METHODS FOR IMPROVING NEUROLOGICAL RECOVERY AFTER HYPOTHERMIC CIRCULATORY ARREST

Fructose-1,6-bisphosphate and hypertonic saline dextran in a surviving porcine model

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OU LU 2005
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2005
Oulu, Finland

Abstract

During surgery of the aortic arch and pediatric heart surgery, the blood flow to the brain has to be interrupted at times to allow a bloodless operation field and adequate conditions for surgical repair. During this no-flow period the brain is exposed to a high risk of ischaemic injury, as it will become irreversibly damaged after 5 minutes of circulatory arrest at 37°C. Additional time can be gained by cooling the patient with an extracorporeal heart-lung machine, as hypothermia reduces the cerebral metabolic rate and allows longer safe periods of circulatory standstill. This method of cerebral protection, called hypothermic circulatory arrest (HCA), is widely used in clinical practice. Thus the brain becomes susceptible to ischaemic injury after 30 minutes of HCA at 15°C. Lower temperatures than this are not practicable, however, as they require longer periods of cardiopulmonary bypass, which may further aggravate cerebral injury. To ensure a better outcome for patients undergoing these operations, additional ways of protecting the brain are required.

The present work focuses on neuroprotective biochemical and fluid therapy methods for use during HCA, employing a surviving porcine model. Fructose-1,6-bisphosphate (FDP), a high-energy intermediate of glycolysis, was examined for potential neuroprotective properties in two cerebral injury settings associated with HCA. First, FDP was administered before and after a 75-minute period of HCA at a brain temperature of 18°C. This led to better survival, neurological recovery and brain histopathological findings and had supportive effects on brain metabolism (I). Second, a 25-minute period of HCA along with an iatrogenic embolic load produced by microsphere injection was used to generate a massive ischaemic injury to the brain. In this setting FDP did not affect the neurological outcome but had a clear supportive impact on cerebral metabolism (II). In addition, cerebral histopathological samples taken during the first study were analysed by electron microscopy, which revealed significant preservation of the ultrastructure in the FDP-treated animals (III).

Hypertonic saline dextran (HSD) is a novel fluid therapy method which has been shown to enhance the outcome after hypovolaemic shock with or without head injury and is potentially very effective in reducing ischaemia-reperfusion injury. Its administration led to a decrease in intracranial pressure, improved brain metabolism, faster and better recovery and less histopathologically observable morphological damage (IV).

The findings indicate that both FDP and HSD have significant neuroprotective properties and should be assessed in humans as well.

Keywords: cerebral protection, fructose-1,6-bisphosphate, hypertonic saline dextran, hypothermic circulatory arrest
to my family and friends
Acknowledgements

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Oulu, November 2005

Timo Kaakinen
# Abbreviations

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<tr>
<td>AIF</td>
<td>Apoptosis-inducing factor</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CBF</td>
<td>Cerebral blood flow</td>
</tr>
<tr>
<td>CK</td>
<td>Creatine kinase</td>
</tr>
<tr>
<td>CK-BB</td>
<td>Creatine kinase (BB isoenzyme)</td>
</tr>
<tr>
<td>CK-MB</td>
<td>Creatine kinase (MB isoenzyme)</td>
</tr>
<tr>
<td>CK-MM</td>
<td>Creatine kinase (MM isoenzyme)</td>
</tr>
<tr>
<td>CMRO₂</td>
<td>Cerebral metabolic rate of oxygen</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
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<tr>
<td>CPB</td>
<td>Cardiopulmonary bypass</td>
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<tr>
<td>EEG</td>
<td>Electroencephalogram</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FDP</td>
<td>Fructose-1,6-bisphosphate</td>
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<tr>
<td>HCA</td>
<td>Hypothermic circulatory arrest</td>
</tr>
<tr>
<td>Hct</td>
<td>Haematocrit</td>
</tr>
<tr>
<td>HES</td>
<td>Hydroxyethyl starch</td>
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<tr>
<td>HSD</td>
<td>Hypertonic saline dextran</td>
</tr>
<tr>
<td>HTS</td>
<td>Hypertonic saline</td>
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<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
</tr>
<tr>
<td>ICP</td>
<td>Intracranial pressure</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>MCA</td>
<td>Middle cerebral artery</td>
</tr>
<tr>
<td>MPT</td>
<td>Mitochondrial permeability transition</td>
</tr>
<tr>
<td>MPTP</td>
<td>Mitochondrial permeability transition pore</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide reduced form</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>O₂</td>
<td>Oxygen molecule</td>
</tr>
<tr>
<td>O₂⁻</td>
<td>Superoxide</td>
</tr>
<tr>
<td>ONOO⁻</td>
<td>Peroxynitrite</td>
</tr>
<tr>
<td>pCO₂</td>
<td>Partial pressure of carbon dioxide</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet-activating factor</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>pO₂</td>
<td>Tissue oxygen partial pressure</td>
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<tr>
<td>rCBF</td>
<td>Regional cerebral blood flow</td>
</tr>
<tr>
<td>RCP</td>
<td>Retrograde cerebral perfusion</td>
</tr>
<tr>
<td>RLFP</td>
<td>Regional low-flow perfusion</td>
</tr>
<tr>
<td>SCP</td>
<td>Selective cerebral perfusion</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
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<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
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List of original publications

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:


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1 Introduction

Surgery of the aortic arch and pediatric heart surgery often require interruption of the blood flow to make the operation field bloodless and surgical repair possible. The brain is exposed to a high risk of ischemic injury during heart arrest, however. It was the cardiopulmonary bypass (CPB) technique, introduced by Gibbon in 1954 (Gibbon, Jr. 1978), that made modern aortic and cardiac surgery possible, and soon afterwards it was found that hypothermia could protect the brain from ischaemia. In this way hypothermic circulatory arrest (HCA) was invented (Niazi & Lewis 1957). DeBakey performed the first aortic arch replacement using normothermic CPB with isolated perfusion of the carotid and brachiocephalic arteries (DeBakey et al. 1957), a technique that was in effect an early version of selective cerebral perfusion (SCP). The latter is widely used in aortic surgery, but has the minor drawbacks of being technically complicated and possibly inducing embolization. Thus HCA was adopted for use in aortic arch surgery in the 1970s (Gschnitzer 1973, Griepp et al. 1975), enabling the procedures to be performed with acceptable mortality and morbidity rates (Griepp et al. 1991, Kouchoukos et al. 1995).

Despite advances in surgical and anaesthetic techniques, patients undergoing aortic arch surgery are still potentially susceptible to considerable ischaemic injury to the brain. HCA itself is accompanied by global ischaemia of the brain, and hypothermia is the sole major factor that can improve the outcome greatly after HCA. At the normal physiological temperature of 37°C, the cerebral metabolic rate of oxygen (CMRO₂) runs at its full value and irreversible brain injury occurs 5 minutes after the cessation of cerebral blood flow, whereas at 15°C the CMRO₂ is reduced to 16% of the normothermic value, which makes the safe duration of HCA about 30 minutes (McCullough et al. 1999).

Patients undergoing aortic arch surgery also run a marked risk of cerebral embolization due to extracorporeal circulation and the manipulation of sclerotic vessels (Kawachi et al. 2003). Thus the outcome of aortic surgery is not only dependent on the duration of HCA or the temperature used but on several other factors such as pH and the haematocrit during CPB (Sakamoto et al. 2004b). Other measures such as neuroprotective drugs, novel fluid therapies, cardiopulmonary bypass strategies and surgical techniques such as SCP or retrograde cerebral perfusion (RCP) have been invented to improve the outcome after HCA.
The mechanisms of ischaemic brain injury are well documented nowadays. It seems that numerous mechanisms lie behind the cellular destruction that takes place, the main ones among which are the loss of membrane potential, a biochemical cascade that includes glutamate excitotoxicity, and reperfusion injury (Janardhan & Qureshi 2004).

Fructose-1,6-bisphosphate (FDP) is a high-energy intermediate of glycolysis that enhances the functioning of the key enzymes in the glycolytic chain and bypasses a key reaction in the pathway which consumes adenosine triphosphate (ATP). Its neuroprotective properties may include support for brain metabolism before and during ischaemia and mitigation of reperfusion injury. The potential mechanisms by which FDP prevents ischaemic injury are many, and the main one among them has not been fully defined yet. Both animal and clinical studies support its neuroprotective properties, and it also has protective properties with respect to ischaemia/reperfusion injury in several other organs. No research has yet been carried out, however, to evaluate the perioperative and postoperative efficacy of FDP in mitigating brain injury after HCA.

In the first paper arising from the present work, the efficacy of FDP in reducing ischaemic cerebral injury is evaluated in a surviving porcine model of prolonged HCA (I), while the second focuses on the potential beneficial effect of FDP on embolic brain injury associated with HCA (II) and the third presents an analysis of tissue samples collected during the experiments of paper I by electron microscopy in order to elucidate the impact of ischaemic injury on the neuronal ultrastructures and to assess whether FDP may be able to prevent cellular damage at the ultrastructural level (III).

Hypertonic saline combined with dextran (hypertonic saline dextran, HSD) is a modern small-volume fluid therapy which was introduced initially in the 1980s as a treatment for hypovolaemic shock (Velasco et al. 1980). HSD is a potent vasodilator, immunomodulator and anti-oedemagenic agent as well as having positive inotropic effects on the heart. It is potentially very effective for mitigating reperfusion injury after ischaemia. Previous studies have indicated that it has significant neuroprotective properties, but no evaluation has been made of it in an HCA setting. Thus the fourth paper investigates the effectiveness of HSD after prolonged HCA (IV).
2 Review of the literature

2.1 Cerebral metabolism and ischaemia

2.1.1 Cerebral metabolism

The brain accounts for only 2% of the total body mass, but it is very well supplied with blood, since 20% of the cardiac output in humans is taken up by the brain. Under aerobic conditions, the metabolism of one mole of glucose generates 38 moles of ATP, whereas only two moles of ATP will be formed per one mole of glucose in anoxic tissue. Although the brain has some ability to metabolize glucose anaerobically, it is usually almost totally dependent on the oxygen, glucose and other nutrients supplied to it in the blood (Ljunggren et al. 1974a).

2.1.2 Cerebral blood flow

Under physiological conditions, the cerebral blood flow (CBF) is almost 50 ml/100 g/min, while the regional cerebral blood flow (rCBF) in the grey matter is approximately 80 ml/100g/min. The white matter does not usually require as much energy, so that its normal rCBF is approximately 20 ml/100g/min (McHenry, Jr. et al. 1978). The brain circulation has a complex system of autoregulation which ensures that the parts of the brain requiring the most energy are perfused most efficiently. If the systemic mean arterial pressure in healthy subjects is kept within 50 to 170 mmHg, the cerebral blood flow will remain constant (Harper 1966).
2.1.3 Cerebral ischaemia

Cerebral ischaemia occurs when rCBF falls below 20 ml/100 g/min in the most vulnerable areas, such as the cortex (Symon et al. 1977). The white matter is more resistant to failure in oxygen delivery, but it will also be damaged at an rCBF below 10 ml/100 g/min. Hypothermia causes a reduction in CMRO₂ and lowers the threshold for ischaemia, increasing the resistance to hypoxic injury.

The area that is subject to complete or nearly complete ischaemia is called the ischaemic core, while the surrounding, less severely affected area is called the ischaemic penumbra (Iadecola 1998). The penumbra is defined as brain tissue in which the rCBF has decreased to a level causing electrophysiological silence and transient but recurrent losses of membrane ion gradients and energy metabolites (Ginsberg & Pulsinelli 1994). It has been shown by positron emission tomography (PET) that the rCBF in the ischaemic core is below 7 ml/100 g/min while the penumbra has a rCBF of 7 to 17 ml/100 g/min (Baron 1999).

If reperfusion occurs in the ischaemic core within 60 minutes of the onset of ischaemia the neurons can survive the insult (Garcia et al. 1996), and although the beneficial outcome of reperfusion in the penumbra dramatically worsens after 3 hours of ischaemia, PET studies have shown that some areas can survive for up to 24 hours (Baron 1999). Thus even delayed attempts at protecting the brain may save neurons in the ischemic penumbra.

The type of ischaemic injury caused by HCA is global, theoretically affecting the entire brain. Another type of cerebral hypoxia is focal ischaemic injury, as occurs in stroke. From the clinical point of view the injury incurred in HCA is a combination of the two, as will be discussed below.

2.1.4 Depletion of brain energy sources

The cessation of blood flow results in a rapid drop in the concentrations of high-energy phosphates such as phosphocreatine and adenosine triphosphate (ATP) (Ljunggren et al. 1974a). Glucose and glycogen are converted to ATP via anaerobic glycolysis, but the availability of these fuel sources in the brain is limited, as they run out in only 1-3 minutes at 37°C (Siesjo 1978). If reperfusion is not achieved within approximately 5 minutes, the most vulnerable cells will be permanently damaged (Astrup et al. 1981). In hypothermia, the rate of metabolic activity of the brain is depressed and the fuel sources are depleted more slowly (Shin’oka et al. 2000).
Fig. 1. The glycolysis pathway. Only the relevant enzymes and reactions of the cascade are shown. The enzymes are indicated in italics. 1,3-BPG: 1,3-bisphosphoglycerate, ADP: adenosine dinucleotide, ATP: adenosine triphosphate, FDP: fructose-1,6-bisphosphate, NAD: nicotinamide dinucleotide, NADH: reduced form of NAD, PFK: phosphofructokinase. Modified from Murray et al. (1996).
When the rCBF falls below 50% of the normal values, the production of lactate in the brain tissue is increased, most probably secondarily to anaerobic glycolysis (Iadecola 1999). One molecule of glucose is converted to pyruvate, as seen in Figure 1, generating 2 molecules of ATP. Under aerobic conditions, the pyruvate is converted to acetyl-CoA, which enters the citric acid cycle with the eventual formation of 36 molecules of ATP, but under anaerobic conditions it is converted to lactate and no more ATP is formed. This excess formation and metabolism of lactate results in acidosis, as one molecule of nicotinamide dinucliotide releases one hydrogen ion during the process (Murray et al. 1996). Lactic acidosis is worse when the plasma glucose concentration is high (Ljunggren et al. 1974b). Acidosis exacerbates ischaemic injury, although the exact mechanisms are still unclear. Recent data suggest that astrocytes up-regulate their capability to metabolize glucose anaerobically during ischaemia (Marrif & Juurlink 1999). After perfusion is restored, the reaction catalysed by lactate dehydrogenase is reversed, as oxygen becomes available again, and the lactate produced anaerobically by the astrocytes is metabolized aerobically back to pyruvate by the neurons, which have only a limited capacity for anaerobic metabolism (Schurr et al. 1997). The newly formed pyruvate can now enter the citric acid cycle again to provide ATP for the damaged cells. Thus some controlled formation of lactate during ischaemia is not considered as detrimental to neurons nowadays as was previously thought (Schurr 2002).

### 2.2 Pathogenesis of ischaemic brain injury

When the blood flow to the brain is interrupted, loss of consciousness occurs in seconds. After ten seconds all electrical activity in the brain ceases, and in approximately 90-120 seconds the cell membranes lose their ionic gradients due to energy failure. This phenomenon, called depolarization, is the first major step in the process of ischaemic brain injury. If the blood flow is not restored in 5 minutes, the injury will quickly become irreversible and the ischaemic cells enter the second phase, biochemical cascade. If the blood flow is eventually restored, the cells will start to receive oxygen and nutrients again. This resumption of flow is a double-edged sword, however, since the damaged tissue becomes exposed to the third harmful part of the process, reperfusion injury. The three phases are interrelated and often share the same mechanisms, so that the above classification is somewhat arbitrary. The three steps are discussed in detail below.

### 2.2.1 Depolarization

In normal circumstances the sodium-potassium (Na⁺/K⁺) ATPase ion pump located in the plasma membrane consumes ATP to pump three sodium ions from the intracellular space to the extracellular space in exchange for two potassium ions passing in the opposite direction. This energy-demanding process generates a negative ion gradient between the
cytoplasm and the extracellular matrix which is inherent in the normal functioning of the neurons (Pocock & Richards 1999). In ischaemia, unless the blood flow is rapidly restored, the neuronal plasma membrane will start to lose its ionic gradient, leading to depolarization. This is a result of the loss of energy supply to the Na\(^+\)/K\(^-\) pump. Ischaemic depolarization occurs when the rCBF falls below 10 ml/100 g/min (Symon 1985) and causes an influx of sodium ions to the cells via voltage-gated Na\(^+\) channels. These sodium ions draw water from the extracellular space into the cells, thus generating cytotoxic oedema.

If the ischaemic depolarization is extremely short, 1 to 2 minutes, reperfusion results in neuronal salvage, in addition to which this short ischaemic insult will trigger the activation of mechanisms that make the cells more resistant to further ischaemic episodes, a phenomenon known as ischaemic preconditioning, or ischaemic tolerance (Kitagawa et al. 1991).

More prolonged depolarization (5 to 30 minutes) followed by reperfusion will partly or completely restore the cellular oxidative metabolism, but at this stage the reperfusion injury will affect tissue recovery from the interruption of the blood flow, as discussed below. An extended period of membrane depolarization (>30 minutes) will result in neuronal death despite reperfusion, and a depolarization period of more than 60 minutes will lead to death of the glial cells as well, which are normally more resistant to ischaemia than the neurons (Neumar 2000b).

Depolarization occurs in approximately 70 to 100 seconds under normothermic conditions, but takes longer in hypothermia, occurring after 194 to 310 seconds at 28°C (Bart et al. 1998). In the presence of deep hypothermia, depolarization takes time up to 10-20 minutes according to calculations by McCullough (McCullough et al. 1999).

