Management of Ischemia and Brain Death-Associated Injuries in Porcine Kidney Grafts

AMIR SEDIGH
Dissertation presented at Uppsala University to be publicly examined in Gustavianum Auditorium Minus, Akademigatan 3, uppsala, Saturday, 31 May 2014 at 09:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in Swedish. Faculty examiner: Professor Styrbjörn Friman (Avdelningen för kirurgi, Institutionen för kliniska vetenskaper, Sahlgrenska akademin, Göteborgs universitet).

Abstract

Organs from deceased donors after brain death (BD) remain the major source of organs for transplantation. The catastrophic event of BD and the inevitable consequences of ischemia reperfusion injury (IRI) are linked to impaired graft quality and transplantation outcome. The aim of this thesis was to create a BD model in pigs to assess early effects on IRI in kidneys preserved with an oxygenated solution and to evaluate the protective effects of coating the renal vessel walls with a heparin conjugate during hypothermic machine perfusion (HMP).

Brain death was achieved by raising the intracranial pressure (ICP) through stepwise increasing the volume of an epidurally placed balloon to the point of exceeding the mean arterial pressure (MAP) creating a negative cerebral perfusion pressure (CPP). This reproducible, clinically relevant experimental model makes evaluation of potential targeted methods to protect the organs possible. Kidneys retrieved from brain-dead pigs were preserved either in an oxygenated emulsion composed of 75% histidine-tryptophan-ketoglutarate (HTK) and 25% perfluorohexyloctane F6H8 or HTK alone. After 18h of cold storage the kidneys were transplanted into allogeneic pigs. F6H8 was associated with replenishment of adenosine triphosphate and lower gene expression of hypoxia inducible factor (HIF)-1α, vascular endothelial growth factor (VEGF), interleukin (IL)-1α and tumour necrosis factor (TNF)-α. F6H8 reduced early IRI at both the cellular and molecular level.

Kidneys from BD pigs were evaluated for the feasibility of coating the vessel walls with the heparin conjugate CHC (Corline Systems AB, Uppsala, Sweden) to restore glycocalyx. Porcine kidneys were preserved by HMP for 20h with 50 mg biotinylated CHC added to the perfusion solution. CHC was detected on the inner surface of the kidney vessels by immunofluorescence, and its uptake in kidneys was confirmed by reduced content in the perfusate. An ex vivo normothermic perfusion circuit was developed to assess kidney function. Perfusion with CHC during HMP was associated with lower creatinine levels, increased urine volume and reduced tubular injury. Modifying renal vessels walls using CHC during HMP improved early graft function. Preservation with the oxygenated F6H8 solution or CHC could be used to improve graft quality and ameliorate IRI in kidneys retrieved from deceased donors.

Amir Sedigh, Department of Surgical Sciences, Transplantation Surgery, Akademiska sjukhuset, Uppsala University, SE-75185 Uppsala, Sweden.

© Amir Sedigh 2014

ISSN 1651-6206
ISBN 978-91-554-8939-7
urn:nbn:se:uu:diva-222020 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-222020)
To My Family, Sophie, Michael, Emma and Cecilia
Studying the brain gives you nothing, unless you study it in relationship to the environment!
List of Papers

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

I Karlis Purins, Amir Sedigh, Christian Molnar, Leif Jansson, Olle Korsgren, Tomas Lorant, Gunnar Tufveson, Lars Wennberg, Lars Wiklund, Anders Lewén, Per Enblad

* Equal contribution.


IV Amir Sedigh, Sofia Nordling, Rolf Larsson, Erik Larsson, Bo Norlin, Norbert Lübenow, Fredrik Lennmyr, Gunnar Tufveson, Peetra U Magnusson, Tomas Lorant.
Perfusion of porcine kidneys with macromolecular heparin ameliorates early ischemia reperfusion injury. (Manuscript)

