) lowered the number of adherent leukocytes in cerebrocortical vessels in piglets after prolonged DHCA. Also, the RER preservation was present in TEM analysis. In study II, the RIPC lowered ischaemia-reperfusion-related oxidative stress, and showed statistically non-significant changes in regulators of cellular oxidative balance, after 7 days of follow-up. In study III the attenuation of oxidative stress by RIPC could already be seen during CPB, indicating that the neuroprotective pathways triggered by RIPC become functional more rapidly than previously thought.

The lower oxidative stress is known to result in lower cytokine production, thus mediating lower levels of cerebrocortical vascular endothelial activity and adherent leukocytes, which also contribute to I/R injury. Better RER preservation in preconditioned piglets might partly explain the lower level of oxidative stress.

6.7 Clinical implications

The findings of this study strengthen the understanding of pathophysiology and the role of remote ischemic preconditioning (RIPC) behind ischemia reperfusion injury. Remote ischemic preconditioning is an inexpensive intervention that is easily applicable to cardiac operations. It is well tolerated and safe (Bilgin-Freiert et al. 2012), and it lacks risks usually related to invasive and pharmacological interventions.

Cardiac and aortic surgery-related ischemia reperfusion injury to the central nervous system is an obvious target for intervention. The surgical operations are often elective and allow administration of RIPC stimulus to be correctly timed. Since there is always equipment available for non-invasive blood pressure measurement at anesthesia induction, there is no need for new technologies to be introduced to the operating theatres.

For emergency operations, such as the Stanford type A acute aortic dissection, hemodynamic stability is often a luxury that the surgeon cannot afford. The findings in study III indicate the possible protective effects of RIPC taking place rapidly. Thus, even emergency-operated patients could receive RIPC intervention.
during anaesthesia induction and sternotomy, and gain benefit from this intervention during the life-saving operation.
7 Conclusions

I Remote ischaemic preconditioning prior to prolonged deep hypothermic circulatory arrest result in the form of decreased amount of adherent leukocytes in cerebrocortical vessels, providing better mitochondrial respiratory chain function, and appearing to preserve endoplasmic reticulum.

II RIPC lowers cerebral oxidative stress and might alter redox-regulating enzymes after prolonged DHCA in a chronic porcine model.

III RIPC significantly lowers oxidative stress during cardiopulmonary bypass, but does not demonstrate relevant cardiac-related effects.
References


Original publications


* = equal contribution in article


Original publications are not included in the electronic version of the dissertation.
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