**2.2.2 The biochemical cascade**

Ischaemia activates an interrelated biochemical cascade leading to cellular injury. The main pathways are summarized in Figure 2.

**2.2.2.1 Glutamate release**

Glutamate, an excitatory neurotransmitter in the brain, activates receptors of three types: N-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate (Gwag et al. 2002). Under normal circumstances, glutamate is rapidly cleared from the synaptic cleft by the neurons and glial cells and stored in vesicles in the cytoplasm, ready to be used again at times of action potential (Santos et al. 1996).

Loss of cellular energy results in depolarization, which releases neurotransmitters such as glutamate into the synapse, although this energy depletion also leads to the failure of glutamate uptake from the synapse. Thus the receptors in the presynaptic and postsynaptic membranes are activated continuously, resulting in excitotoxicity (Lipton & Rosenberg 1994). The activation of NMDA and AMPA receptors results in an influx of
calcium and sodium ions into the cells, respectively. The glutamate-mediated influx of sodium adds to the cytotoxic oedema brought about initially by depolarization, and the influx of calcium ions triggers a complex series of cytotoxic events discussed below. Glutamate excitotoxicity has been confirmed as playing a major role in ischaemic injury after HCA (Redmond et al. 1994).

Fig. 2. The biochemical cascade during cerebral ischaemia. AIF: apoptosis-inhibiting factor AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, ATP: adenosine triphosphate, CyC: cytochrome c, ER: endoplasmic reticulum, IP$_3$: inositol triphosphate, MPT: mitochondrial permeability transition, NMDA: N-methyl-D-aspartate, NO: nitric oxide, PLA$_2$: phospholipase A$_2$, PLC: phospholipase C, ROS: reactive oxygen species.
2.2.2.2 Loss of calcium homeostasis

The intracellular free Ca$^{2+}$ concentration is kept within narrow limits under physiological conditions, but it will rise via several pathways during ischaemia. Depolarization leads to opening of the voltage-gated Ca$^{2+}$ channels, and loss of the sodium gradient reverses the Na$^+$/Ca$^{2+}$ exchanger in the plasma membrane, resulting in further Ca$^{2+}$ influx into the cytosol (Lobner & Lipton 1993, Carini et al. 1994). Glutamate release leads to an influx of calcium ions into the cells, as discussed above. Moreover, glutamate acts on the endoplasmic reticulum (ER) via the activation of phospholipase C to generate inositol-1,4,5-triphosphate, which in turn causes the release of Ca$^{2+}$ from the ER into the cytoplasm (Schoepp & Conn 1993). In vivo microelectrode measurements have confirmed that the concentration of free Ca$^{2+}$ in the cytosol increases 2-3-fold within 8 minutes of global ischaemia in the hippocampal CA1 neurons, cerebellar Purkinje cells and cortical neurons, the most vulnerable of all the neurons to ischaemia (selective vulnerability). At the same time the extracellular calcium concentration falls from 1.2 - 1.5 mmol/l to 0.2 - 0.5 mmol/l (Silver & Erecinska 1992). The influx of Ca$^{2+}$ into the cells activates a number of mechanisms resulting in the degradation of cellular structures, as discussed further below.

2.2.2.3 Calpains

Calpains are cysteine proteases that play a role in dendrite outgrowth, long-term potentiation and synaptic remodelling under physiological conditions (Song et al. 1994, Vanderklish et al. 1996). They require Ca$^{2+}$ for their proteolytic activity and undergo overactivation during ischaemia (Croall & DeMartino 1991). Calpains degrade key cellular structure proteins and key signalling proteins such as tubulin, α-spectrin and protein kinase C (Billger et al. 1988, Kishimoto et al. 1989, Roberts-Lewis et al. 1994). The degradation of cellular structure proteins most likely leads to necrotic cell death, but the cleavage of signalling proteins may trigger delayed neuronal destruction by apoptosis (Neumar 2000c).

2.2.2.4 Phospholipase A$_2$

Activation of phospholipase A$_2$ produces free fatty acids and arachidonic acid from membrane phospholipids. Arachidonic acid is further metabolized to reactive oxygen species (ROS) by the enzyme cyclo-oxygenase, a process that damages the cellular structures as well, contributing to neuronal injury (Dumuis et al. 1990). Free fatty acid metabolites aggravate cerebral oedema, produce vasoconstriction and induce platelet aggregation, effects that may be mediated by platelet-activating factor, which is a product of phospholipid metabolism and may reduce the rCBF in the ischaemic brain (Braquet et al. 1989).
Oxygen free radicals, or reactive oxygen species (ROS), are normal by-products of the metabolism of oxygen in the mitochondrial respiratory chain. Another important pathway is the breakdown of arachidonic acid, as mentioned above. Under normal conditions, 2% to 3% of the body’s oxygen consumption is diverted to the production of ROS (Floyd 1997), the main physiological function of which is the oxidative burst of leukocytes, which cells use as an adjunct in their inflammatory reactions to foreign entities such as viruses or bacteria. Some insults such as ischaemia or sepsis may overactivate ROS production and therefore damage the host system unintentionally. ROS, which are normally held at bay by antioxidative enzymes such as superoxide dismutase, catalase and glutathione peroxidase, are known to damage cellular DNA, proteins and lipids. The free radical scavengers glutathione, α-tocopherol and ß-carotene also counteract the action of ROS (Halliwell 1987). Ischaemia and reperfusion cause massive ROS production via increased intracellular Ca²⁺ concentrations, which overwhelm the capacity of the endogenous protective mechanisms to control the damage. The longer the duration of ischaemia, the greater the production of ROS (Hall et al. 1993), the main species of which that are formed during ischaemia/reperfusion are superoxide (O₂⁻) and nitric oxide (NO) (Love 1999).

### 2.2.2.6 Nitric oxide

Neuronal nitric oxide synthase (nNOS), which is activated by an increased cytosolic Ca²⁺ concentration, transforms L-arginine to L-citrulline (Lipton et al. 1993). Under ischaemic conditions the reaction generates excess NO, which is a highly toxic ROS. NO reacts with O₂⁻, i.e. superoxide, to form peroxynitrite (ONOO⁻), yet another toxic ROS (Kooy et al. 1994), which is fairly stable and can traverse several cell diameters and cause damage over a much wider range than either NO or O₂⁻. ONOO⁻ is converted to a hydroxyl radical and nitrogen dioxide, which can cause damage to cellular proteins via the nitration of tyrosine residues (Koppenol 1998). The activation of nNOS may also induce apoptosis (Tseng et al. 1997).

Endothelium-derived NO is produced by endothelial NO synthase (eNOS). Upon formation in the vascular endothelium, NO diffuses into the adjacent cells and activates soluble guanylate cyclase, which in turn mediates many of the beneficial effects of NO. NO is a potent vasodilator in vascular smooth muscle, and regulates regional blood flow (Samdani et al. 1997). In addition, it is anti-thrombic, anti-inflammatory and anti-proliferative. By contrast, its loss contributes to impaired vascular relaxation, increased platelet aggregation and vascular smooth muscle cell proliferation, enhanced endothelial-leukocyte adhesion and increased blood pressure (Huang et al. 1995, Hudetz et al. 1999). Studies using animal models of cerebral ischaemia have demonstrated that eNOS and vascular NO play a prominent role in maintaining cerebral blood flow and preventing neuronal injury (Iadecola 1997).

Hypoxia has been associated with both the up-regulation and down-regulation of steady-state eNOS mRNA expression. Exposure of human or bovine endothelial cells to
low oxygen tension results in a profound decrease in the transcript for eNOS and a corresponding fall in eNOS protein levels (Phelan & Faller 1996). In contrast, up-regulation of eNOS mRNA has been detected in hypoxic bovine aortic endothelial cells (Arnet et al. 1996).

Inducible NOS (iNOS), which is not normally present in tissue, is induced after ischaemia and contributes to secondary late-phase damage (Endres et al. 2004). In contrast to nNOS and eNOS, iNOS expression is delayed, starting 6-12 hours after the ischaemic insult (Iadecola et al. 1996).

2.2.2.7 *Mitochondrial permeability transition (MPT)*

Both the increased Ca$^{2+}$ concentration and the increased ROS formation in the cytosol activate MPT which can also initiated by numerous other factors, such as ATP depletion, receptor-mediated cell death signals etc. MPT opens a multiprotein channel, the mitochondrial permeability transition pore (MPTP), that bridges the inner and outer mitochondrial membranes (Zamzami et al. 1995). The result is mitochondrial swelling, membrane depolarization, uncoupling of oxidative phosphorylation and increased ROS production. Mitochondrial Ca$^{2+}$ flows into the cytosol, adding to the vicious circle of increased cytosolic Ca$^{2+}$. The MPTP also allows the passage of mitochondrial proteins such as cytochrome $c$ and apoptosis-inducing factor (AIF) into the cytosol to induce apoptosis and caspases (Kroemer et al. 1997). If the amount of mitochondria in the damaged cell is large enough and ATP levels are low, the cell will undergo necrosis via the massive release of mitochondrial Ca$^{2+}$. On the other hand, smaller numbers of degrading mitochondria induce apoptosis via the outflow of CyC and AIF, a process that consumes ATP (Leist et al. 1997).

2.2.2.8 *Apoptosis*

Apoptosis, or programmed cell death, occurs when the injury to the cell has been mild enough to avoid immediate necrosis. Apoptotic cells are condensed and small in appearance and the nuclear chromatin is cleaved and condensed as well. The organelles remain fairly intact and the presence of specific cytoplasmic entities called apoptotic bodies can be observed (Kerr et al. 1972). Apoptosis requires ATP and some intact ribonucleic acid (RNA) and protein synthesis capability in the cells. Cells that have undergone apoptosis are phagocytized by macrophages, *i.e.* their cytoplasm is never in contact with the extracellular space. This is the reason why apoptosis does not induce an inflammatory response as necrosis does (Savill et al. 1993).

The activation of apoptosis occurs via three pathways: 1) caspases, a family of cysteine proteases, are activated by MPT via an *intrinsic* pathway, with mitochondrial cytochrome $c$ acting as the initiating factor; 2) caspases are activated by specific cell membrane receptors (mostly Fas and TNF receptors) via an *extrinsic* pathway; and 3) apoptosis is activated by MPT through the release of AIF and CyC via caspase-independent mechanisms (Graham & Chen 2001).
The ischaemic core is usually characterized by necrotic cell death, whereas apoptotic changes have been found in the ischaemic penumbra, indicating a role for ATP in the process (Graham & Chen 2001). It has been demonstrated that apoptotic activity is increased after HCA (Mennander et al. 2002).

2.2.3 Reperfusion injury

Ischaemia is a potent inductor of systemic inflammation, which is the main contributor to reperfusion injury after a cerebral ischaemic insult. The most striking feature on the cellular level is the adhesion of activated leukocytes to the endothelium in the cerebral microvasculature and their infiltration into the brain tissue. Autoregulation of cerebral blood flow is impaired after ischaemia, leading initially to hyperperfusion, with subsequent normoperfusion or even hypoperfusion of the injured areas. The main mechanisms of reperfusion injury are discussed below and shown in Figure 3.

2.2.3.1 Leukocyte adhesion

Ischaemia and reperfusion cause the cerebral cells (microglia, endothelial cells and neurons) to up-regulate the expression and release of proinflammatory cytokines such as tumour necrosis factor (TNF)-α and interleukin (IL)-1β. These stimulate the expression of other cytokines, mainly proinflammatory IL-6 and IL-8 and anti-inflammatory IL-4 and IL-10 (Barone & Feuerstein 1999). The proinflammatory cytokines promote the expression of adhesion molecules, including selectins (L, P and E-selectin), intercellular adhesion molecules (ICAM) 1 and 2 and integrins (β1 and β2-integrins or CD11/CD18) on the endothelial cells and leukocytes (Lipsky 1993). The role of TNF-α is contradictory, as a single dose extends the cerebral injury after middle cerebral artery (MCA) occlusion (Barone et al. 1997a), but TNF-receptor knockout mice generate larger infarcts than controls after a similar ischaemic insult, suggesting neuroprotective properties (Rothwell & Luheshi 1996).

The result of this activation cascade is the adhesion of leukocytes (primarily neutrophils) and red blood cells and platelets to the capillary endothelium. This obstructs the blood flow in the capillary bed, leading to secondary ischaemia (del Zoppo et al. 1991, Garcia et al. 1994, Mazzoni et al. 1995). There are four phases in the activation of neutrophils: 1) rolling, which is characterized by weak interaction between the neutrophils and endothelium mediated by surface selectins (Bevilacqua & Nelson 1993); 2) adherence, in which stronger adhesion of β2-integrins to the endothelial ICAM molecules plays a major role (Etzioni 1999); 3) activation and 4) emigration (Schaller & Graf 2004). Once the neutrophils have infiltrated the brain tissue, they release toxic products such as proteases and ROS, which damage the neurons and endothelial cells directly (Neumar 2000a).
2.2.3.2 Alterations in brain tissue perfusion

After perfusion to the ischaemic brain is restored, there is apparent hyperperfusion of the injured tissue (Muller et al. 1994). This is a result of impaired autoregulation of cerebral blood flow. After a variable length of time, this hyperperfusion is followed by hypoperfusion or normal rCBF. PET studies have shown that the most severely damaged areas of the brain receive marked hyperperfusion (up to 300% of normal rCBF) and it is there that the extent of the infarcted tissue is greatest. When hyperperfusion is modest (up to 125% of normal rCBF), the tissues generally resist the injury and the infarcts remain small in size (Heiss et al. 1997). It is not clear, however, whether it is the actual duration of ischaemia or the aggravated reperfusion injury due to hyperperfusion that damages the tissues. Most likely the areas receiving the greatest hyperperfusion are those that have been subjected to longer periods of ischaemia, so that they have a badly injured vascular bed that is incapable of any regulative functions such as vasodilation or constriction and they also have increased endothelial permeability (Schaller & Graf 2004).

![Diagram of reperfusion injury](image-url)

Fig. 3. Reperfusion injury.
2.2.3.3 Additional consequences of reperfusion injury

Neutrophil activation and the release of cytotoxic substances lead to endothelial dysfunction and increased permeability due to the loss of integrity in the blood-brain barrier (Cooper et al. 2000). This leads to sequestration of plasma proteins and water into the brain tissue, creating oedema. The increased brain water content elevates the intracranial pressure (ICP) (Pappius 1974), and the microvessels become compressed, which further aggravates the decrease in rCBF. In addition, the damaged endothelium secretes excessive amounts of vasoconstrictors such as endothelin and thromboxane A2, which constrict the cerebral vessels to an abnormal extent (Tsui et al. 1997).

2.3 Strategies for mitigating ischaemic cerebral injury

2.3.1 Perioperative hypothermia

Hypothermia is the most effective method for reducing ischaemic injury to the brain. The CMRO$_2$ falls by 7% for every 1°C reduction in temperature (Govier et al. 1984), so that hypothermia increases the high-energy phosphates and intracellular pH in tissues, thus delaying the loss of energy metabolites and the detrimental effects of acidosis (Kramer et al. 1968, Shin'oka et al. 2000). Hypothermia delays the onset of membrane depolarization and also prevents the excitotoxic release of glutamate (Rokkas et al. 1995, Bart et al. 1998), which means that the cells can manage with less energy and/or blood flow than during normothermia. The customarily recognised levels of hypothermia are mild (32-34°C), moderate (25-32°C), deep (15-25°C) and profound (<15°C).

2.3.1.1 Hypothermic circulatory arrest

HCA has been used for nearly 50 years as an adjunct to aortic surgery, and it is often required for the repair of congenital heart anomalies as well. As modern research has set time limits for the use of HCA (Svensson et al. 2001), morbidity and mortality after these operations have become acceptable. The correlation between temperature and the duration of safe HCA is described in Table 1. The Boston Circulatory Arrest Study suggested that deep HCA lasting for longer than 41 minutes will lead to excess deficits in neurological development in neonates operated on for congenital heart defects (Wypij et al. 2003), and Svensson et al. (1993) reported that the use of HCA is associated with a mortality rate of 10% and the occurrence of permanent neurological deficits in 7% of patients. The main causes of these adverse sequelae are brain complications (Crawford et al. 1989). The duration of HCA correlates with the occurrence of brain complications, and nearly all patients have been reported to suffer temporary cognitive dysfunction after HCA (Svensson et al. 2001).
Table 1. Calculated safe duration of HCA (McCullough et al. 1999).

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Cerebral metabolic rate (% of 37°C)</th>
<th>Safe duration of HCA (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td>100</td>
<td>5</td>
</tr>
<tr>
<td>30</td>
<td>56</td>
<td>9</td>
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<td>25</td>
<td>37</td>
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<td>31</td>
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<tr>
<td>10</td>
<td>11</td>
<td>45</td>
</tr>
</tbody>
</table>

Profound hypothermia is superior to higher temperatures if only metabolic suppression is addressed (Gillinov et al. 1993) but it is not practicable from a clinical point of view as it requires extended periods of CPB. Both extended CPB and low temperatures induce blood clotting disorders, which may increase bleeding during and after surgery (Wilde 1997). CPB also activates a systemic inflammatory response, and prolonged CPB time is an independent risk factor for cerebral injury (Taylor 1998).

It was concluded in a recent study by Sakamoto et al. (2004b) that several factors that may lead to an adverse cerebral outcome after HCA, including higher temperature, a lower haematocrit, a more alkaline pH and the duration of HCA (Sakamoto et al. 2004b).