Reprints were made with permission from the respective publishers.
Contents

Introduction .................................................................................................................. 11
Types of donors ........................................................................................................... 13
  Donation after brain death (DBD) ................................................................. 13
  Extended criteria donors (ECD)................................................................. 14
  Donation after cardiac death (DCD) .......................................................... 14
  Controlled DCD ....................................................................................... 14
  Uncontrolled DCD .................................................................................... 14
Multiple-organ retrieval ........................................................................................... 15
Pathophysiology of brain death ............................................................................. 16
Organ preservation ................................................................................................. 17
  History of hypothermic perfusion .............................................................. 18
  Preservation solution .................................................................................. 20
  Static cold storage (CS) ........................................................................... 21
  Hypothermic machine perfusion (HMP) .................................................. 21
  Oxygenation during cold storage ............................................................... 23
  Normothermic machine perfusion (NMP) .................................................. 23
Ischemic reperfusion injury (IRI) ......................................................................... 24
  Molecular and cellular events associated with IRI .................................. 25
  Immunogenicity of the transplant organ ...................................................... 26
  Oxygen free radicals ................................................................................. 26
Acute kidney injury (AKI) and delayed graft function (DGF) .......................... 26
Endothelial glycocalyx (GCX) ............................................................................. 28
Aims ......................................................................................................................... 29
Materials and Methods ......................................................................................... 30
  Ethics (papers I-IV) .................................................................................... 30
  Animals and anaesthesia (papers I-IV) ....................................................... 30
  Induction of brain death (papers I-IV) ........................................................ 31
  Validation of the brain death model (paper I) .......................................... 32
Surgery ....................................................................................................................... 32
  Kidney recovery (papers II-IV) ................................................................. 32
  Kidney transplantation (paper II) .............................................................. 33
Kidney Storage ........................................................................................................ 33
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen-presenting cells</td>
</tr>
<tr>
<td>ATN</td>
<td>Acute tubular necrosis</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BD</td>
<td>Brain death</td>
</tr>
<tr>
<td>BE</td>
<td>Base excess</td>
</tr>
<tr>
<td>$B_iP_2$</td>
<td>Brain tissue oxygenation</td>
</tr>
<tr>
<td>CHC</td>
<td>Corline heparin conjugate</td>
</tr>
<tr>
<td>CPP</td>
<td>Cerebral perfusion pressure</td>
</tr>
<tr>
<td>Cr</td>
<td>Creatinine</td>
</tr>
<tr>
<td>CS</td>
<td>Cold Storage</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DBD</td>
<td>Donation after brain death</td>
</tr>
<tr>
<td>DCD</td>
<td>Donation after cardiac death</td>
</tr>
<tr>
<td>DGF</td>
<td>Delayed graft function</td>
</tr>
<tr>
<td>DIC</td>
<td>Disseminate intravascular coagulation</td>
</tr>
<tr>
<td>ECD</td>
<td>Extended criteria donor</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>$F_2H_8$</td>
<td>Perfluorohexyloctane</td>
</tr>
<tr>
<td>Gcx</td>
<td>Endothelial glycocalyx</td>
</tr>
<tr>
<td>GRP</td>
<td>Glucose related protein</td>
</tr>
<tr>
<td>HES</td>
<td>Hydroxyethyl starch</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia inducible factor</td>
</tr>
<tr>
<td>HMP</td>
<td>Hypothermic machine perfusion</td>
</tr>
<tr>
<td>HR</td>
<td>Heart rate</td>
</tr>
<tr>
<td>HS</td>
<td>Heparan sulphate</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>HTK</td>
<td>Histidine-tryptophan-ketoglutarate</td>
</tr>
<tr>
<td>IC</td>
<td>Intracranial compliance</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intracellular adhesion molecule</td>
</tr>
<tr>
<td>ICP</td>
<td>Intracranial pressure</td>
</tr>
</tbody>
</table>
IFN Interferon
IL Interleukin
im Intramuscular
IRI Ischemia reperfusion injury
iv Intravenous
MAP Mean arterial pressure
MW Molecular weight
NMP Normothermic machine perfusion
NP Normothermic perfusion
NV Neurovent
PAS Periodic acid-Schiff
PEG Polyethylene glycol
PFC Perfluorocarbon
PNF Primary non-function
RBF Renal blood flow
RT-PCR Real-time polymerase chain reaction
ROS Reactive oxygen species
RR Renal resistance
SCD Standard criteria donors
SD Standard deviation
SEM Standard error of the mean
TB Toluidine blue
TEG Thromboelastography
TLR Toll-like receptor
TNF Tumour necrosis factor
UFH Unfractionated heparin
UW University of Wisconsin solution
VCAM Vascular cell adhesion molecule
VEGF Vascular endothelial growth factor
vWF Von Willebrand factor
Introduction