### 2.3.1.2 Mechanisms of cerebral ischaemia during HCA

There are two major mechanisms behind the cerebral complications that follow HCA. First, HCA causes global hypoxia in the brain, influencing all areas, the most vulnerable of which are the hippocampus and its CA1 neurons (Tabuchi et al. 1995). The hippocampus acts as a processor of memory functions, and the first findings of mild cerebral ischaemic injury can be found by means of neuropsychiatric memory tests (Buss et al. 1996). If the duration of HCA remains within safe limits, these subtle deficits may pass over, but any increase in the length of HCA may entail more severe neurocognitive disorders (Reich et al. 1999), and prolonged HCA results in irreversible brain injury (McCullough et al. 1999).

Second, aortic surgery with or without HCA carries a marked risk of cerebral embolism. Although stroke is a possible complication of all cardiac surgery, the risk is highest in surgery of the aortic arch. Retrospective studies have shown the incidence of stroke in operations on the thoracic aorta to vary between 5.5% and 12% (Ergin et al. 1994, Ergin et al. 1996, Goldstein et al. 2001, Budillon et al. 2001, Kawachi et al. 2003). The causes of stroke include manipulation of the heart, aorta and other major vessels, which in elderly patients usually contain atheroma bodies on account of systemic atherosclerosis, so that emboli may be launched into the cerebral circulation, and also postoperative atrial fibrillation (Hogue, Jr. et al. 1999). Despite advances in surface materials and anticoagulant drugs, cardiopulmonary bypass circuits may generate macro or microemboli, particles that can vary in size from microscopic air bubbles to clearly visible blood clots. Microbubbles can lead to mild cognitive derangements, whereas larger clots and particles may cause stroke (Hsu 2001, Kurusz & Butler 2004).
It can be concluded in the light of the above information that HCA alone is not sufficient to protect the brain during cardiothoracic surgery. The other strategies that have been developed are presented in brief in the sections below.

### 2.3.2 Cerebral perfusion strategies

#### 2.3.2.1 Selective cerebral perfusion

Modern SCP was developed in the 1980s (Frist et al. 1986). In this technique the innominate artery on the right, the left common carotid artery and the left subclavian artery are each individually cannulated, along with clamping of the distal aorta. Another approach is to cannulate only the right axillary artery, so that the brain receives perfusion via the right common carotid artery. The left cerebral hemisphere receives blood via the circulus Willisii. This means that the brain can be perfused during HCA, reducing the risk of cerebral ischaemia (Matsuda et al. 1989). The use of SCP also has the advantage of preserving cerebral autoregulation. SCP seems to be superior to RCP in terms of cerebral outcome (Midulla et al. 1994), but it is technically more complex than HCA, requiring more time and manipulation for the cannulations, and this increased duration of the operation and manipulation of the vessels results in an increased risk of embolization. SCP can be performed with good neurological results, but correct selection of the patients is mandatory (Veeragandham et al. 1998, Harrington et al. 2004).

#### 2.3.2.2 Retrograde cerebral perfusion

RCP was first used as a treatment for massive air embolism, a severe complication of CPB (Mills & Ochsner 1980). This technique uses the cerebral venous system to perfuse the brain in a retrograde manner during HCA, via the superior vena cava. RCP is simpler to perform than SCP and its neuroprotective mechanisms include the preservation of cerebral hypothermia (Anttila et al. 2000a) and the expelling of both air and particle emboli from the cerebral vasculature (Juvonen et al. 1998a), in addition to it may provide direct metabolic support by supplying oxygen and nutrients to the brain (Li et al. 2002, Li et al. 2004a). RCP must be used with caution, since it may induce cerebral oedema at higher flow rates (Juvonen et al. 1998b). A study using intravital microscopy was able to show that oedema occurs after prolonged periods of RCP, but it did not detect any significant cerebral metabolic support (Duebener et al. 2003). Thus, the efficacy of RCP in supporting the brain metabolism during HCA remains controversial.

#### 2.3.2.3 Regional low-flow perfusion

Regional low-flow perfusion (RLFP) of the brain has produced more favourable results in terms of neurological recovery from neonatal cardiac surgery than deep HCA alone.
DeCampli et al. (2003) placed a cannula in the brachiocephalic artery of neonatal piglets and observed that brain oxygenation was higher with RLFP at a flow rate of 20 ml/kg than with HCA alone. A flow rate of 40 ml/kg, however, produced significant upper body oedema. Using a surviving neonatal piglet model, the same group was able to show that RLFP resulted in less histopathological damage and apoptosis than HCA alone and led to better neurological recovery (Myung et al. 2004). The use of HCA in neonates has decreased markedly due to advances in cerebral perfusion techniques and deeper knowledge of the limitations of the technique.

### 2.3.3 Cardiopulmonary bypass strategies

#### 2.3.3.1 pH management

The solubility of carbon dioxide (CO₂) in the blood increases in hypothermia, which in turn reduces the CO₂ partial pressure, resulting in an alkaline shift in pH as the temperature falls. This forms the basis for two pH management techniques for use in CBP, i.e. the alpha-stat and pH-stat strategies.

*Alpha-stat strategy.* This method allows the alkaline shift in pH to occur during cooling by keeping the pH at 7.4 and CO₂ at 5.3 kPa, corrected to a temperature of 37°C. This preserves cerebral autoregulation, and as the CMRO₂ falls with temperature, CBF diminishes and the blood vessels constrict (Murkin et al. 1987, Duebener et al. 2002). The alpha-stat strategy is associated with higher cerebral oxygen saturation during mild hypothermia than the pH-stat strategy (Li et al. 2004b).

*pH-stat strategy.* Here the alkaline shift in pH is counteracted by adding CO₂ to the inflowing gas. The pH is measured by correcting it to the actual core temperature of the patient. This leads to a markedly different setting from the alpha-stat strategy as a state of relative hypercarbia and acidemia is generated. During the pH-stat strategy cerebral autoregulation is impaired and the vessels dilate due to hypercarbia (Duebener et al. 2002). This phenomenon is called the “luxury perfusion”, as the CBF is a lot higher than would be required for the maintenance of CMRO₂ (Griepp & Griepp 1992). The CBF is markedly higher, the metabolic suppression more efficient and cerebral oxygenation better than in the alpha-stat strategy, especially during deep hypothermia (Li et al. 2004b). Also, the cooling of the brain improves, possibly on account of the more evenly distributed blood flow than in the alpha-stat strategy (Kurth et al. 1997). The recovery of cerebral energy metabolites and oxygenation in the rewarming phase is faster when the ph-stat strategy is used rather than the alpha-stat strategy (Aoki et al. 1993), and this technique has indeed been shown to provide a superior outcome after experimental HCA in pigs (Pokela et al. 2003a).

The “luxury perfusion” brought about by the pH-stat strategy has traditionally been thought to involve an increased risk of embolic loading on the brain, and some evidence has been found for this in animal studies (Cook et al. 2000). Therefore the alpha-stat strategy has been the predominating regimen for aortic arch surgery in adults. The increased embolic load detected in animal models could not be demonstrated in humans,
however (Plochl et al. 2001), and (Dahlbacka et al. 2005) were able to show that the pH-stat strategy in connection with an experimental embolization during HCA in swine was associated with a slightly better outcome and significantly improved metabolic recovery. It may be that the pH-stat strategy could also be used for human adults without increasing the risk of emboli.

A combination of the pH-stat and alpha-stat strategies has been found to provide better neuroprotection than either strategy alone. Skaryak et al. (1995) cooled their animals down to 14°C employing the pH-stat strategy and then switched to the alpha-stat strategy of pH management. The choice of this approach supports the theory that the ph-stat strategy entails a harmful worsening of intracellular acidosis at very low temperatures, which can be eliminated with the alpha-stat strategy. Additional research is needed to confirm these findings.

A comparison of the alpha-stat and pH-stat strategies has also been performed in an SCP setting, leading to the conclusion that the pH-stat strategy was superior in a canine model, the dogs having been first subjected to brain infarction (Ohkura et al. 2004). This study was hardly conclusive, however, as it employed limited methods and small number of animals. Further research is needed to elucidate whether the beneficial impact of SCP on the brain during HCA can be further enhanced by optimal pH management.

2.3.3.2 Haematocrit

Haemodilution during CBP has traditionally been thought to be advantageous because higher haematocrit (Hct) levels have been assumed to cause red blood cell sludging and a reduction in CBF. Recent studies have shown, however, that higher levels of Hct do not result in an adverse outcome. 10% Hct is adequate for providing metabolic support for the brain during CPB, and an Hct of 30% seems to be superior in terms of cerebral outcome (Duebener et al. 2001, Sakamoto et al. 2002). A recent study using near-infrared spectroscopy showed that if the Hct is maintained at 10-15% CBF will be increased as compared with Hct levels of 25-30%, and the authors suggest that in the presence of haemodilution the lower oxygen content of blood is at least partly compensated for by the increased CBF. However, CMRO₂ may rise deleteriously at lower haematocrit levels, leading to an adverse outcome after HCA (Sakamoto et al. 2004a). In conclusion, lower haematocrit levels (<30%) seem to be disadvantageous during CPB and HCA (Sakamoto et al. 2004b).

2.3.3.3 Oxygenation strategies

It has been proposed that reperfusion injury in the myocardium may be aggravated by ROS when 100% oxygen is used with CPB (Ihnken et al. 1996). In an acute, normothermic model of cerebral ischaemia in pigs, a positive impact on the neurological outcome was found when the animals were ventilated with hypoxic gas after ischaemia (10% O₂ content) rather than with 100% oxygen (Douzinas et al. 2001a). In another study by the same group, the histopathological injury was significantly attenuated by
hypoxaemic reperfusion in a similar setting (Douzinas et al. 2001b). In a study using normoxic ventilation in pigs after reversible occlusion of the carotid arteries, however, there were more areas of hypoperfusion and higher levels of glutamate in the brain (Solas et al. 2001). CPB was not used in any of these cases. Nollert (Nollert et al. 1999) used some replacement of the inflowing oxygen with nitrogen to provide a normoxic perfusion, but this resulted in an adverse outcome. The idea of using normoxic or even hypoxic reperfusion to protect the brain from ischaemia is an interesting one, but additional research is needed to confirm whether the methods are usable with CPB.

2.3.3.4 Other methods

Leukocyte depletion. A leukocyte-depleting filter that selectively removes activated neutrophils from the circulating blood without affecting the entire leukocyte population (Thurlow et al. 1995) has been shown to reduce reperfusion injury after HCA (Rimpiläinen et al. 2000).

Biocompatibility. CPB circuits induce a systemic inflammatory reaction, and advances in the creation of heparin or phosphorylcholine-coated circuits have diminished the activation of the complement system and leukocytes (Hamada et al. 2001, Rubens & Mesana 2004). It remains unclear whether these developments can improve the cerebral outcome, however (Taylor 1998).

2.3.4 Postischaemic hypothermia

Patients resuscitated after cardiac arrest benefit from 24 hours of induced mild hypothermia, as the mortality figures and neurological outcomes are better (The Hypothermia after Cardiac Arrest Study Group 2002). This landmark study was performed, because significant neuroprotection had been reported as a result of subsequent hypothermia after ventricular fibrillation-induced cerebral ischaemia in animal experiments (Weinrauch et al. 1992). Significant protection of the brain has also been reported in other animal models of cerebral ischaemia (Corbett et al. 2000). The beneficial impact on outcome has been demonstrated using mild hypothermia, and lower temperatures may entail increased mortality (Steen et al. 1979).

The mechanisms by which postischaemic hypothermia may prevent ischaemic injury in the brain are mostly similar to those that apply to perioperative hypothermia during HCA. Hypothermia during reperfusion reduces the production of ROS (Thoresen et al. 1997) and stabilizes the cellular membranes, which become more impermeable to ions and water, so that the formation of oedema is diminished. This latter is a consequence of the reduction in ATP depletion, which provides more energy for the Na⁺-K⁺ ionic pump. There is also some evidence that postischaemic hypothermia reduces ICP (Ehrlich et al. 2001), and it may also inhibit cerebral glutamate release, Ca²⁺-related protein degradation and postischaemic hypoperfusion (Colbourne et al. 1997, Corbett et al. 2000).

There is increasing evidence that postischaemic hypothermia may not be applicable to ischaemic insults other than global ischaemia during and after resuscitation. Hypothermia
has several adverse effects, such as increased bleeding, acidosis, impairment of cardiac and hepatic function and susceptibility to sepsis and pneumonia, which may complicate the therapy (Krause et al. 2000, Ishikawa et al. 2000). In addition, it seems that patients with traumatic head injury do not benefit from postischaemic hypothermia (Harris et al. 2002). Extended postoperative hypothermia after HCA is reported to have led to a poor outcome (Romsì et al. 2002a), and topical cooling for 2 hours after HCA, which resulted in mild core hypothermia as well, did not affect the outcome at all (Pokela et al. 2003b).

2.3.5 Pharmacological agents

2.3.5.1 Overview

There are throngs of potentially neuroprotective drugs available which have been tested in small animal experiments or in vitro (Table 2), but only a few of them are actually in clinical use. Over 50 different neuroprotective compounds were tested in animal stroke models in the 1990s, for example, but only tissue plasminogen activator successfully improved the outcome for stroke patients in clinical trials (Kidwell et al. 2001). There are many reasons why the majority of candidate medications do not work in humans or in more complex animal models:

1. Animal experiments often use short-term end-points, whereas it is the long-term recovery that determines the final outcome in human clinical trials. One way to counteract this pitfall at least in part is to use surviving animal models, which assess the behavioural outcome, i.e. neurological recovery (Valtysson et al. 1994). On the other hand, it is not ethically justifiable to prolong the lives of test animals unnecessarily, as the experiment may cause considerable pain and suffering.

2. Most animal studies are acute models, in which the animals are sacrificed immediately after the experiment. The histopathological changes resulting from cerebral ischaemia develop in 4-7 days, and harvesting the brain earlier may produce false results. The NMDA antagonist dizocilpine, for example, seemed to reduce the infarct volume in rats after MCA occlusion on day 3, but infarct sizes were similar in the drug and control groups after 4 weeks. This may lead to a mismatch between the reported acute findings and the eventual outcome, as ascertained in a surviving animal model (Gladstone et al. 2002).

3. Animal models of ischaemia are homogeneous, with highly controlled environmental factors. The MCA occlusion model, for example, uses young, healthy rats, whereas the clinical trials in humans often include patients of varying ages, comorbidities, medications etc. The heterogeneous nature of human trials may therefore mask the potential neuroprotective efficacy of the drugs. On the other hand, a drug found to be neuroprotective in young animals with a healthy circulation may have no effect in human adults. The surviving porcine model developed by Randall Griepp and colleagues in New York and further developed by Tatu Juvonen et al. in Oulu is a compromise between the clinical and preclinical settings, but it also has the “drawback” of using young, healthy piglets (Midulla et al. 1994, Juvonen et al.
Perhaps the use of adult minipigs with some atherosclerosis would provide a model that was even closer to the actual clinical setting (Xi et al. 2004).

4. The timing of administration is of paramount importance. A potentially neuroprotective drug may even be deleterious in terms of outcome if given at the wrong time, i.e. outside its time window of efficacy. NMDA antagonists such as dizocilpine, for example, may provide neuroprotection in the acute phase of injury when glutamate excitotoxicity is present in the cells. Later, the dendrite outgrowth necessary during recovery from ischaemia may be inhibited, since both NMDA and Ca\(^{2+}\) channel activation is needed in the process (Maletic-Savatic et al. 1999).

5. The neuroprotective drug therapies may have to be extended. A single bolus or therapy during the first 24 hours may not be enough, since the cell death processes may only be delayed, resuming again after the effect of the drug has ceased. A minimum duration of 72 hours has been proposed for stroke patients (Dyker & Lees 1998).

6. A trial with a small number of test subjects (animals or patients) has a limited ability to generate significant differences in outcome. Drugs expected to have a positive but relatively small impact on ischaemic injury may generate false negative results if the statistical power is insufficient (Lees 2001). Tests with such drugs need larger numbers of subjects, which in turn is less practicable and more expensive, especially with patients or with larger animals in surviving models. One way to overcome this problem is to use multiple end-points simultaneously in order to maximize the power of a single experiment.

7. The patient population may be too sick to benefit from the potential therapy. If the brain damage is too extensive, for example, resulting in increased mortality, smaller beneficial effects will not affect the outcome (Price et al. 2003).

8. The mechanism of action is not relevant in humans, the drugs are toxic in humans, or the drugs have not been evaluated sufficiently to be used properly in a clinical setting (Gladstone et al. 2002).

2.3.5.2 Fructose-1,6-bisphosphate

As mentioned above, ischaemic cerebral injury includes a cascade of harmful events, beginning from the depletion of ATP stores and followed by a biochemical cascade in which the elevated cytosolic Ca\(^{2+}\) concentration plays a key role. The cells may then be damaged even further by ROS during reperfusion.

Fructose-1,6-bisphosphate (FDP) is a high-energy intermediate involved in glycolysis. FDP increases the activity of phosphofructokinase and pyruvate kinase, which are considered to be “rate-limiting” enzymes in anaerobic glycolysis (Murray et al. 1996). Its molecular weight is 336.082 Da. When administered exogenously, FDP can be converted to energy, with one mole of FDP generating 4 moles of ATP under anaerobic conditions, since the compound enters glycolysis after the reaction catalysed by phosphofructokinase, which requires ATP. In the anaerobic metabolism of glucose only 2 moles of ATP is formed per mole of glucose. The glycolytic pathway is presented in detail in Figure 1.
Table 2. Pharmacological agents having neuroprotective properties after ischaemia. Modified from Rimpiläinen (2001) and Romsi (2003).

<table>
<thead>
<tr>
<th>Mechanism of action</th>
<th>Drug</th>
<th>Model and references</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMPA receptor antagonism</td>
<td>NBQX dog, rat</td>
<td>(Redmond et al. 1995, Pitsikas et al. 2001)</td>
</tr>
<tr>
<td></td>
<td>GYKI 52466 rat</td>
<td>(Nisim et al. 1999)</td>
</tr>
<tr>
<td></td>
<td>CNQX rat</td>
<td>(Nisim et al. 1999)</td>
</tr>
<tr>
<td>NMDA receptor antagonism</td>
<td>Dizocilpine</td>
<td>dog (Redmond et al. 1994, Aoki et al. 1994b)</td>
</tr>
<tr>
<td></td>
<td>Cerestat rat</td>
<td>(Pitsikas et al. 2001)</td>
</tr>
<tr>
<td></td>
<td>Ketamine rat</td>
<td>(Proescholdt et al. 2001)</td>
</tr>
<tr>
<td></td>
<td>Magnesium</td>
<td>small human trials (Muir 2001)</td>
</tr>
<tr>
<td>Na⁺ channel antagonism</td>
<td>Lamotrigine</td>
<td>pig (Anttila et al. 2000c)</td>
</tr>
<tr>
<td></td>
<td>Lubelazole</td>
<td>rat (Haseldonckx et al. 1997)</td>
</tr>
<tr>
<td></td>
<td>Sulfoxide</td>
<td>human (Karaca et al. 2002)</td>
</tr>
<tr>
<td>Ca²⁺ channel antagonism</td>
<td>Nimodipine</td>
<td>rat, human (Korenkov et al. 2000, van Gijn &amp; Rinkel 2001)</td>
</tr>
<tr>
<td></td>
<td>Nicardipine</td>
<td>rat (Amenta &amp; Tomassoni 2004)</td>
</tr>
<tr>
<td>ROS inhibition</td>
<td>PBN piglet, rat</td>
<td>(Langley et al. 2000, Yang et al. 2000)</td>
</tr>
<tr>
<td></td>
<td>Allopurinol</td>
<td>human (Clancy et al. 2001)</td>
</tr>
<tr>
<td>Protease inhibition</td>
<td>Aprotinin</td>
<td>piglet (Aoki et al. 1994a)</td>
</tr>
<tr>
<td>Thromboxane A2 receptor</td>
<td>Vapiprost</td>
<td>piglet (Tsui et al. 1997)</td>
</tr>
<tr>
<td>blockade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAF receptor antagonism</td>
<td>BN 52021 rat</td>
<td>(Liu et al. 1996, Langley et al. 1999)</td>
</tr>
<tr>
<td></td>
<td>BN 50730 rat</td>
<td>(Liu et al. 2001)</td>
</tr>
<tr>
<td>Cytokine inhibition</td>
<td>Corticosteroids</td>
<td>piglet (Shum-Tim et al. 2001, Shum-Tim et al. 2003)</td>
</tr>
<tr>
<td>TNF-α converting enzyme</td>
<td>DPH-067517 rat</td>
<td>(Wang et al. 2004)</td>
</tr>
<tr>
<td>inhibition</td>
<td></td>
<td></td>
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<tr>
<td>Inhibition of cerebral</td>
<td>Thiopental</td>
<td>human (Hirotani et al. 1999)</td>
</tr>
<tr>
<td>metabolism</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Propofol</td>
<td>rat (Gelb et al. 2002, Bayona et al. 2004)</td>
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<tr>
<td></td>
<td>Lidocaine dog</td>
<td>(Wang et al. 1999)</td>
</tr>
<tr>
<td>Inhibition of apoptosis</td>
<td>Cyclosporine A</td>
<td>pig, gerbil (Tatton et al. 2001, Domanska-Janik et al. 2004)</td>
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<td></td>
<td>Erythropoietin</td>
<td>rat, pig (Siren et al. 2001, Romsi et al. 2002b)</td>
</tr>
<tr>
<td>Preconditioning</td>
<td>Isoflurane</td>
<td>rat (Xiong et al. 2003, Zhao &amp; Zuo 2004)</td>
</tr>
<tr>
<td></td>
<td>Erythropoietin</td>
<td>human (Ehrenreich et al. 2002)</td>
</tr>
<tr>
<td>Angiogenesis</td>
<td>VEGF rat</td>
<td>(Sun et al. 2003)</td>
</tr>
<tr>
<td>Multiple mechanisms</td>
<td>Mannitol</td>
<td>human (Roberts et al. 2003)</td>
</tr>
<tr>
<td></td>
<td>Dexanabinol</td>
<td>rat (Belayev et al. 1995)</td>
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<tr>
<td></td>
<td>FDP</td>
<td>see section 2.3.5.2</td>
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</table>

Abbreviations: AMPA = α-amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid; NMDA = N-methyl-D-aspartate; nNOS = neuronal nitric oxide synthase; PAF = platelet activating factor; PBN = α-phenyl-N-t-butyl-nitrone; ROS = reactive oxygen species; TNF = tumour necrosis factor; VEGF = vascular endothelial growth factor.
Markov et al. (2000), evaluating the physiological effects of FDP administration, found that an infusion of FDP resulted in slightly decreased heart and respiration rates in healthy adults, with rises in the serum concentration of inorganic phosphate and the intraerythrocytic concentration of ATP. The energy derived from carbohydrates was highly increased, but lipid metabolism was decreased, suggesting enhanced carbohydrate metabolism.

Mechanisms of action. 1) FDP may provide better preservation of neuronal energy stores during ischaemia and/or re-supply the depleted energy pools more effectively during reperfusion by the mechanisms mentioned above (Tavazzi et al. 1992). 2) FDP has been shown to inhibit the rise in intracellular Ca^{2+} concentration during ischaemia (Hassinen et al. 1991, Bickler & Kelleher 1992), an effect mediated by the activation of phospholipase-C, which modulates intracellular Ca^{2+} activity (Donohoe et al. 2001). 3) FDP enhances the cellular uptake of potassium, which results in a decrease in intracellular sodium concentration, thus reducing cellular cytotoxic oedema (Cattani et al. 1980). 4) FDP inhibits the formation of ROS and the activation of neutrophils (Markov et al. 1987, Tavazzi et al. 1990, Sola et al. 2003). The ROS inhibition may be partly mediated by the stabilization of intracellular glutathione (Vexler et al. 2003). ROS and neutrophil inhibition may also protect the endothelium from ischaemia, thus leading to increased CBF (Gobbel et al. 1994). 5) T lymphocyte activity is also lowered by FDP (Markov et al. 2002, Bordignon et al. 2003). 6) FDP enhances the ability of astrocytes to take up glutamate and metabolize it to glutamine (Kelleher et al. 1994), and protects them during hypoxia, enabling them to support the neurons during and after ischaemia (Kelleher et al. 1996). 7) FDP has been shown to reduce glutamate excitotoxicity by inhibiting glutamate release and the subsequent formation of NO by NOS (Bickler & Buck 1996, Edde et al. 1998, Cardenas et al. 2000, Zeng et al. 2003, Rogido et al. 2003). 8) FDP seems to increase red blood cell deformability, possibly reducing blood viscosity (Cacioli et al. 1988). 9) Increased ATP formation may be enough to prevent necrosis and guide the cell towards apoptosis instead (Fortes Aiub et al. 2003). 10) Total CBF and rCBF in the parietal region of rats increased relative to controls receiving normal saline, suggesting beneficial effects on the cerebral vasculature as well (Gan'shina et al. 2004). 11) FDP has been shown to inhibit apoptosis, perhaps by up-regulating protein APE/Ref-1, which is involved in cellular repair functions (Long et al. 2002).

Protection from ischaemic injury outside the brain. It has been shown on the strength of animal models that FDP reduces ischaemic injury in many organs, such as the heart (Tavazzi et al. 1990), kidney (Didlake et al. 1989), intestine (Sawchuk et al. 1986), striated muscle (Odero A et al. 1985) and liver (Mihas et al. 2003). It has also been beneficial in reducing injury to the transplant heart (Markov et al. 2002) and intestine (Sola et al. 2004) upon addition to the storage fluid. A randomized, controlled clinical trial in patients undergoing coronary artery bypass grafting reported lower CK-MB levels, a higher cardiac index and greater left ventricular stroke work, suggesting myocardial protection in a clinical setting as well (Riedel et al. 2004).

Neuroprotection. FDP has mostly been assessed in animal models, with only a single study involved stroke patients completed to date. In this study, FDP was combined with a ROS inhibitor and a Na^+ channel blocker, sulphoxide, and the combination was found to be neuroprotective (Karaca et al. 2002). On the other hand, there is a plethora of preclinical studies to support the efficacy of FDP in mitigating ischaemia/reperfusion
injury (Farias et al. 1990, Sola et al. 1996, Yang & Zhou 2000, Yang et al. 2003), including the use of stroke models such as reversible MCA occlusion (Kuluz et al. 1993) and experimental unilateral cerebral embolization in rats (Trimarchi et al. 1997).

Negative results. There are some instances in which FDP has not proved to be neuroprotective (LeBlanc et al. 1991a, LeBlanc et al. 1991b, LeBlanc et al. 1992, Tortosa et al. 1993, Behringer et al. 2001, Fujii et al. 2001). As mentioned above, it causes elevation of serum phosphate levels, and phosphate toxicity occurs more easily in foetal or newborn animals due to slower excretion. This may explain why such models have failed to show any neuroprotective effect of FDP. In any case, neonatal phosphofructokinase may not be stimulated as easily by FDP as that in adults (Hamano et al. 1989). Moreover, lactate formation may be exceedingly deleterious to the neonatal brain (LeBlanc et al. 1991a). The young age of the animals, different animal models and differences in the timing of administration and schedules of ischaemia may explain the negative results.

2.3.6 Hypertonic fluid therapy

2.3.6.1 Overview

Isotonic crystalloid fluid therapy forms the basis for intravenous volume support. The predominant crystalloid solutions used nowadays are normal saline (0.9% NaCl) and Ringer acetate or lactate solutions. The Ringer solutions mimic the extracellular fluid in terms of their electrolyte content, including Ca$^{2+}$ and K$. Isotonic crystalloids become distributed throughout the entire extracellular space, and less than 25% of the initial volume remains in the circulation (Tollofsrud et al. 1993). Crystalloid fluid therapy alone leads to oedema due to plasma dilution and loss of colloid osmotic pressure.

Colloids are macromolecular solutions (plasma expanders, hydroxyethyl starch [HES], dextran, gelatin, albumin) which bind water and cannot escape from the intravascular space due to their size. They are made in various concentrations, which along with the specific molecule determine their colloid osmotic pressure, i.e. their ability to bind water. A rule of thumb concerning colloids is that they expand plasma volume to an extent equal to the infused volume, although minor differences exist between the molecules (Lamke & Liljedahl 1976).

2.3.6.2 Hypertonic saline

Although hypertonic saline (HTS) has been used either experimentally or clinically for decades, it emerged as a topic of modern research when Velasco et al. (1980) conducted their first experiments on it. Using a canine haemorrhagic shock model, they reported rapid and effective restoration of blood pressure and cardiac output following administration of 7.5% NaCl, resulting in 100% survival in the HTS group, whereas all the controls who received normal saline died. The volume used for resuscitation was only
10% of the volume bled. These dramatic results reflect the potency of HTS for volume expansion. Its haemodynamic effects are summarized in Figure 4.

**Effect on plasma volume.** The cellular membranes are semipermeable in the sense that while water may pass freely, solutes such as sodium may not. Sodium is an extracellular electrolyte, its intracellular concentration being kept near zero by the Na\(^+/\)K\(^-\) ion pump in the cellular membrane. It is also the most important electrolyte in determining water distribution, as it is responsible for most of the extracellular osmolality (Pocock & Richards 1999). A rapid intravascular infusion of HTS will elevate plasma osmolality, resulting in a marked gradient in osmolality between the plasma and the extracellular and intracellular space. This means that extracellular and intracellular water is drawn to the intravascular space, expanding the plasma volume. The initial water is derived from the erythrocytes and endothelial cells, while additional water comes from certain tissues (Mazzoni et al. 1988).

**Cardiovascular effects.** HTS has a direct positive inotropic effect on the heart (Kien & Kramer 1989), perhaps by virtue of increased Ca\(^{2+}\) influx through the sarcolemma (Mouren et al. 1995). The elevated plasma osmolality results in vasodilation of both the systemic and pulmonary arterioles, mediated by direct relaxation of the vascular smooth muscle (Gazitua et al. 1971). Increased preload, cardiac contractility and heart rate, along with reductions in the pulmonary and systemic vascular resistances (afterload) increase cardiac output (Smith et al. 1985). The mean arterial pressure (MAP) is affected only slightly in normovolaemic subjects, but a rapid, effective rise in MAP has been observed in haemorrhagic test animals (Kramer et al. 1986). At the microcirculatory level, HTS reduces endothelial swelling and opens compressed capillaries (Mazzoni et al. 1990), improving regional blood flow and tissue oxygenation (Behrman et al. 1991, Corso et al. 1998). Renal perfusion pressure rises along with glomerular filtration, which with decreased sodium absorption leads to increased diuresis (Cox et al. 1994). In the lung, HTS improves gas exchange by reducing the interstitial volume in the alveoles (Rabinovici et al. 1996).

**Effects on the immune system.** HTS has been shown to have immunomodulatory properties. It suppresses the activation of neutrophils by down-regulating the expression of key adhesion molecules such as L-selectin and CD11b (Thiel et al. 2001). Endothelial expression of ICAM-1 has also been shown to be reduced by HTS (Oreopoulos et al. 2000). Intravital microscopy studies have shown diminished amounts of rolling or adherent leukocytes in trauma or ischaemia/reperfusion animal models (Barone et al. 1997b, Spera et al. 1998). HTS is reported to have ameliorated lung injury (Rizoli et al. 1998, Yada-Langui et al. 2000, Powers et al. 2005), thermal injury (Barone et al. 1997b) and spinal cord injury (Spera et al. 1998) in this manner, and it may also reduce susceptibility to sepsis after hypovolaemic shock (Coimbra et al. 1997).
2.3.6.3 Hypertonic saline with dextran

The haemodynamic improvement brought about by HTS is of transient duration, lasting only 30 to 60 minutes (Smith et al. 1985, Järvelä et al. 2003), as the mobilized water is quickly redistributed, negating the osmolality gradient between the plasma and tissues and returning the plasma volume to normal. Smith et al. (1985) added dextran to HTS, creating HSD, which has an effect of more prolonged duration, up to 3-4 hours (Smith et al. 1985, Velasco et al. 1989), as the dextran binds the mobilized water to the intravascular space. The same effect has been achieved with HES as well (Boldt et al. 1990), but dextran may be unique among the colloids in terms of immunomodulation, since it has been shown to reduce neutrophil adhesion in an isotonic environment after ischaemia (Menger et al. 1993).

HSD is a commercial product that is accepted legally in more than 15 countries. Currently its principal indication is pre-hospital fluid resuscitation for trauma patients, for which purpose a single 250 ml infusion given in 5-20 minutes is considered safe and effective for restoring blood pressure (Kramer 2003). Emerging evidence for its beneficial effects in the operating room and intensive care unit may widen its therapeutic range in the future, however.
2.3.6.4 Neuroprotective effects of hypertonic fluid therapy

The first experiment with HTS in the field of neuroprotection was performed in 1919, when an intravenous injection of 30% saline resulted in immediate shrinkage of the brain tissue parenchyma in a feline model (Weed & McKibben 1919). Since those days, the mechanisms of ischaemia/reperfusion injury have been revealed and HTS has received increasing attention in the field of neuroprotection, since its effects in terms of improved microcirculatory flow, the recruiting of cellular and interstitial water and immunomodulation make it a promising therapy for treating the reperfusion injury or brain oedema. There are no reports available on the use of hypertonic solutions with HCA, although HSD has been observed to lower ICP during experimental CPB in pigs (McDaniel et al. 1994).

*Animal studies.* The first animal studies involved haemorrhagic shock models, in which the outcome for the animals receiving HTS/HSD was overwhelmingly better than that for the controls (Velasco et al. 1980, Nakayama et al. 1984). As the brain damage incurred during the low-flow period is a controlling factor for the eventual outcome after major haemorrhage, investigators realized that these solutions could also have neuroprotective properties. It was found that ICP did not rise during HTS resuscitation, whereas resuscitation with Ringer lactate, normal saline, dextran or HES led to an early rise in ICP. The effect of HTS in reducing ICP lasted no more than 2 hours (Prough et al. 1985, Ducey et al. 1989), but rCBF and the cerebral oxygen supply improved at the same time (Schmoker et al. 1991).

Models of cerebral ischaemia without haemorrhagic shock have also shown that hypertonic therapy can be useful. HTS provided a longer and more effective reduction in ICP and a greater improvement in rCBF than did either whole blood or HES in a rabbit model of global ischaemia (Kempski et al. 1996). Likewise, when rats were subjected to cortical vein occlusion, the sizes of the brain infarctions were smaller in the hypertonic group than in the controls. The primary mechanism behind the positive result, in authors’ opinion, was the elevated rCBF, which reduced the areas of hypoperfusion (Heimann et al. 2003). Administration of a single bolus of 23.4% HTS reduced focal intraparenchymal pressures in various brain regions along with ICP in a canine model of intracerebral haemorrhage (Qureshi et al. 2002), and a continuous infusion of 3% HTS provided lower ICP and increased survival in a rat model of heat stroke (Kuo et al. 2003). Rats subjected to experimental subarachnoid haemorrhage benefited from HSD therapy, as indicated by improved behavioural recovery, lower ICP and lesser histopathological signs of injury (Zausinger et al. 2004). In a rat model of traumatic brain injury, HTS with HES reduced ICP and improved rCBF in the cortical areas, leading to a smaller contusion area than with normal saline (Thomale et al. 2004).