The improvement of organ transplantation took a major step forward with the development of the clinical diagnosis of brain death, which was first described by the Harvard Ad Hoc Committee on Brain Death in 1968. This led to a growing number of deceased donor organs becoming available for transplantation. In the early years of organ transplantation, however, the short- and long-term effects of brain death (BD) on the quality of donor organs were not fully recognized. Donor selection was based on the premise that only high-quality organs could be considered for transplantation, and the importance of donor maintenance and organ preservation was underestimated. Due to a significant and growing organ shortage, a gradual shift has taken place. As a result, utilization of older donors with comorbidities has intensified and become common practice. Further, many centers are revisiting donation after cardiac death as a way to expand the pool of deceased donors.

Clinical observations and experimental research have revealed the direct organ injury associated with the onset of BD. Triggered by marked physiological instability, such injuries can lead to deterioration of organ function prior to organ retrieval, and consequently harmful effects on the transplanted organs. The accelerated immune responses in the organs recovered from BD donors have been linked with exposure to a pro-inflammatory milieu and ischemic injury in the context of BD. As a result, organs from BD donors seem to experience more episodes of rejection after transplantation. The consistent inferiority, as well as the different outcomes of such organs, exemplifies the effects of diseased-donor organ quality in comparison to organs derived from living donors. The different phases of organ transplantation from the ischemic phase, through the reperfusion phase, are accompanied by cell and tissue damage. Pathophysiological processes associated with graft injuries, defined as ischemia/reperfusion injury (IRI) and constituting the most important antigen-independent factors linking other intracellular events, orchestrate many of processes involved in delayed graft function (DGF), representing acute kidney injury in the immediate postoperative period after transplantation, primary non-function (PNF), long term graft dysfunction and impaired graft survival. The events surrounding brain
death, occurring before organ removal, ischemic preservation and ultimately the reperfusion phase in the recipient may initiate an inflammatory response, which in turn may acutely increase host immunological activity. The observation that IRI entails risk factors even for delayed immunological sequelae and late allograft failure suggests that long-term changes may be programmed early on in the process. Organs from older and suboptimal donors may not only demonstrate a compromised physiological capacity but also a limited capacity to repair IRI. The accelerated immune responses in the organs recovered from older donors have been associated with changes due to aging per se and exposure to a pro-inflammatory milieu in the context of brain death. 

Deceased donor management is of critical importance and has been cited as one of the most neglected areas in transplantation medicine. The negative effect on organ quality caused by the abnormal physiological state and ongoing inflammation associated with brain death needs to be avoided. Future expansion of organ transplantation demands new approaches. One major area of advancement is the modulation of an organ during its ischemic transfer from donor to recipient. Pre-emptive treatment strategies and reversal of the detrimental effects of brain death and preservation injury on existing impaired organs has an immense potential. There is, however, a growing awareness that the graft is not only the target, but may also trigger the host immune response. Graft quality and immunogenicity determines, at least in part, success of organ transplantation. This calls for a change of philosophy and therapy in order to positively influence the quality of the organs from deceased donors prior to transplantation. Such strategies should be delivered reliably and safely in order to improve both early and long-term outcome. Suppression of IRI by applying strategies to prevent these deleterious processes may possibly improve both short- and long-term graft survivals.

Figure 1. Flow diagram of organ storage from donation to transplantation.
Understanding the mechanisms of brain death, with respect to IRI, may allow us to define novel targets for managing organ quality, ways to manipulate immunogenicity, and provide a conceptual framework of specific aspects of preservation, which may be adapted for suboptimal donors.

Types of donors

An allograft is a transplant of an organ or tissue between two genetically non-identical members of the same species. Most human tissue and organ transplants are allografts. Organs may be retrieved from deceased or living donors but the majority of organ supply comes from deceased donor. The various categories of deceased donors are described below.