*Clinical trials.* The impressive data obtained from animal models of haemorrhagic shock led investigators to plan clinical trials. The results of a randomized, controlled trial suggested that trauma patients with a score below 8 on the Glasgow Coma Scale would benefit from HSD therapy, since a subgroup analysis suggested that such patients have significantly higher survival following hospital discharge (Vassar et al. 1993). This beneficial impact on the outcome was later confirmed in a meta-analysis. Head trauma
patients with hypotension and a GCS score below 8 who received HSD were about twice as likely to survive as those with isotonic fluid resuscitation (Wade et al. 1997).

The use of hypertonic solutions has also been successful for the treatment of elevated ICP. Only a few prospective, randomized clinical trials exist, however, as most of the literature consists of uncontrolled case reports. The earliest report is by Worthley et al. (1988), who administered 29.2% HTS to two patients in whom furosemide and mannitol therapies had failed. HTS resulted in a sustained reduction in ICP along with improved diuresis. Later, repeated administration of 7.5% HTS with HES was effective in bringing about a sustained reduction in ICP (Hartl et al. 1997), and eight patients with a refractory rise in ICP that had proved resistant to conventional treatment were given 23.4% HTS, which reduced ICP by over 50% in 16 of 20 episodes of administration (Suarez et al. 1998). 10% HTS has been shown to be effective in controlling a rise in ICP in patients who could not be treated effectively with mannitol (Schwarz et al. 2002). A randomized, controlled study of paediatric patients with head trauma showed that HTS was more effective than Ringer lactate for treating elevated ICP and also entailed fewer complications, a shorter mechanical ventilation time and a shorter time in the intensive care unit (Simma et al. 1998). A recent comparison of HSD and 20% mannitol in equimolar doses showed the decrease in ICP to be more sustained and more effective in the HSD group (Battison et al. 2005). The optimal concentration, dosing, timing of administration and patient selection for hypertonic fluid therapy are not known, and no specific guidelines can be laid down at the present time (Bhardwaj & Ulatowski 2004).

Controversial studies. A recent well-planned, randomized, controlled clinical trial did not find any significant impact of 7.5% HTS on the outcome, when the solution was given to patients suffered from severe head trauma (GCS below 8) and hypotension as a single 250 ml bolus in a pre-hospital setting, the control group receiving the same volume of Ringer lactate. The fluid chosen in this case was not HSD as in the earlier clinical trial and meta-analysis mentioned above, and thus the negative result may be explained by the short duration of the haemodynamic response to HTS (Cooper et al. 2004). Shackford et al. (1998) reported that HTS and Ringer lactate achieved similar reductions in ICP in a randomized, prospective study, but the two groups differed in that the HTS group had a lower GCS on admission and a higher initial ICP, and required more interventions.

2.3.6.5 Safety of hypertonic solutions

Rapid changes in serum osmolality may result in coma and seizures (Schell et al. 1996), and abrupt alterations in serum sodium levels are reported to have caused subdural and intracerebral haemorrhages in small children (Finberg 1967). Central pontine myelinolysis is triggered by rapid correction of chronic hyponatraemia with isotonic or hypertonic saline (Laureno & Karp 1997), but there is not a single report of the occurrence of myelinolysis in connection with the use of HTS/HSD. Patients with cardiac dysfunction may develop acute heart failure after the administration of hypertonic solutions (Prien et al. 1993), and hypernatraemia is a potential complication, especially in the presence of renal insufficiency. The colloids may also trigger allergic reactions,
ranging from urticaria to anaphylaxis (Hernandez et al. 2002). The potential adverse effects of hypertonic solutions are summarized in Table 3.

<table>
<thead>
<tr>
<th>Complication</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central pontine myelinolysis</td>
<td>Rapid correction of pre-existing chronic hyponatraemia</td>
</tr>
<tr>
<td>Encephalopathy</td>
<td>Delirium, diminished level of consciousness, seizures, coma</td>
</tr>
<tr>
<td>Hypernatraemia</td>
<td>Requires strict monitoring of serum sodium levels, especially with renal insufficiency</td>
</tr>
<tr>
<td>Subdural or intracerebral haemorrhage</td>
<td>Reported in infants</td>
</tr>
<tr>
<td>Transient hypotension</td>
<td>May occur when the infusion is rapid</td>
</tr>
<tr>
<td>Heart failure, pulmonary oedema</td>
<td>The heart is incapable of supporting rapid plasma volume expansion</td>
</tr>
<tr>
<td>Hypokalaemia</td>
<td>Associated with large infusion volumes of HTS with no replacement of potassium</td>
</tr>
<tr>
<td>Hyperchloraemic acidosis</td>
<td>The solutions are unbuffered, may occur with large infusion volumes.</td>
</tr>
<tr>
<td></td>
<td>May be avoided by adding acetate</td>
</tr>
<tr>
<td>Bleeding disorders</td>
<td>Caused by dilution of plasma proteins, or by the colloid, especially dextran. Occurs only if large volumes are used</td>
</tr>
<tr>
<td>Haemolysis</td>
<td>May happen during rapid changes in plasma osmolality, slower infusions recommended</td>
</tr>
<tr>
<td>Phlebitis</td>
<td>Peripheral veins may be affected. Central administration recommended</td>
</tr>
<tr>
<td>Rebound cerebral oedema</td>
<td>May occur after sudden withdrawal of therapy</td>
</tr>
<tr>
<td>Allergic reactions</td>
<td>Ranging from mild skin irritation to anaphylaxis, to colloids only</td>
</tr>
</tbody>
</table>

**Table 3. Potential adverse effects of hypertonic fluid therapy. Modified from (Qureshi & Suarez 2000, Bhardwaj & Ulatowski 2004)**

### 2.4 Electron microscopy

The wavelength of light limits the use of light microscopes to magnifications of 500-1000x, in that the green light that is used in light microscopy has a wavelength of 500 nanometers (nm) and a theoretical resolution of 200 nm, so that structures smaller than 200 nm cannot be identified. The invention of electron microscopy (EM) made scientific research of ultrastructural cellular organelles possible, as this technique can identify structures as small as 1 nm. Electron microscopes function in exactly the same way as their optical counterparts except that they use a focused beam of electrons instead of light to image the specimen and gain information on its structure and composition (Johannessen 1984).

There are two variations on this technique: transmission electron microscopy (TEM) and scanning electron microscopy. As the latter is not relevant to the present work, it will not be discussed further here. The principle of TEM is as follows. An electron gun produces a stream of monochromatic electrons, which is focused to a thin, coherent beam by a condenser lens. The beam is restricted by the condenser aperture, which eliminates electrons that are located too far away from the optic axis, thus reducing interference.
After the aperture, the beam penetrates the sample. Some parts of the beam pass through the sample, while others encounter matter and are reflected or dissipated, so that they do not pass through the sample. The beams that pass through then reach the objective lens and projection lens, which enlarge the image, and finally the phosphor image screen, which generates light and makes it possible to see the image. The image on the phosphor screen is usually photographed by a camera. The image is monochromatic, the darker areas representing the parts of the sample which reflected electrons and the lighter areas the parts through which the electrons passed more easily (Johannessen 1984).

Disadvantages. Single electrons are volatile in nature and eager to react with matter. When they encounter any type of matter, they are reflected or dissipated, creating lower forms of energy such as x-rays, ultraviolet rays, heat, etc. Thus their penetrance through matter is very low, so that EM requires very thin samples. As even gaseous substances react with electrons, EM has to be performed in a vacuum. Also, since the water in living cells would capture all the electrons fired through it, special fixation techniques have to be used to remove all the water. This means that every EM sample is dead and no additional staining is possible. These limitations have caused EM to be replaced with other more practicable methods for histopathological purposes. No other method allows such a high magnification and resolution as EM, however, making it the method of choice for ultrastructural studies (Johannessen 1984).

2.4.1 EM signs of ischaemic injury

2.4.1.1 Glycogen depletion

Depletion of glycogen, which is normally stored in irregular granules or clearly visible glycogen rosettes (Burkitt et al. 1993), is the earliest sign of ischaemic injury, beginning almost immediately after the onset of ischaemia, as the cells start to utilize their secondary sources of energy. The glycogen stores in neurons are very limited, lasting only a few minutes (Silver 1991).

2.4.1.2 Mitochondrial oedema

The mitochondrial matrix is tightly packed together and usually laminar in shape under physiological conditions (Burkitt et al. 1993). The key ultrastructural finding related to ischaemia is mitochondrial swelling, which begins 10 to 20 minutes after the onset of ischaemia. Fluid enters the intercristal space, forcing the inner and outer mitochondrial membranes to separate and thus causing disruption of oxidative phosphorylation. Mitochondrial vacuoles may occur, and electron-dense deposits representing intramitochondrial calcium appear within 2 hours. The swelling leads to rupture of the mitochondrial membranes, which may occur 1-2 hours after the onset of ischaemia and constitutes a sign of irreversible injury (Silver 1991, Solenski et al. 2002, Kumar et al. 2004). Rupture of the mitochondria leads to either cell apoptosis or necrosis, depending
on the amount of damaged mitochondria, as discussed in section 2.2.2.8. The characteristics of mitochondrial damage during hypoxia are shown in Figure 5.

### 2.4.1.3 Nuclear changes

The cell nuclei are visible in EM as large organelles separated from the cytoplasm and other organelles by a nuclear membrane. They contain chromatin, which is represented by a combination of dense, electron-capturing areas and lucent areas that the electrons have passed through more easily. The dense areas are not active in ribonucleic acid synthesis and form tightly packed zones called heterochromatin, while the electron-lucent areas, euchromatin, represent the part of the deoxyribonucleic acid that is active in ribonucleic acid synthesis (Burkitt et al. 1993).

In ischaemia, the chromatin forms larger clumps which move towards the nuclear membrane. The first changes appear in 15 minutes. After 3-4 hours, the nuclear membrane ruptures, a sign of severe, irreversible injury (Silver 1991, Kumar et al. 2004). Ischaemic injury to neuronal nuclei is depicted in Figure 6.

### 2.4.1.4 The cytoplasm and other organelles

As ischaemia worsens, cellular oedema becomes more severe. This is represented by large areas of electrolucent spaces between the organelles. Swelling occurs approximately 30 minutes after the onset of ischaemia. ER As ischaemia produces oedema, the cristae of the ER, a major component of the cytoplasm and the site of protein synthesis, separate, constituting the first sign of injury. Then the ribosomes attached to the ER begin to tear off, a sign of the cessation of protein synthesis. Eventually the ER ruptures after severe ischaemia, another sign of irreversible injury (Silver 1991, Kumar et al. 2004). An example of the extent of the ultrastructural signs of ischaemic damage to these organelles is also given in Figure 6. Another crucial step leading to irreversible injury is the breakdown of the lysosomal membranes and the release of structure-degrading enzymes into the cytosol. It seems that the threshold between reversible and irreversible cellular injury at the ultrastructural level is marked by the breakdown of the cellular membranes. The other signs of injury are reversible should the ischaemic insult on the cell come to an end (Farber 1982, Kumar et al. 2004).

### 2.4.1.5 Effect of reperfusion

Irreversible ischaemia and transient ischaemia followed by reperfusion produce different findings at the ultrastructural level. Irreversible ischaemia extended to 24 hours produces findings similar to those mentioned above, but after 24 hours the cells and mitochondria are condensed in appearance, without any oedema, and with well-preserved shape and matrix density. By contrast, reversible severe ischaemia and reperfusion lead to extended degeneration of the organelles and severe oedema, most likely representing the extent of
the additional damage caused by the reperfusion injury (Solenski et al. 2002, Kumar et al. 2004).

Fig. 5. Left: Mitochondria with relatively normal intercristal spaces. A sample from the FDP group in paper III, magnification x30000. Right: Two mitochondria with severe oedema, the lower on the verge of membrane rupture. A sample from the control group in paper III, magnification x30000.
Fig. 6. Left: A normal nucleus with an intact nucleolus and dispersed chromatin. The cytoplasm shows no oedema, and the endoplasmic reticulum is intact with ribosomes attached to it. Normal/mildly swollen mitochondria can be seen throughout the cytoplasm. A sample from the FDP group in paper III, magnification x5000. Right: A badly damaged nucleus, with chromatin clumped in the proximity of the membrane. The nucleolus is absent and the cytoplasm is characterized by severe oedema. The endoplasmic reticulum is partly ruptured, and detached ribosomes can be seen floating freely in the cytoplasm. A sample from the control group in paper III, magnification x5000.
3 Aims of the present studies

The general hypothesis to be tested here states that by employing a randomized, controlled and blinded study setting with a relevant animal model, a significant improvement in the neurological outcome attainable by a certain intervention can be demonstrated. The specific hypotheses for the individual papers were that:

1. Fructose-1,6-bisphosphate improves the neurological outcome after hypothermic circulatory arrest.
2. Fructose-1,6-bisphosphate mitigates the embolic brain injury associated with short hypothermic circulatory arrest.
3. The impact of fructose-1,6-bisphosphate on the outcome after hypothermic circulatory arrest is reflected in enhanced preservation of the cerebral ultrastructures.
4. Hypertonic saline dextran is effective in reducing ischaemic brain injury and improving neurological recovery after hypothermic circulatory arrest.
4 Materials and methods

4.1 The surviving porcine model

The surviving porcine model used here was first employed by Professor Randall Griep and his group at the Mount Sinai School of Medicine in New York, and was later adopted and further developed by Professor Tatu Juvonen (Juvonen et al. 1998a). The first experiments at the Cardiothoracic Research Laboratory in Oulu were performed in the autumn of 1997, and more than 520 individual experiments had been performed by the end of June 2005. The model is under continuous, critical improvement. The protocol used in the present experiments is that depicted in Figures 7 and 8.

Fig. 7. The surviving porcine model as used in the present experiments.
4.2 Test animals

The animals involved in these experiments were crossings from a native stock of Yorkshire pigs and were provided by a nearby piggery in Oulu, Finland. No cloned animals were used. The pigs were aged 8-10 weeks and their median weight was 27.0 kg (25th and 75th percentiles 25.3-30.0 kg).

4.3 Preoperative management

All the animals received humane care in accordance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, National Research Council (published by the National Academy
Press, revised in 1996). The protocol was approved by the Research Animal Care and Use Committee of the University of Oulu.

The humane end-points of the test animal permit granted by the committee defined the circumstances under which a single experiment had to be terminated prematurely. Some but not all of these end-points caused complete exclusion of the animal from the present material. The humane end-points included the following: 1) experiments that fail for various reasons (i.e. massive iatrogenic bleeding, pleuritis/pericarditis, lethal iatrogenic intracranial haemorrhage, fever <40°C preoperatively), 2) a comatose animal not accepting fluids and/or food on the 4th postoperative day despite maximal analgesia, 3) an animal unable to move on the 4th postoperative day, despite of maximal analgesia, 4) a significant disturbance following an initially normal recovery, 5) an animal suffering from severe cardiac failure that does not respond to feasible medical interventions, and 6) any situation which causes significant pain and suffering to the animal that cannot be relieved with feasible interventions.

4.4 Drug administration

In the first series of experiments (I), the FDP (Esafosfina®; Biomedica Foscama Industria Chimico-Farmacutaica SpA, Ferrentino, Italy), molecular weight 336.082 Da, was stored at room temperature in 100 ml bottles containing FDP 100g/L, Pi2+ 46 mmol/L (1.43 g), Na+ 44 mmol/L (2.64 g) and fructose 22 mmol/L (4.0 g). The liquid was sterile and colourless, and its pH was 3.5-3.8 and osmolarity 620 mmol/L. The dose was set at 500 mg/kg per infusion, the volume of each infusion being 5 ml/kg. Before infusion, the drug was neutralized with NaOH (34 ml NaOH/200 ml FDP) to a pH of 6.3. The NaOH solution contained Na+ 2 mmol/L. The placebo consisted of a corresponding volume (5 ml/kg) of isotonic NaCl (Na+ 150 mmol/L) with a pH of 6.0-6.5.

In the second experiment (II), the FDP® (Esafosfina®; Biomedica Foscama Industria Chimico-Farmacutaica SpA, Ferrentino, Italy) was stored at room temperature in 50 ml injectable powder bottles, 5 g FDP in each bottle. The dose was the same as that used in experiment I (500 mg/kg, 5 ml/kg). Normal physiological saline was used as a solvent. The liquid was pale yellow in colour and its pH was 5.6. No neutralization with sodium hydroxide was required. The placebo consisted of a corresponding volume (5 ml/kg) of isotonic sodium chloride with a pH of 6.0 to 6.5. The slightly yellow colour of the FDP was masked by using bright yellow infusion bags.

The drug and placebo solutions, prepared by a nurse who did not take part in any other stages of the work, were administered intravenously in a randomized, blinded fashion in both FDP experiments (I, II). The solutions were prepared after the animals had been selected in a randomized manner, and no other person knew of the randomization protocol. The first, 15-minute infusion was given immediately before HCA and the second, 30-minute infusion immediately after HCA. The intravenous dose of FDP was estimated from the results of pilot studies that showed that higher doses of FDP led to toxic levels of serum phosphate and thus a fatal decrease of serum ionized calcium levels.

The hypertonic saline dextran used in the fourth experiment (IV) (RescueFlow®; Biophausia AB, Uppsala, Sweden) was a hypertonic solution containing 7.5 g NaCl and 6
g dextran (70 kDa) in 100 ml of solvent. The preparation was unbuffered, its pH being 3.5-7.0. The osmolarity of HSD is 2567 mOsm/L. The control solution consisted of normal saline, 0.9 g NaCl in 100 ml. The osmolarity of normal saline is approximately 290 mOsm/L and the pH of an unbuffered solution is 4-7. The fluids were randomized and prepared by a nurse in the same randomized, blinded manner as described above for FDP. The colourless solutions were administered using identical syringes. The dose was set at 4 ml/kg, this being the most widely used in the literature (Kramer 2003). The first 5-minute infusion was given just after the start of rewarming and the second 4 hours after the start of rewarming. Both groups received additional Ringer acetate according to standard fluid therapy protocols.

4.5 Anaesthesia and haemodynamic monitoring

Anaesthesia was induced with ketamine hydrochloride (10 mg/kg intramuscularly) and midazolam (1 mg/kg intramuscularly). A peripheral venous catheter was inserted into the right ear for the administration of the drugs and to maintain the fluid balance with Ringer acetate.

In the first experiment anaesthesia was deepened with thiopental sodium (125-250 mg intravenously), and pancuronium bromide (4 mg intravenously) was given for muscular paralysis, while in experiments II and IV anaesthesia was deepened with an intravenous bolus injection of thiopental (25-125 mg) and fentanyl (25 µg/kg). Cefuroxime, 1.5 g, was given as an antibiotic prophylaxis at the induction of anaesthesia and before extubation.

After endotracheal intubation, the animals were maintained on positive-pressure ventilation with 50% oxygen. Anaesthesia was maintained with isoflurane (1.2%-1.3%) in experiment I and with a continuous infusion of fentanyl (25 µg/kg/h), midazolam (0.25 mg/kg/h) and pancuronium (0.2 mg/kg/h) in addition to isoflurane (0.5%) in experiments II and IV. The animals were kept under anaesthesia throughout the experimental period until extubation in all cases, but not during HCA. Electrocardiographic monitoring took place throughout the experiment, and an arterial catheter was positioned in the left femoral artery for arterial pressure monitoring and blood sampling. A thermodilution catheter (CritiCath®, 7F, Ohmeda GmbH & Co, Erlangen, Germany) was placed through the left femoral vein to allow blood collection and pressure monitoring in the pulmonary artery and to record blood temperature and cardiac output. A 10F catheter was inserted into the urinary bladder for monitoring urine output. Blood, rectal, oesophageal (only in experiment I), epidural and intracerebral temperatures were monitored continuously.

4.6 Cardiopulmonary bypass

A membrane oxygenator (Midiflow D 705; Dideco, Mirandola, Italy) was primed with 1 L of Ringer acetate and heparin (5000 IU). The right thoracic vessels were ligated and cut via a right thoracotomy performed in the fourth intercostal space, the pericardium was opened and the heart and great vessels were exposed. After baseline measurements,
systemic heparinization (500 IU/kg) was performed. The ascending aorta was cannulated with a 16 French arterial cannula and the right atrial appendage with a single 24 French atrial cannula. Non-pulsatile CPB was initiated at a flow rate of 90 to 110 mL/kg/min, and the flow was adjusted to maintain an arterial pressure of 50 to 70 mmHg. A 10 French intracardiac sump cannula was positioned in the left ventricle through the apex of the heart for decompression of the left side of the heart during CPB. A heat exchanger was used for core cooling or warming. In experiment I the CPB was performed using the alpha-stat strategy, with the PaCO₂ level maintained at 5.3 kPa, uncorrected for temperature, while in experiments II and IV, the pH-stat strategy was adopted during CPB, by adding CO₂ to the inflowing gas and maintaining the PaCO₂ level at 5.3 kPa, corrected for the actual core temperature of the animal. After weaning from CPB, the systemic heparinization was ended by means of an infusion of protamine sulphate.

### 4.7 Experimental protocol

The protocol is presented in Figure 9. A cooling period of 60 minutes was employed to attain a brain temperature of 18°C. Once the target temperature had been reached, the ascending aorta was cross-clamped just distal to the aortic cannula, and cardiac arrest was induced by injecting potassium chloride (40 mmol) through the aortic cannula. The 75-minute period of HCA was then initiated in experiments I and IV. The cerebral embolization in experiment II was performed before initiation of the 25-minute period of HCA. Cardiac cooling with topical ice slush was maintained throughout the HCA period. Similarly, intracerebral temperatures were controlled and maintained at 18°C with ice packs placed over the head.

![Fig. 9. The experimental protocol.](image)
The embolization in experiment II was performed as follows. Immediately before initiation of HCA, the descending and ascending aorta (clamp shifted proximally to the aortic cannula) were cross-clamped and the CPB flow rate, designated as pre-injection flow, was adjusted to maintain an aortic arch pressure of 50 mmHg. This pressure level was stabilized for one minute before the injection of 200 mg of polystyrene microspheres (250-750 µm in diameter) into the isolated aortic arch. After embolization, the post-injection flow rate was again adjusted to maintain a perfusion pressure of 50 mmHg and stabilized for 5 min. The 25-minute period of HCA was then initiated.

Reperfusion was started after the HCA period. Furosemide (40 mg), mannitol (15 g), methylprednisolone (80 mg), lidocaine (40 mg) and calcium glubionate (2.25 mmol Ca²⁺) were administered five minutes after the start of rewarming, the left ventricular sump cannula was removed after 50 minutes of rewarming, and weaning from CPB occurred at about 60 minutes. Temperatures during rewarming and after weaning from CPB were regulated using a heat-exchanger mattress, heating lamps, paracetamol infusions (1000 mg x1-2 intravenously, Perfalgan®; Bristol-Myers Squibb, New York, USA) and ice packs. Intravenous dopamine infusion was started if the fluid therapy was insufficient to maintain the mean arterial pressure above 65 mmHg. The animals were extubated 8 hours (I and II) or 16 hours (IV) after the start of rewarming and were then moved to a recovery room.

The diuresis and fluid balance were observed continuously during the experiment, and haemodynamics (pulse rate, systemic and pulmonary arterial pressures, central venous pressure, pulmonary capillary wedge pressure, cardiac output), respiratory gases and blood, rectal and epidural temperatures were monitored continuously with a Datex® AS/3 anaesthesia monitor (Datex Inc., Espoo, Finland).

4.8 Electroencephalographic monitoring

EEG monitoring was employed in experiments I and II. Cortical electrical activity was recorded using four stainless steel screw electrodes 5 mm in diameter implanted in the skull over the parietal and frontal areas of the cortex attached to a digital EEG recorder (Nervus®; Reykjavik, Iceland) and an amplifier (Magnus® EEG 32/8; Reykjavik, Iceland). The sampling frequency was 1024 Hz and the bandwidth 0.03 to 256 Hz. All four EEG electrodes were referenced to another screw electrode, which, together with a ground screw electrode, was implanted over the frontal sinuses.

In experiment I, the isoflurane level was adjusted in such a way that the EEG showed a steady burst-suppression pattern. The isoflurane end-tidal concentration was then kept at this steady level (1.2-1.3 vol.%) until the end of monitoring. EEG recordings were made for 10 minutes before the cooling perfusion to obtain a baseline level of steady burst-suppression activity. Recordings were restarted after HCA and continued until extubation. The duration of the bursts was measured from 5-minute EEG samples at 1-hour intervals. Artifact periods were excluded from each 5-minute sample, after which the sum duration of the bursts was calculated as a percentage of the sum of artifact-free EEG passages in the sample (BSR, burst-suppression ratio). This percentage was used as a measure of EEG activity in the analysis.
4.9 Microdialysis technique

An intracerebral microdialysis catheter was inserted through a hole made in the right side 0.5 cm posteriorly to the coronal suture. The microdialysis catheter (CMA 70; CMA/Microdialysis, Stockholm, Sweden) was inserted into the brain cortex to a depth of 15 mm below the dura mater, connected to a 2.5 mL syringe attached to a microinfusion pump (CMA 106 [CMA/Microdialysis]) and perfused with a special solution at a rate of 0.3 μl/min (Perfusion Fluid CNS;CMA/Microdialysis). Samples for immediate measurement of brain glucose, lactate, pyruvate, glutamate and glycerol concentrations with a microdialysis analyzer (CMA 600; CMA/Microdialysis) by ordinary enzymatic methods were collected at various time intervals.

4.10 Intracranial monitoring

All the intracranial parameters were monitored continuously. A probe for the measurement of epidural temperature was inserted into the epidural space through a cranial hole made on the left side anteriorly to the coronal suture, and a catheter for the measurement of intracerebral tissue oxygen partial pressure (Revodorex Brain Oxygen Catheter-Micro-Probe, REF. CC1.SB; GMS, Mielkendorf, Germany) was inserted through a hole on the right side anteriorly to the coronal suture. Another temperature probe (Thermocouple Temperature Catheter-Micro-Probe, REF. C8.B, GMS) for the measurement of intracerebral temperature and an intracranial pressure-monitoring catheter (Codman Micro-Sensor ICP Transducer; Johnson & Johnson, Piscataway, New Jersey, USA) were introduced through a hole located on the left side posteriorly to the coronal suture. Intracerebral temperature and brain tissue oxygen partial pressure were monitored with a Licox CMP Monitor (GMS) and intracranial pressure with a Codman ICP Express Monitor (Johnson & Johnson).

4.11 Additional intraoperative measurements

Systemic arterial and venous blood samples were collected at baseline (after the thermodilution catheter had been applied), at the end of cooling (immediately before the institution of HCA) and at 30 minutes, 2 hours, 4 hours, 8 hours and 16 hours (in experiment IV) after the start of rewarming for determination of the following parameters: pH, oxygen and carbon dioxide partial pressures, oxygen saturation, haematocrit, haemoglobin, sodium, potassium, glucose (II, IV) and ionized calcium (I, II, IV) (i-STAT Analyzer; i-STAT Corporation, East Windsor, NJ), lactate (I, II) (YSI 1500 analyzer; Yellow Springs Instrument Co, Yellow Springs, Ohio, USA), leukocyte differential count (Cell-Dyn analyzer; Abbot, Santa Clara, Calif, USA), phosphate (I, II) (Cobas Integra; Roche Diagnostics, Basel, Switzerland), pyruvate (I) (Hitachi 911; Roche Diagnostics, Mannheim, Germany) and creatine kinase (CK) and its isoenzymes CK-
MM, CK-MB and CK-BB (I) (Hyd rasys LC-electrophoresis, Hyr ys-densitometry; Sebia, France).

Venous blood samples were also collected at 1 hour 15 minutes, 1 hour 30 minutes, 2 hours 30 minutes, 3 hours, 3 hours 30 minutes, 4 hours 30 minute, 5 hours, 5 hours 30 minute, 6 hours, 7 hours, 10 hours, 12 hours and 14 hours in experiment IV for determination of pH, oxygen and carbon dioxide partial pressures, oxygen saturation, haematocrit, haemoglobin, sodium, potassium, glucose and ionized calcium (i-STAT Analyzer; i-STAT Corporation).

4.12 Postoperative evaluation

All the animals were observed continuously by the investigators for 24 hours postoperatively and after that by a veterinary nurse. A transdermal fentanyl plaster (Durogesic®; Janssen-Cilag, Berchem, Belgium) with a dose of 50 µg/h applied to the skin was used for 72 hours after surgery in addition to boluses of fentanyl (100 µg intramuscularly 1-3 times a day). After 72 hours, buprenorphin (Temgesic®; Schering AG, Berlin, Germany) was injected intramuscularly 1-3 times a day. The animals were evaluated daily in terms of a species-specific quantitative behavioural score by an experienced observer who was blinded to the experimental group to which they belonged. The quantified assessments of mental status (0 = comatose, 1 = stuporous, 2 = depressed, and 3 = normal), appetite (0 = refuses liquids, 1 = refuses solids, 2 = decreased, and 3 = normal), and motor function (0 = unable to stand, 1 = unable to walk, 2 = unsteady gait, and 3 = normal) were summed to obtain a final score, with the maximum score of 9 denoting apparently normal neurological function and lower values indicating substantial brain damage. A cumulative total behavioural score for each animal was calculated as the sum of all the postoperative daily scores and taken to represent overall recovery.

4.13 Perfusion fixation

Each surviving animal was electively sacrificed on the seventh postoperative day. Immediately after an intravenous injection of pentobarbital (60 mg/kg) and heparin (500 IU/kg), the thoracic cavity was opened and the descending thoracic aorta clamped. Ringer solution (1 L) was infused through the upper body via the ascending thoracic aorta, and blood was suctioned from the superior vena cava until the perfusate was clear of blood. Then a 10% formalin solution (1 L/15 min) was perfused through the brain in the same manner to achieve perfusion fixation, after which the entire brain was immediately harvested, weighed and immersed in 10% neutral formalin. The same method of fixation was used for the animals that died prematurely before the seventh postoperative day.
4.14 Histopathological analysis

After fixing of the brain in formalin for one week en bloc, 3-mm coronal samples were sliced from the left frontal lobe, the thalamus (including the adjacent cortex) and the hippocampus (including the adjacent brainstem and temporal cortex), and sagittal samples were obtained from the posterior brainstem (medulla oblongata and pons) and cerebellum. The specimens were fixed in fresh formalin for another week and then processed by rinsing in water for 20 minutes and immersion in 70% ethanol for 2 hours, 94% ethanol for 4 hours and absolute ethanol for 9 hours. They were then placed in an absolute ethanol-xylene mixture for 1 hour and in xylene for 4 hours and embedded in warm paraffin for 6 hours. 6-µm sections from these brain specimens stained with haematoxylin and eosin were screened by an experienced pathologist who was unaware of the experimental design and of the identity and fate of the individual animals. He carefully examined for the presence or absence of ischaemic or other tissue damage.

Signs of injury were scored visually as follows: 1 = dark or eosinophilic neurons or cerebellar Purkinje cells; 2 = moderate oedema; and 3 = severe oedema or infarct foci (local necrosis), a histopathological sum score for all the specific brain areas (cortex, thalamus, hippocampus, posterior brainstem and cerebellum) being taken for semiquantitative comparison between the animals. In experiment IV this statistical analysis was performed twice, with or without the exclusion of early deaths.

4.15 Electron microscopy

Biopsies from the left parietal cerebral cortex of 6 animals in the FDP group and 5 in the control group in experiment I were chosen for analysis by electron microscopy for the purposes of paper III. The animals in the FDP group were selected at random. All the animals selected had survived for the required seven days postoperatively. Sections of 1-2-mm from the left parietal cortex samples collected after termination of the animal on the 7th postoperative day were immediately fixed in a mixture of 1% glutaraldehyde and 4% formaldehyde in 0.1 M phosphate buffer, postfixed in 1% osmium tetroxide, stained en bloc with uranyl acetate, dehydrated, and embedded in Epon (Epon Embed 812, Electron Microscopy Sciences, Fort Washington, PA, USA). Semi-thin (1 µm) sections were stained with toluidine blue, and ultrathin sections were double-stained with uranyl acetate and lead citrate and examined under a Jeol 100S or Jeol 1200 EX electron microscope (Jeol Co., Tokyo, Japan) in a blinded manner by a senior cell biologist, who looked for at least 10 typical, representative neurons in each tissue sample.

The electron micrographs were examined and analysed in a blinded fashion by a senior pathologist, using an objective grading system. The mitochondria, nuclei, cytoplasm and endoplasmic reticulum of the neurons were analysed for injury, the signs of which were scored as follows: 0 (normal mitochondria, intact nuclei, no oedema in the cytoplasm, normal endoplasmic reticulum); 1 (mild and/or moderate mitochondrial oedema, chromatin aggregation in the nuclei, mild and/or moderate oedema in the cytoplasm, endoplasmic reticulum swollen but still intact); 2 (severe mitochondrial swelling, disintegrated nuclei, severe cytoplasmic oedema, ruptured endoplasmic
reticulum); and 3 (ruptured mitochondria). A total score was calculated for each sample by summing the scores for the individual areas.

4.16 Statistical analysis

The statistical analysis was performed using standard commercially available statistical programs: SPSS, version 10.0 (paper I), or version 11.5 (papers II-IV) (SPSS Inc, Chicago, Ill, USA) and SAS, version 8.02 (papers II-IV) (SAS Institute Inc., Cary, NC). The values were expressed as medians with 25th-75th percentiles. The differences in survival outcome between the groups were evaluated using the two-tailed Fisher’s exact test, and other differences between the groups were evaluated by means of the t-test for normally distributed data or the Mann-Whitney U-test if the normality assumption failed. When there were several measurement points, the analysis of variance for repeated measurements was used in paper I and the SAS procedure Mixed in papers II-IV. Since the measurement intervals were uneven, the spatial exponential covariance structure was defined in the repeated statement. Repeated measurement analysis was not used for the lactate/pyruvate ratio or the lactate/glucose ratio in paper IV because of huge variation in the variables, so that only cross-sectional analysis was performed. Complete independence was assumed between the animals in view of the randomization. The p-values reported are as follows: p-between groups indicates the level of difference between the groups, and p-time*group indicates the behaviour between groups over time. Spearman correlation coefficients with their significance levels were calculated to estimate the correlations between variables in paper III. Two-tailed significance levels are reported.
5 Results

5.1 Exclusion criteria

Out of the total of 87 pigs used in these experiments, 68 were included in the eventual series. The reasons for exclusion are shown in Table 4. The three animals used in pilot studies at the beginning of experiment I, to determine the exact dose of FDP, and the four pigs used as pilots at the beginning of experiment IV, to evaluate the effects of HSD on the haemodynamic parameters, are not shown in Table 4. The final numbers of animals accepted were 24 in experiment I, 20 in experiment II and 24 in experiment IV. The series discussed in paper III consists of 11 animals from experiment I.

Table 4. Indications for the exclusion of animals.