Donation after brain death (DBD)

Donation after brain death is formally known as donation from heart-beating donors. It includes ICU patients who have irreversible loss of brain and brainstem function and who fulfill the legal criteria for brain death, which was first described by the Harvard Ad Hoc Committee on Brain Death in 1968.

Brain death can be the end result of major trauma due to intracerebral hemorrhage or a cerebrovascular event leading to cerebral ischemia and/or compression due to hypoxia or metabolic deregulation. The diagnosis of BD is based on repeated comprehensive neurological evaluation coupled with the absence of brainstem reflexes and an apnea test under standardized conditions. This includes unawareness of and unresponsiveness to external stimuli, no spontaneous movements or no response to pain, and no cranial nerve reflexes. The reflexes absent include pupillary response (fixed pupils), oculocephalic reflex and corneal reflex, as well as no response to the caloric reflex test and no spontaneous respiration.

A complementary test such as electroencephalography, transcranial duplex sonography or cerebral angiography is required in cases of uncertainty during the clinical examination. The heart is beating and the intra-thoracic and intra-abdominal organs are perfused with the donor’s own oxygenated blood until procurement. Brain-dead donors can further be divided into standard criteria donors (SCDs) and extended criteria donors (ECDs).
Extended criteria donors (ECD)

ECDs are diseased donors who have characteristics beyond the standard criteria for organ donation. An ECD is defined by a donor of over 60 years or of an age between 50 and 60 years combined with additional risk factors such as cerebrovascular cause of death, a history of hypertension, or a final serum creatinine above 132 μmol/L. There is a significantly higher risk of graft failure with organs from ECDs as compared to SCDs 25,26.

Donation after cardiac death (DCD)

The concept of DCD is not new. In the early days of organ transplantation, all organs from diseased donor were recovered from donors after cardiac death 27. This concept was replaced after the concept of brain death was established 1. Donors after cardiac death or non-heart-beating donors constitute a heterogeneous group of deceased donors with a common characteristic being that the organs are recovered after cessation of circulation due to cardiac arrest. Death is declared on classic cardiocirculatory criteria instead of neurologic criteria. Four sub-categories of DCD donors were defined according to a consensus on donor management reached in Maastricht in 1995 (Table 1) 28.

Controlled DCD

Controlled DCD donors are usually ICU patients with global neurological injuries but simultaneously functioning brainstem, but who maintain spontaneous ventilation whereby they do not meet the criteria for brain death. After comprehensive medical evaluation in conjunction with a dialogue with the family, a consensus is reached that there is no possibility of recovery and a withdrawal of therapy may be planned. Organ recovery can occur immediately following the cessation of the heart.

Uncontrolled DCD

These donors are patients who have died suddenly and unexpectedly of a cardiac event or from trauma and are hence generally in an emergency department. Following an unsuccessful attempt to resuscitate, the patient is declared dead. After a few minutes or a “hands-off period”, organ recovery can take place.
Table 1. Maastricht classification of donors after cardiac death

<table>
<thead>
<tr>
<th>Category</th>
<th>Description</th>
<th>Organ recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Dead on arrival</td>
<td>Uncontrolled</td>
</tr>
<tr>
<td>II</td>
<td>Unsuccessful resuscitation</td>
<td>Uncontrolled</td>
</tr>
<tr>
<td>III</td>
<td>Awaiting cardiac arrest (withdrawal of treatment)</td>
<td>Controlled</td>
</tr>
<tr>
<td>IV</td>
<td>Cardiac arrest while brain dead</td>
<td>Uncontrolled</td>
</tr>
</tbody>
</table>

Multiple-organ retrieval

After declaration of brain death, in combination with the informed consent from the next of kin, a deceased person may be identified as an organ donor and the organs may be retrieved. The multiple organ retrieval method enables the removal of the heart, lungs, kidneys, liver, intestine and pancreas from a single donor for transplantation into six or more recipients. The process can be categorized in several subsequent stages: incision, exploration and control, mobilization of each organ, in situ perfusion, removal of the organs, and closure of the incision. The final steps include the back-table processing, packaging and transport of the organs to the recipient centers. The principles are the same regardless of which organ is removed. Wide exposure and little mobilization of each organ is necessary up to disconnection from the circulation while the heart is still beating.