<table>
<thead>
<tr>
<th>Indication for exclusion</th>
<th>Experiment I</th>
<th>Experiment II</th>
<th>Experiment IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>FDP Control</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Experiment I</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Experiment II</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Experiment IV</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Indication for exclusion</th>
<th>FDP Control</th>
<th>FDP Control</th>
<th>HSD Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fever (Rectal temperature &gt; 40°C at baseline)</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unable to get out of perfusion</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lethal arrhythmia after perfusion</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Iatrogenic circulatory air embolism</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extubation-related lethal laryngeal spasm</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Decannulation bleeding</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Massive postoperative bleeding</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Pleuritis/pericarditis</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unidentified treatment-resistant hypotension</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Loss of vascular permeability, uncontrolled</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>oedema</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total number of animals excluded</td>
<td>4 of 28 (14.3%)</td>
<td>4 of 24 (16.7%)</td>
<td>4 of 28 (14.3%)</td>
</tr>
</tbody>
</table>
5.2 Comparability of the groups

All the animals included in these series were stable before, during and after surgery and survived at least until the first postoperative day. There were no significant differences between the groups in terms of CPB cooling or rewarming times, total CPB times, weight of the animals or temperatures during HCA. All the baseline values were similar between the groups in experiment I, and although the baseline central venous pressure and arterial oxygen partial pressure were significantly higher in the FDP group in the embolization experiment (II), the overall differences were not significant ($p=0.5$ and $p=0.3$, respectively), so that this was most likely a random phenomenon. The baseline venous oxygen saturation was significantly higher in the HSD group ($p=0.03$) in experiment IV, which was reflected in significantly lower oxygen extraction in the same animals ($p=0.05$), but these differences were short-lasting and the overall differences between groups with regard to these parameters were not significant ($p=0.7$ and $p=0.2$, respectively).

5.3 Summary of the results

The primary and secondary end-points of experiments I, II and IV are summarized in Tables 5 and 6, respectively. The reader is advised to consult the original articles reproduced at the end of the present volume for detailed results.

Table 5. Primary end-points of the experiments.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Survival</th>
<th>Median behavioural score on day 7</th>
<th>Median cumulative behavioural score</th>
<th>Median total histopathological score</th>
</tr>
</thead>
<tbody>
<tr>
<td>FDP (I)</td>
<td>12</td>
<td>10 (83%)</td>
<td>8</td>
<td>44.4</td>
<td>3.0</td>
</tr>
<tr>
<td>Control (I)</td>
<td>12</td>
<td>5 (42%)</td>
<td>7</td>
<td>34.6</td>
<td>5.5</td>
</tr>
<tr>
<td>$p$-value (FDP I vs. Control I)</td>
<td>0.09</td>
<td>0.004</td>
<td>0.01</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>FDP (II)</td>
<td>10</td>
<td>5 (50%)</td>
<td>6</td>
<td>28.0</td>
<td>12.5</td>
</tr>
<tr>
<td>Control (II)</td>
<td>10</td>
<td>4 (40%)</td>
<td>7</td>
<td>33.0</td>
<td>11.4</td>
</tr>
<tr>
<td>$p$-value (FDP II vs. Control II)</td>
<td>&gt;0.9</td>
<td>0.62</td>
<td>0.56</td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td>HSD (IV)</td>
<td>12</td>
<td>9 (75%)</td>
<td>9</td>
<td>46.0</td>
<td>7.0</td>
</tr>
<tr>
<td>Control (IV)</td>
<td>12</td>
<td>8 (66%)</td>
<td>8</td>
<td>40.5</td>
<td>10.0</td>
</tr>
<tr>
<td>$p$-value (HSD vs Control IV)</td>
<td>&gt;0.9</td>
<td>0.25</td>
<td>0.067</td>
<td>0.01</td>
<td></td>
</tr>
</tbody>
</table>
Table 6. Important secondary end-points of the experiments. One arrow: mild increase/decrease, two arrows: significant increase/decrease.

<table>
<thead>
<tr>
<th>End-point</th>
<th>FDP with prolonged HCA (I)</th>
<th>FDP with embolic brain injury (II)</th>
<th>HSD with prolonged HCA (IV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain lactate</td>
<td>† up to 4 hours after HCA</td>
<td>no change</td>
<td>†</td>
</tr>
<tr>
<td>Brain lactate-pyruvate ratio</td>
<td>†</td>
<td>††</td>
<td>†</td>
</tr>
<tr>
<td>Brain pyruvate</td>
<td>†</td>
<td>††</td>
<td>no change</td>
</tr>
<tr>
<td>Brain glucose</td>
<td>†</td>
<td>††</td>
<td>†</td>
</tr>
<tr>
<td>Brain tissue oxygen</td>
<td>no change</td>
<td>no change</td>
<td>†</td>
</tr>
<tr>
<td>Intracranial pressure</td>
<td>no change</td>
<td>no change</td>
<td>†† up to 8 hours after HCA</td>
</tr>
<tr>
<td>Cerebral perfusion pressure</td>
<td>no change</td>
<td>no change</td>
<td>†† up to 3 hours after HCA</td>
</tr>
<tr>
<td>EEG burst-suppression ratio</td>
<td>no change</td>
<td>no change</td>
<td>not measured</td>
</tr>
<tr>
<td>pH</td>
<td>†† during administration</td>
<td>†† during administration</td>
<td>no change</td>
</tr>
<tr>
<td>Blood CO₂ tension</td>
<td>†† during administration</td>
<td>†† during administration</td>
<td>no change</td>
</tr>
<tr>
<td>Mean arterial pressure</td>
<td>no change</td>
<td>no change</td>
<td>short-lasting †† related to administration</td>
</tr>
<tr>
<td>Cardiac index</td>
<td>no change</td>
<td>no change</td>
<td>†† related to administration</td>
</tr>
<tr>
<td>Vascular resistance</td>
<td>†</td>
<td>no change</td>
<td>†† related to administration</td>
</tr>
<tr>
<td>Fluid balance</td>
<td>††</td>
<td>††</td>
<td>††</td>
</tr>
<tr>
<td>Serum sodium</td>
<td>††</td>
<td>††</td>
<td>††</td>
</tr>
<tr>
<td>Serum phosphate</td>
<td>††</td>
<td>††</td>
<td>not measured</td>
</tr>
<tr>
<td>Serum ionized calcium</td>
<td>††</td>
<td>††</td>
<td>no change</td>
</tr>
<tr>
<td>Haemoglobin</td>
<td>no change</td>
<td>no change</td>
<td>short-lasting †† related to administration</td>
</tr>
</tbody>
</table>

5.4 Electron microscopy (paper III)

The total neuronal score was lower in the FDP group, as were the nucleus and cytoplasm scores, and the mitochondrion score was somewhat lower (Table 7). There was a significant positive correlation between the conventional histopathological total score of paper I and the total EM score of paper III ($r=0.73$, $p=0.01$).
Table 7. Neuronal electron microscopy scores. For the scoring system, see section 4.15.

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Pig #</th>
<th>Mitochondria</th>
<th>Nuclei</th>
<th>Cytoplasm</th>
<th>Total score</th>
</tr>
</thead>
<tbody>
<tr>
<td>FDP</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>1.2</td>
<td>0.7</td>
<td>1.0</td>
<td>2.7</td>
</tr>
<tr>
<td>Control</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>2.2</td>
<td>1.8</td>
<td>2.0</td>
<td>6.0</td>
</tr>
<tr>
<td>p-value</td>
<td></td>
<td>0.059</td>
<td>0.01</td>
<td>0.011</td>
<td>0.002</td>
</tr>
</tbody>
</table>
6 Discussion

6.1 Preface

The present work is part of the effort by our research group to contribute scientifically to improving the outcome after HCA. Aortic surgery and other forms of surgery requiring HCA entail significant risks and/or high morbidity and mortality rates. Moreover, due to the nature of the diseases affecting the thoracic aorta, such as aneurysm rupture or dissection, the operations often take place outside normal working hours and there may not be much time to prepare the patient thoroughly for surgery. Easy access to simple yet effective methods of brain protection at all times is therefore of paramount importance.

Our team has found several effective forms of brain protection and methods of predicting the outcome after HCA over the years. Cold RCP improved cerebral protection as compared with HCA alone (Anttila et al. 1999), perhaps by improving cerebral cooling (Anttila et al. 2000a), and intermittent RCP may lead to a lower risk of brain oedema than with continuous RCP without affecting the efficacy of RCP as such (Anttila et al. 2000b). A Na⁺ channel blocker, lamotrigine, was found to be neuroprotective (Anttila et al. 2000c), but a NMDA antagonist, memantine, did not improve the outcome after a 75-minute period of HCA (Rimpiläinen et al. 2001). A leukocyte filter connected to the CPB circuit improved the neurological recovery of the animals and resulted in less histopathological damage (Rimpiläinen et al. 2000), while the combination of lamotrigine with leukocyte filtration proved to be even more efficacious in mitigating cerebral injury than either of the methods alone (Rimpiläinen et al. 2002). The administration of erythropoietin in the presence of prolonged HCA did not affect mortality or either behavioural or histopathological recovery, but some intracranial parameters and a lower apoptotic index indicated that erythropoietin may nevertheless be beneficial after HCA (Romsi et al. 2002b). Indeed, apoptotic activity increases in the brain after HCA, as shown by our group (Mennander et al. 2002). Three potentially neuroprotective proteins, which had been shown to possess anti-inflammatory properties in vitro and in vivo did not have any significant neuroprotective effect in our model of prolonged HCA, but the results may have been affected by the small number of animals used (Heikkinen et al. 2004).
Although hypothermia has been a major focus of our research, neither prolonged hypothermia for up to 14 hours after HCA nor topical cooling for 2 hours provided any clear benefit with regard to recovery (Romsi et al. 2002a, Pokela et al. 2003b). Another of our team’s interests is to determine which perfusion strategy is most beneficial for protecting the brain during operations requiring HCA. The pH-stat strategy seems to be more beneficial, since its use improves the outcome relative to the alpha-stat strategy (Pokela et al. 2003a), and it even seems to be superior in the context of cerebral embolization (Dahlbacka et al. 2005).

Predictors of the outcome after HCA are important in enabling a rapid, effective response to possible threats to patient survival. The microdialysis technique provides a virtually on-line method of monitoring cerebral metabolism, and signs of ischaemia may be taken to indicate a poorer outcome after HCA (Pokela et al. 2001). Increased intracranial pressure (Pokela et al. 2002), decreased EEG burst-suppression activity (Pokela et al. 2003c) and higher levels of serum S-100β protein (Pokela et al. 2000) are other important markers of severe cerebral injury following HCA.

The present experiments point to additional means of cerebral protection during and after HCA. FDP and HSD both proved to be beneficial in terms of the outcome. FDP also provided some support for cerebral metabolism in the presence of irreversible cerebral ischaemia caused by particle embolization, and it was effective in preserving the neuronal ultrastructures. The present findings will be discussed further in the sections below, along with the primary and secondary end-points of our model.

6.2 The surviving porcine model

The ischaemic injury caused by a 75-minute period of HCA at an intracerebral temperature of 18°C is severe, as the present mortality rates clearly show. Such injuries could obviously not be studied in humans for ethical reasons, but the use of test animals allowed us to explore such issues. The dog has traditionally been the animal of choice in cardiothoracic research, since these are easily available and their vascular system resembles that of man, even the use of dogs is considered unethical nowadays, presumably because their status as pets has made them too precious to us.

Primates are close relatives to humans, and their use as test animals would be ideal, but their poor availability, high price and the fact that primate pathogens may pose a serious threat to researchers complicate their use also in addition to ethical issues.

Pigs have gained greatly in popularity for modern experimental cardiothoracic surgery. They are easily produced in normal piggeries, making their cost quite low, and the anatomy of their vascular system closely resembles that of humans. Although the surviving porcine model used here represents an effort to mimic the actual clinical setting, it is not completely without problems. The pigs used here were 8-10 weeks old, while patients undergoing aortic arch surgery are mostly adults or elderly, with comorbidities and significant atherosclerosis. The use of adult pigs is impractical due to their size, and thus the results reported here are only able to give some indications with regard to clinical applications. The model comes somewhat closer to the truth when considering pediatric cardiac surgery, however. When evaluating animal studies, the
reader must keep in mind that each species has its own unique attributes. The blood of pigs, for example, coagulates much faster than that of humans. One must therefore be cautious when extrapolating results based on an animal model to humans.

The native porcine stock, i.e. the heterogeneity of the pig genome, also raises some questions. Animals may react differently to external impulses. If the present work were to focus solely on preclinical issues such as the mechanisms of cerebral ischaemic injury, this would be a problem. The use of cloned animals, a very common practice in preclinical studies, is based on idea to controlling the heterogeneity aspect to the maximum degree possible. As the animals share the same genome, they should react similarly to interventions. Some cloned minipig populations are being developed, but their cost will be exceedingly high at first. From a clinical point of view, however, the use of a native stock allows us to mimic humans, since patients naturally do not have the same genome. The use of non-cloned adult minipigs, perhaps with accelerated atherosclerosis, may further improve the current surviving porcine model in the future, as mentioned in section 2.3.5.1.

6.3 Study design

The first, clear benefit of the surviving porcine model is the possibility to collect cerebral biopsies for histopathological analysis. Histopathology is widely accepted as a reliable method of assessing tissue injury when performed in a blinded manner by an experienced pathologist. The second benefit of the model is the fact that most pigs survive the procedure and can be monitored neurologically afterwards. The primary end-points of the present work are therefore histopathology and the neurological outcome, including survival. The follow-up period of one week is a compromise between the resources of our team and the development of cerebral ischaemic lesions. A longer follow-up period could produce more information, as the animals would have more time to recover from HCA, but it was not possible during the experimental phase of the present work to prolong the follow-up period.

As presented in the Materials and Methods section, the present work has many secondary end-points. Individually, they all are too weak or specific to have any significant power, but when analysed together and found to be in line with each other, they may quite emphatically support the primary end-points. On the other hand, as only a few of the secondary end-points show a difference between the groups, the actual difference must be very small. Experiment II is a good example: the lactate-pyruvate ratios and pyruvate levels favoured the FDP group, but the histopathology and neurological recovery results were the same in both groups.

The fact that no power analyses were conducted is a limitation that must be kept in mind, even though clear differences between the groups were observed in terms of various end-points. We could have produced more reliable results if we had optimized the sizes of the groups by means of power analysis. On the other hand, the model is a very demanding one in terms of time and resources, so that it would not have been practicable to increase the group sizes significantly. A randomized, controlled and blinded study design was nevertheless adopted in order to make the results more reliable.
The primary end-points can be analyzed either including or excluding the animals which died before the 7th postoperative day. In paper IV the histopathology scores are shown twice, with and without these early deaths, while elsewhere the analyses include all the animals. This may create some mismatching and confusion when making conclusions, as the brains of the animals were harvested and fixed at different stages of cerebral injury. The behavioural scores are in any case analysed with the early deaths excluded, even though one could argue that we may in this way have altered the actual differences between the groups. In our opinion, however, neurological recovery as recorded by a scoring system applies only to living animals, since it measures life and its quality, not death as an alternative to this as survival does.

6.4 Survival outcome

There were no significant differences in survival, although survival was somewhat higher in the FDP group in experiment I. Such a difference could be expected, as the other end-points also favoured the FDP group. The use of a larger number of animals would most likely have resulted in a significant difference, but research using the surviving porcine model is expensive and time-consuming as well as requiring several collaborators. Thus no significant increase in the number of animals could be contemplated.

The finding in experiment II that the survival rates did not differ significantly between the groups is consistent with the histopathological and behavioural data. The embolization with polystyrene microparticles created an irreversible occlusion in the cerebral vessels which did not permit any reperfusion of the ischaemic core distal to the occlusion. Also, the embolization was performed by injecting the particles via the aortic cannula, thus affecting both hemispheres of the brain, so that hardly any adjunct reperfusion from the contralateral side could have occurred. Cerebral complications have a major impact on the eventual outcome after HCA, and it seems that in the presence of irreversible arterial occlusion FDP cannot do much to alleviate the outcome. However, after major cerebral embolization by non-degradable particles of the kind employed here, hardly any currently known drug would make any difference, and therefore tissue reperfusion is mandatory in order to make any major impact on the outcome during FDP therapy.

Trimarchi et al. (1997) report significantly better neurological recovery after particle embolization, but their rats were subjected only to a unilateral brain injury, as the small particles (48.4 µm) were injected into the right common carotid artery. Thus the injured areas of the brain most likely received blood and FDP from the collateral circulation via the circulus Willisii.

The survival rates in experiment IV similarly did not differ significantly between the groups, although the rate was slightly higher in the HSD group. A significant difference in survival could have been expected in terms of the other end-points, however. This HSD study used the pH-stat protocol during CPB, as it had been shown to have significant neuroprotective properties relative to the alpha-stat protocol (Pokela et al. 2003a), which was used in experiment I, and it may be that this protocol prevented any significant survival differences from emerging with this number of animals, as the
controls survived quite well too. The model may have to be altered in future in order to generate larger differences in mortality, e.g. by using a higher temperature during HCA, using the alpha-stat protocol during CPB or prolonging the period of HCA. Durations of 90 to 120 minutes have been used successfully elsewhere (Allen et al. 2003, Hagl et al. 2004, Li et al. 2004a).

The survival rates are not comparable between the experiments, as the protocols differ considerably from each other. Besides employing an advanced CPB strategy, experiment IV involved a different anaesthesia protocol from experiment I, while experiment II entailed embolization, use of the pH-stat protocol during CPB and a shorter period of HCA. The above applies to all the other end-points as well.

6.5 Neurological outcome

The findings in experiment I that the administration of FDP led to significantly higher neurological scores on all the postoperative days except day 2 and that the cumulative score representing overall recovery after HCA was also significantly higher in the FDP group are in line with the other data. In experiment II there were no differences in the behavioural scores on any postoperative day and the cumulative scores were also similar between the groups. As mentioned above, it is not possible to compare the results of these individual experiments directly, but it is evident in the light of the behavioural data that the irreversible embolization resulted in a far more severe brain injury than the reversible global ischaemia induced in the other studies. This is exemplified by the fact that only one pig in experiment II achieved complete behavioural recovery.

In experiment IV the animals in the HSD group recovered faster, as indicated by their significantly higher behavioural scores on day 2. The cumulative score was also higher in the HSD group, although the classical borderline of a significant difference was not reached. Once again, the use of more animals would probably have corrected this small detail. The fact that the behavioural scores match the other end-points also points to the superiority of the HSD group in this comparison.

6.6 Histopathological analysis

As the development of cerebral histopathological lesions in response to anoxia takes time (Dowden & Corbett 1999), the use of surviving animal models is mandatory in order to produce reliable histopathological results. The first signs of cellular injury include generalized swelling and/or shrinkage, but the borders of the affected tissue are not well demarcated during the first 2-3 hours. After 12 hours, eosinophilic neurons and astrocytic “ghost” cells appear, which may indicate irreversible injury (Garcia et al. 1993). Infarct volume expansion is likewise rapid up to 12 hours after ischaemia, after which this development slows considerably (Dereski et al. 1993). It seems, however, that there are late-activated forms of cell death that take place in the brain, ones which come into play 3 days after ischaemia. Apoptosis may play a role in the late development of ischaemic brain injury (Du et al. 1996). Cell death in the hippocampal CA1 region has been
observed 3-7 days after ischaemia (Bottiger et al. 1998), and the final development of ischaemic lesions may take a matter of weeks, as the injury site becomes scarred with fibroblast growth after clearance of the debris by macrophages (Kumar et al. 2005).

A postoperative period of 7 days before elective sacrifice seems to have been long enough to allow enough histopathological injury to develop in our model. Another consideration of paramount importance is that the pathologist should remain unaware of the experimental setting, as histopathological analysis is always vulnerable to a certain amount of subjectivity.

The findings in experiment I that the FDP group had significantly lower total histopathological scores and regional scores in the thalamus and hippocampus are once again in line with the other data, suggesting an improved outcome brought about by FDP administration. In experiment II, however, there were no significant differences between the groups in the histopathological analysis. On the other hand, the scores in this embolization experiment were markedly higher than elsewhere, and since the same scoring methods were used throughout, it can be concluded that the injury to the brain was probably more severe in this case. As mentioned above, however, direct comparison between the results of these individual experiments should be avoided.

There was significantly less morphological damage to the brain in the HSD group than in the controls in experiment IV, a finding which supports the beneficial results observed in outcome, behavioural data, intracranial measurements and microdialysis.

One drawback of the histopathological analysis used here is that no effort was made to measure apoptosis, which can be assessed with relatively simple and accessible methods such as TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP end labelling) staining and caspase-3 activity staining. The analysis of apoptosis would make an excellent end-point for use with this surviving porcine model in the future.

### 6.7 Haemodynamics and metabolism

Serum phosphate increased significantly after the administration of FDP in experiments I and II, leading to a significant drop in serum ionized calcium levels, which may be caused by its calcium-chelating effect. This has also been reported elsewhere (Hassinen et al. 1991, Sola et al. 1996, Takeuchi et al. 1998). The peripheral vascular resistance was slightly lower in the FDP group in experiment I, but no differences could be seen between the groups in experiment II. The lower vascular resistance could be a consequence of lower calcium levels, but the small difference observed in experiment I may equally well have been caused by chance. The isoflurane levels differed between experiments I and II, but this may have been affected by the fact that isoflurane is a potent vasodilator. The lower ionized calcium levels in the FDP groups did not give rise to any significant differences in cardiac output or other haemodynamic parameters.

Phosphate toxicity has been found to be associated with serum phosphate concentrations averaging 6.5 mmol/L (LeBlanc et al. 1991a), and the maximum median serum phosphate level of 6.3 mmol/L recorded for the FDP group at 2 hours after the start of rewarming in experiments I and II suggests that the dose used here approximated the maximal tolerable dose.
The lower serum calcium levels in may have protected both the brain and the myocardium from ischemic injury, however. Calcium overload during ischaemia and reperfusion is a well-documented mechanism leading to cell injury, and the lower calcium levels may have attenuated this overload. Cerebral calcium uptake correlates directly with serum calcium levels (Trimarchi et al. 1990), indicating that the effects of FDP on the calcium levels may have provided the brain with additional protective mechanisms. CK-MB levels were lower in the FDP group during the postoperative period in experiment I, indicating myocardial protection brought about by this drug as well.

The increased levels of sodium and osmolarity brought about by the administration of FDP in experiment I were a consequence of the high sodium content of the trisodium salt of FDP, which probably led to an improved fluid balance in the FDP group. In experiment II, however, FDP did not require neutralization of its pH and thus did not contain excess sodium ions. Even so, the administration of FDP still resulted in a better fluid balance, the sodium and osmolarity levels being lower in the FDP group than in the control group, in contrast to experiment I. Thus the increased diuresis induced by FDP may involve mechanisms other than sodium loading, but our methods and research goals do not allow further discussion on this matter.

The clear yet brief differences in pH, PaCO₂ and PvCO₂ observed in experiment II were obviously linked to the administration of FDP, as the low pH of the drug presumably led to increased levels of CO₂. Although high PaCO₂ dilates the cerebral vessels and itself has protective properties with regard to the brain (Pokela et al. 2003a), the difference between the groups is scarcely significant from a clinical point of view and the effect was short-lasting. The FDP group overcompensated for the initially lower arterial and venous pH levels in the postoperative hours, it had more alkaline values later in the postoperative period. The FDP group had significantly lower pH levels and higher PaCO₂ levels at the end of cooling in experiment I, which also coincided with the administration of FDP.

The administration of HSD in experiment IV elevated the cardiac output, especially after the second infusion, but as the vascular resistance was lower at the same time, HSD did not have any significant impact on the mean arterial pressure. Immediately after the rewarming phase, however, the mean arterial pressure was briefly higher in the HSD group, as was the cardiac output. As the vascular resistance levels in the two groups were similar at that time, the increased cardiac output in the HSD group must have led to the higher arterial pressure. The first infusion was given at the start of reperfusion, and the controlled extracorporeal circulation may have dampened the impact of HSD on the cardiac output during perfusion, as the cardiac output in the HSD group tended to rise immediately after weaning from CPB. The second infusion had a slight but non-significant impact on the central venous pressure, indicating a subsequent increase in plasma volume.

Sodium levels did not rise adversely in the HSD group. We performed some pilot experiments before the initiation of the series proper in order to find out how the sodium levels would behave with two infusions, and since they remained mostly below 150 mmol/L, we decided to use two infusions rather than a single one in order to prolong the beneficial effect of the solution, as the effect of HSD lasts 3-4 hours at most (Smith et al. 1985). Caution is recommended with regard to repeated administrations at the moment, as the therapy may be complicated by hypernatraemia, although there is no actual
evidence of a poorer outcome related to hypernatraemia (Stapley et al. 2002). Nevertheless, lowered excretion of sodium due to renal failure may be a contraindication for HSD therapy in critically ill patients, as additional dosing or higher concentrations may lead to coagulation disorders (Coats & Heron 2004). Repeated administration was successful in patients with refractory rises in ICP, with no apparent side effects from hypernatraemia or bleeding (Hartl et al. 1997). Further research would be of great importance to find out whether sustained hypernatraemia or bleeding disorders are actually complications of repeated HSD therapy.

The fact that administration of HSD resulted in significantly lower levels of mean arterial pressure at the beginning of the rewarming period is due to vasodilation caused by HSD. The administration of unbuffered HSD probably caused a significantly lower pH at 5 minutes after HCA, and vasodilation most likely also explains the significantly lower vena line temperatures in the HSD group at 15 minutes and 30 minutes. The tissues were perfused more efficiently in the HSD group and thus received more heat from the blood. Indeed, the rectal and epidural temperatures were significantly higher in the HSD group 30 minutes after HCA.

HSD led to a temporary but significant decrease of the haematocrit, which in turn has been shown to reduce mean arterial pressure and to increase cerebral blood flow. A low haematocrit is also associated with increased cerebral metabolism, however, and thus with possible deleterious effects on the brain during HCA (Sakamoto et al. 2004a). Despite this, the results clearly demonstrate the neuroprotective effects of HSD, and any adverse effect related to a temporary decrease in the haematocrit should be assessed in a separate study in which haematocrit values are both uncorrected and corrected to optimal levels.

### 6.8 Microdialysis

The administration of FDP led to significantly lower lactate-pyruvate ratio and higher pyruvate levels at end of rewarming in experiment I, although in general glucose and pyruvate levels tended to be higher in the FDP group throughout the postoperative period and brain lactate levels were higher in this group during the first postoperative hours. These findings point to increased cerebral energy metabolism from carbohydrates, both aerobic and anaerobic. Increased levels of glucose and lactate immediately after reperfusion have been associated with an improved outcome after HCA, but later increases in brain lactate levels are a sign of a poor outcome (Pokela et al. 2001). Lactate levels were quite similar in both groups later in the postoperative period. The higher lactate and glucose levels in the FDP group may derive from the fact that astrocytes metabolize glucose anaerobically to produce lactate after ischaemia, thereby supporting the injured neurons (Schurr et al. 1997, Bliss & Sapolsky 2001). Aerobic lactate utilization instead of glucose may augment neuronal recovery after ischaemia (Schurr 2002). In severe ischaemia, however, the formation of lactate in this manner becomes uncontrolled and the ensuing lactate accumulation will lead to cellular acidosis and a poor outcome.
The theory of the beneficial effects of FDP on cerebral carbohydrate metabolism received further support in experiment II, where the lactate-pyruvate ratios were significantly lower and pyruvate levels significantly higher in the FDP group during the postoperative period, while glucose levels were non-significantly higher. Lactate levels were significantly higher in the control group 8 hours after HCA, a detrimental finding at this time (Pokela et al. 2001). An increased lactate/pyruvate ratio is a reliable marker of cerebral ischaemia, and high pyruvate levels indicate adequate reperfusion (Persson & Hillered 1992). No significant impact on the outcome was seen, but this was probably prohibited by the irreversible nature of the vessel occlusion. The microdialysis findings indicate that the results of paper II do not negate the neuroprotective efficacy of FDP. The beneficial effects in terms of supporting brain metabolism are clear, suggesting that certain areas receiving some perfusion, i.e. the penumbra, were affected in a positive way by FDP. Whether this actually happened cannot be proved directly by our current methods. FDP may perhaps have improved neurocognitive recovery in humans, but such fine-tuned end-points cannot be recognized in a rough model involving animals.

Hypertonic solutions have been shown to increase rCBF and improve cerebral oxygenation (Tseng et al. 2003, Krep et al. 2004). Our methods cannot produce direct evidence of increased rCBF, but the beneficial microdialysis findings related to the use of HSD in experiment IV clearly indicate improved tissue oxygenation and perfusion.

6.9 Other intracranial measurements

The fact that intracranial temperatures did not differ between the two groups in any of the experiments may be taken as indicating the high quality of our data. Likewise, there were no differences between the groups in terms of \( p_{\text{O}_2} \) in the FDP experiments (I and II), but the administration of HSD in experiment IV resulted in higher \( p_{\text{O}_2} \) levels. This is in line with the microdialysis findings, suggesting improved cerebral metabolism and oxygenation.

The high sodium content of the FDP solution in experiment I, due to neutralization, resulted in increased osmolarity, a fact that may explain the lower ICP detected in the FDP group during rewarming. This finding could indicate an antioedemagenic effect rather than support for energy metabolism as the principal neuroprotective mechanism of FDP. There were no differences in ICP between the groups at any of the other time points, however, and CPP levels were similar as well.

The FDP solution did not require neutralization in experiment II, and therefore it did not contain any excess sodium, as mentioned above. No differences in ICP could be found between the groups, even though the FDP group did have significantly lower levels of osmolality and sodium in this experiment.

The administration of HSD in experiment IV led to a prolonged period of lower ICP levels in this group, from 45 minutes to 8 hours after HCA. This effect can be regarded as an excellent result, with a beneficial impact on the outcome (Pokela et al. 2002). The reduced levels of ICP, along with beneficial effects on the haemodynamics, led to increased CPP from 45 minutes to 3 hours after HCA, and this in turn improves cerebral oxygenation, as shown by a PET study (Johnston et al. 2005) and in the data presented.
here. The effect of HSD nevertheless wore off after 8 hours, and the ICP levels became similar in both groups. It is possible that a larger dose could have prevented this rebound. In a small porcine experiment reported by Wade et al. (2003), a single dose of 11.5 ml/kg resulted in a slightly better outcome than the 4 ml/kg dose in haemorrhagic dogs, although the difference was not significant. A third infusion 8 hours after HCA or a continuous slow infusion could also have prevented the late increase in ICP. Continuous slow infusions have been effective in lowering ICP, but the reports are few in number and the methods deficient (Qureshi et al. 1998, Suarez et al. 1999, Kuo et al. 2003). Solutions with a higher sodium concentration (10-23.4%) have also proved efficacious in lowering ICP in poor-grade patients and in animal models (Suarez et al. 1998, Mirski et al. 2000, Schwarz et al. 2002). Further research would be needed to evaluate whether a single large dose, repeated dosing or a longer, slow infusion is most effective for obtaining a sustained decrease in ICP and perhaps generating even better outcomes than those reported in paper IV.

6.10 Electroencephalography

The EEG analysis was based on calculating burst-suppression ratios, which is a simple technique to perform even with a low amplitude and interference from artifacts. EEG activity is absent during deep hypothermia and gradually recovers during rewarming (Pokela et al. 2003c). There were no significant differences between the groups in experiment I, although the recovery of EEG activity in the FDP group tended to be quicker, supporting the other findings. In experiment II recovery was similar in both groups, once again matching the other end-points.

6.11 Electron microscopy

The key ultrastructural finding with regard to neuronal ischaemia is mitochondrial swelling with dilation of the intercristal spaces, eventually leading to rupture of the mitochondrial membranes, as mentioned in section 2.4.4.2. This membrane rupture releases mitochondrial Ca\(^{2+}\) into the cytoplasm, which, with the low ATP concentration, is a major factor initiating necrotic cell death. The release of mitochondrial proteins such as cytochrome \(c\) and AIF into the cytoplasm has been shown to induce apoptosis if ATP is still present in the cells in sufficient amounts (Leist et al. 1997, Eguchi et al. 1997). Thus special emphasis was placed on mitochondrial damage in the scoring system used here. Other ultrastructural signs of neuronal injury are clumping of chromatin, often close to the nuclear membrane, and nuclear rupture along with cytoplasmic swelling. Rupture of the endoplasmic reticulum and detachment of ribosomes occur after severe injury. These findings indicate a loss of the ability to synthesize RNA and proteins (Kumar et al. 2004).

As mentioned in section 2.3.5.2, FDP has been found to preserve and re-supply cellular energy pools during ischaemia/reperfusion injury (Tavazzi et al. 1992), to inhibit the intracellular rise in Ca\(^{2+}\) during ischaemia (Hassinen et al. 1991, Bickler & Kelleher 1992) and to reduce the formation of reactive oxygen species during reperfusion (Vexler
Thus FDP counteracts several steps in the mechanism of ischaemia/reperfusion injury, a fact that makes it a very promising neuroprotective agent. The ultrastructural findings of paper III lend further support to the findings of previous studies suggesting significant neuroprotection. The present findings correlate well with the conventional histopathology of paper I, which is proof of the high quality of our data and interpretation. Conversely, this correlation increases the value of the histopathological results, as the findings support each other.

The number of animals considered in paper III is rather low, partly because only 5 animals in the control group survived for seven days postoperatively. We wanted the groups to be as comparable as possible and therefore selected only animals which had survived for the full seven days. Also, electron microscopy is time-consuming and requires highly specialized personnel, as well as being relatively expensive. The low number of cases prevents us from drawing any major conclusions based on the present material alone, but the analysis still points to a clear difference in neuronal scores between the groups, indicating that FDP was successful in preserving the neuronal ultrastructure.
7 Conclusions

The present research using a randomized, controlled and blinded design in a surviving porcine model generated the following results:

5. The high-energy intermediate of glycolysis, fructose-1,6-bisphosphate, improved the neurological outcome after HCA and alleviated the histopathological brain injury. Microdialysis indicated that the beneficial effect was mediated by improved brain metabolism during and after HCA. Lower free Ca\(^{2+}\) concentrations in the plasma may also have had some effect.

6. The microdialysis samples revealed that fructose-1,6-bisphosphate also improved cerebral metabolism in the presence of irreversible ischaemic injury to the brain caused by particle embolization. The neurological outcome was not improved, nor was the histopathological injury alleviated, probably due to the irreversible nature of the vessel occlusion. The number of animals may have been too small to generate significant differences between the groups in terms of the primary end-points.

7. The action of fructose-1,6-bisphosphate in protecting the brain was confirmed by electron microscopy, as the ultrastructures in the cerebral samples were seen to have been preserved. The ultrastructural findings correlated well with conventional histopathology. FDP should be studied further, since it is beneficial with HCA.

8. Hypertonic saline dextran improved the neurological recovery and reduced the histopathological signs of brain injury after HCA. The better recovery was due to amelioration of the reperfusion injury and diminished oedema formation in the brain, leading to improved brain metabolism and oxygenation, as recorded by the microdialysis technique. Additional studies are recommended, since HSD seems to be advantageous with HCA.

The general hypothesis was confirmed in papers I, III and IV, as significant differences were found between the groups. In paper II, however, the general hypothesis was not confirmed, as no differences were found between the groups in the primary end-points, although some cerebral metabolic parameters favoured the FDP group.
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