Maintaining physiological stability during the procedure allows unhurried removal of the organs in optimum state. A complete midline incision from the suprasternal notch to the pubic bone is made, even if thoracic organs are not to be recovered. Preparation for in situ perfusion consists of exposure of the abdominal aorta from the iliac bifurcation distally, to the level of the left renal vein proximally. The inferior vena cava is similarly exposed.

Liver mobilization is carried out first, since this is the most meticulous dissection and attention to hemostasis is strict. The left triangular ligament of the liver is dissected down in order to expose the crural muscle at the aortic hiatus. The aorta is encircled above the celiac axis. If the pancreas or small bowel are to be removed, their dissection likewise proceeds first. Careful inspection of the arterial anatomy is carried out first, and any anomalous arteries must be preserved. First, cannulas are placed in the inferior vena cava and aorta at their respective bifurcations and the vessels divided distally. A single dose of heparin (300 units/kg) is then given intravenously to prevent blood clotting. As soon as the proximal aorta is cross-clamped, in situ perfusion begins through the distal aorta, thereby cooling the organs.
immediately. The organs are retrieved in the following order: the heart and lungs, liver, pancreas, and then the kidneys.

Pathophysiology of brain death

Brain death is the final stage of a series of events that frequently starts with cerebral trauma or cerebrovascular bleeding. These cascades of events trigger hemodynamic, hormonal, metabolic and inflammatory changes, all of which influence the quality and immune activation of the potential donor organs. Once the patient has been declared brain dead, these sequences of events have already affected the organs.

The key to the successful results with organ transplantation is the individual evaluation of the risk index of donors\(^2,31,32\). Systemic and hormonal changes occur directly when the intracranial pressure increases. Thus, brain death is not a solid state as perceived from the outside, but a dynamic, non-physiological course of events affecting a number of pathophysiological processes in the human body\(^18\).

Brain death is usually preceded by a variable period of increasing intracranial pressure (ICP) due to haemorrhage or brain oedema\(^33\). Altered intracranial volume affects venous outflow thereby speeding up the increase in pressure until brain structures are pushed toward to the foramen magnum. This causes herniation of the brain stem through the foramen magnum, which is associated with completely halting of the arterial blood flow and represents a progressive ischemic injury of the central nervous system. It begins supratentorially and proceeds caudally to involve the crucial brain-stem regions of the pons and medulla, eventually reaching the spinal cord.

When the entire brainstem has become ischemic, there is a systemic release of catecholamine, causing an increase in heart rate and vasoconstriction with increased vascular resistance and blood pressure\(^34,35\). The uncontrolled sympathetic activation with increased arterial blood pressure and tachyarrhythmia is followed by a much longer hypotensive period resulting from sympathetic insufficiency, loss of vascular tonus and decreased peripheral resistance\(^36,37\). These are associated with central redistribution of blood volume, increased afterload, and visceral ischemia\(^38\). Sympathetic stimulation, together with the continuing parasympathetic activity, triggers the Cushing reflex, which was previously described in 1902 by the American neurosurgeon Harvey Cushing, including bradycardia, hypertension and irregular breathing\(^18\). Elevated pulmonary hydrostatic pressure results in exacerbated pulmonary edema and is maintained by a capillary endothelial injury triggered by endogenous norepinephrine. If ventilation is not support-
ed, respiratory distress proceeds to apnea and cardiac arrest. Endocrine changes during brain death vary in degree and severity. The function of the posterior pituitary is commonly lost, leading to diabetes insipidus with associated fluid and electrolyte changes. Thyroid hormonal changes may lead to euthyroid sick syndrome. Insulin concentrations decrease, insulin resistance, develops and hyperglycemia is common. The function of the hypothalamus and the control of body temperature are disrupted. Hyperpyrexia occurs first, followed by hypothermia resulting from reduced metabolism and muscle activity in combination with peripheral vasodilatation.

Coagulopathy arises from the release of tissue factor and thromboplastin from necrotic brain tissue, which contributes to disseminated intravascular coagulation (DIC) in donors. The immunological activity that occurs in conjunction with brain death comprises an up-regulation of multiple cytokines and chemokines such as interleukin (IL)-1, IL-2, IL-6, tumor necrosis factor (TNF)-α, interferon (IFN)-γ and vascular endothelial growth factor (VEGF), triggering the immunological response. Amplification of cytokines, chemokines and adhesion molecules results in a chemotactic gradient that promotes infiltration of leukocytes into the organs. T lymphocytes, macrophages and polymorphonuclear neutrophil granulocytes are all found in increasingly quantities in peripheral organs during brain death.

Organ preservation

Knowledge on the preservation of the kidneys has enabled transplantation of the heart, liver, pancreas, lung and small intestine. It is essential to understand basic cell and organ pathophysiology during ischemia and organ dysfunction in order to understand the rationale and effects of varying preservation strategies. Upon organ retrieval, the interrupted circulation leads to ischemia and concomitant depletion of oxygen and nutrients in the organs. This early event promptly leads to a shift from aerobic to anaerobic metabolism, and in turn the production of lactate and development of an acidic environment. Early in this cascade, cell depolarization occurs, which leads to deranged ion homeostasis, a rise in intracellular concentration of protons and calcium, and a combination of other intracellular and membrane-associated events that cause cell death by either apoptosis or necrosis.

The aim of organ preservation is thus to preserve and restore the microvascular, cellular and functional integrity of the organ during the ischemic period. Hypothermia reduces the damaging effects of ischemia by decreasing the metabolic rate and slowing enzymatic degradation of cellular components,
but metabolism is not completely suppressed\textsuperscript{6,60,61}. Energy consumption due to metabolic activity is not halted, only reduced. Cooling an organ from 37 °C to approximately 0 °C slows metabolism by a factor of 12-13. Cooling directly affects plasma membrane lipids, the cytoskeleton and mitochondria by deactivating ion exchange pumps in the cell membrane that cause swelling and cell lysis.

Table 2. Features of ideal preservation

<table>
<thead>
<tr>
<th>Feature</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Longer preservation time</td>
<td>Optimum recipient matching and preparation, long distances, and rested surgical teams</td>
</tr>
<tr>
<td>Reduction of ischemic injury</td>
<td>Limitation of organ damage arising in connection with brain death and during organ retrieval</td>
</tr>
<tr>
<td>Graft and viability assessment</td>
<td>Objective assessment of organ function prior to transplantation to minimize the risk for the organ intended for transplant being discarded</td>
</tr>
<tr>
<td>Intervention</td>
<td>Possibility for intervention and immunomodulation therapy for preservation</td>
</tr>
<tr>
<td>Handling</td>
<td>Technology should be easy to use and demand minimal logistics for organ retrieval teams</td>
</tr>
<tr>
<td>Cost-effectiveness</td>
<td>Increased cost must be proportionate to the increased number of transplants and outcome benefits</td>
</tr>
</tbody>
</table>

The organs are perfused with a special preservation solution to reduce cell swelling and maintain a physiologically intracellular ionic composition\textsuperscript{62-64}. Improvements in preservation have enabled the extension of preservation times, which have further provided logistic benefits and facilitated organ sharing across geographical boundaries. To date, hypothermic preservation is the most widely used method of preservation in clinical practice. Reliance on hypothermia to reverse the effects of ischemia during storage has traditionally involved two types of preservation: simple static cold storage (CS) and hypothermic machine perfusion (HMP).

History of hypothermic perfusion

The ability to maintain organ function outside the body has challenged researchers for more than two centuries and has played a major role in understanding many basic physiological principles. A technological breakthrough was the development of a pump system to maintain a controllable perfusate
flow. Alexis Carrel investigated the effectiveness of tissue preservation under both hypothermic and normothermic conditions. He discovered that cooling allowed the tissue to maintain viability for longer periods compared to normal temperatures, though cooling did have its limitations, and over time the viability was lost. In 1935 Carrel developed, together with Charles Lindberg, the Swedish engineer and pilot, the first organ perfusion device in order to improve tissue viability with maintained functional state. In 1935 Carrel developed, together with Charles Lindberg, the Swedish engineer and pilot, the first organ perfusion device in order to improve tissue viability with maintained functional state. In 1935 Carrel developed, together with Charles Lindberg, the Swedish engineer and pilot, the first organ perfusion device in order to improve tissue viability with maintained functional state.

Figure 2. Organ perfusion pump developed by Carrel and Lindberg in 1935.

The arrival of clinical transplantation as a therapeutic reality in the 1960s called for a reconsideration of isolated perfusion for organ preservation. The benefit of hypothermia was established in the maintenance of renal function and in the reduced demand for oxygenation.

In the early 1960s, Folkert Belzer worked on different hypothermic perfusion techniques for preserving kidneys. Initially, whole blood was utilized as perfusate. His research showed that micro-filtered cryoprecipitated plasma yielded longer preservation times. By 1967, a combination of continuous perfusion and hypothermic storage brought organ preservation to a new level. Belzer describes the successful preservation of dog kidneys over 72 h using oxygenated cryoprecipitated plasma and pulsatile perfusion. Belzer was the pioneer who developed one of the first reliable transportable hypothermic machine perfusion processes and fully understood the future potential of HMP within the broader field of transplant services.
Preservation solution

Different solutions are used for organ preservation, and the most common critical factor is to prevent cellular edema and to produce an appropriate biochemical environment to delay cell destruction and to maximize organ function after reperfusion. Impermeants are agents that remain outside the cells and preserve an extracellular osmotic pressure, which delay the accumulation of water inside the cells (Table 3). Efficient impermeants are saccharides and non-saccharide anions. Hence, impermeants and colloids are added to preservation solutions to prevent cell swelling.

The molecular weight (MW) determines the efficiency of a saccharide to prevent cell swelling. The larger the saccharide – the more effective it is. Non-saccharide impermeants, such as negatively charged gluconate, citrate, and lactobionate, limit cell swelling by electrochemical means. In these anions, MW and charge determine the efficiency. Since impermeants are effective mainly at the cell membrane level, colloids such as hydroxylethyl starch (HES) to prevent interstitial edema are used for the intravascular compartment.

During CS, anaerobic metabolism of glucose results in lactic acid that leads to acidosis. Severe acidosis activates phospholipases and proteases causing lysosomal damage and eventually cell death through apoptosis or necrosis. Adequate correction of the pH by adding a buffer such as phosphate or histidine to the preservation solution prevents intracellular acidosis. There is evidence that CS per se promotes the production of reactive oxygen species (ROS), and this is probably due to mitochondrial damage. Preservation solutions are therefore designed to counteract ROS-mediated injury during preservation and particularly at the time of reperfusion. Antioxidant scavenger compounds, such as allopurinol, tryptophan or glutathione are added to preservation solutions to prevent the formation of ROS.

Further, a balanced electrolyte composition with either high or low sodium (Na+)/potassium (K+) ratio is intended to counteract cellular swelling. Solutions with a high concentration of potassium are classified as intracellular solutions and those with a high concentration of sodium are classified as extracellular solutions. Intracellular type solutions such as University of Wisconsin Solution (UW) were long considered to be pivotal for preservation of cell viability. Recent work, however, has proposed similar results from extracellular-type solutions with a low potassium/high sodium ratio, such as histidine-tryptophan-ketoglutarate solution (HTK).
Table 3. Common composition of organ preservation solutions

<table>
<thead>
<tr>
<th>Category</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Impermeants</td>
<td>Glucose, lactobionate, mannitol, raffinose or sucrose.</td>
</tr>
<tr>
<td>Electrolytes</td>
<td>Calcium, chloride, magnesium, potassium and sodium.</td>
</tr>
<tr>
<td>Buffers</td>
<td>Citrate, histidine, K$_2$HPO$_4$, KH$_2$PO$_4$, NaHCO$_3$, NaH$_2$PO$_4$ and NaHCO$_3$.</td>
</tr>
<tr>
<td>ROS scavenger</td>
<td>Allopurinol, glutathione, mannitol, tryptophan and histidine.</td>
</tr>
<tr>
<td>Colloid</td>
<td>Hydroxyethyl starch (HES) and polyethylene glycol (PEG).</td>
</tr>
<tr>
<td>Additives</td>
<td>Adenosine, glutamic acid and ketoglutarate.</td>
</tr>
</tbody>
</table>

Static cold storage (CS)

Until now static cold storage has been considered the gold standard of organ preservation methods in the majority of transplant centers worldwide. By flushing the organs with cold preservation solution, blood is removed and replaced by a suitable preservative solution. The organ is thereafter placed in a sealed bag or canister immersed in preservation solution and then placed in an icebox. The benefits of simple cold storage are its universal availability and the ease of transportation. CS has contributed to the establishment of many transplant programs worldwide.

Hypothermic machine perfusion (HMP)

HMP is based on a continuous recirculation of cold preservation solution through the vascular system that is achieved by a device that generates either a continuous or an ongoing pulsatile flow. The flow provides complete perfusion of the organ. It promotes extensive removal of blood and the tissue becomes balanced with the preservation solution.
Machine perfusion provides a continuous supply of nutrients to microcirculatory system while metabolic by-products and toxic substrates and free radicals may be flushed out (Fig. 3). It provides additional assessment of viability controls using parameters such as flow and resistance. It further provides a potential opportunity to improve organ quality through pharmacological and gene transfer manipulation in real time. In the era of utilizing a growing number of marginal donors, machine perfusion has garnered regained interest due to a number of beneficial effects (Table 4). A recent prospective multicenter trial showed that HMP led to a lower risk of delayed graft function and lower risk of graft failure of kidney transplants than with CS\textsuperscript{78-87}

Table 4. Benefits of hypothermic machine perfusion (HMP).

<table>
<thead>
<tr>
<th>Outcome benefits</th>
<th>Mechanistic utility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduced primary non-function and delayed graft function.</td>
<td>Patency of the vascular bed.</td>
</tr>
<tr>
<td>Permits use of ECD or DCD donors to increase the donor pool</td>
<td>Washout of metabolic by-products and toxins.</td>
</tr>
<tr>
<td>Improves graft survival</td>
<td>Assessment of viability checks.</td>
</tr>
<tr>
<td>Economic benefit and cost effectiveness.</td>
<td>Provides nutrients at higher metabolic rate.</td>
</tr>
</tbody>
</table>
Oxygenation during cold storage

Historically, oxygenation was considered an essential component of kidney preservation to support mitochondrial resynthesize of adenosine triphosphate (ATP) and to delay the injury process. However, with the introduction of modern preservation solutions oxygen was not thought to be a vital ingredient. Oxygen can be effectively administered by the use of artificial oxygen carriers (Fig. 4). Perfluorocarbons (PFC) are inert solutions that have a high capacity for dissolving oxygen. They release oxygen down a concentration gradient creating a highly oxygenated environment unaffected by temperature. The resulting solution is able to dissolve 20 to 25 times more oxygen than plasma at room temperature.

Figure 4. Hydrocarbon molecules in which hydrogen atoms have been replaced by fluorine.

Oxygen can be continuously added allowing adequate diffusion through the organ, which is particularly beneficial for pancreas preservation. It allows a sufficient amount of ATP to be generated to improve organ viability.

Normothermic machine perfusion (NMP)

Optimal oxygenation by means of blood and blood products as the oxygen-carrying media was an unobtainable challenge with previous technology due to clotting, vascular injury and infection. NMP has recently received regained focus because of the availability of newer solutions and perfusion technology. The NMP technique offers several advantages over conventional preservation techniques. In theory at least, the organs are restored to a physiological and aerobic metabolic state such that the IRI cascade is avoided before transplantation. It may also allow the repair of injuries sustained be-
fore retrieval, including warm ischemic damage, and provides for real-time assessment of viability and function as well as providing a platform for pharmacological and immunological modulation of allografts before transplantation. NMP is complex and its clinical implementation is more expensive than cold preservation. The NMP set-up requires a continuous supply of warm, oxygenated perfusate and any technical failure during preservation of a human organ can have devastating effects that can lead to a non-viable organ. The NMP concept requires having scientific proof that it is safer and more effective than current standard procedure.

Ischemic reperfusion injury (IRI)

Ischemia-reperfusion occurs in a wide variety of clinical situations, such as aortic cross-clamping in major vascular surgery, cardiopulmonary bypass, hemorrhagic shock, stroke, myocardial infarction, liver resection and organ transplantation. IRI in the setting of transplantation is a complex process. It includes a period of anoxia during cold preservation followed by reoxygenation after restoration of the blood flow after implantation, resulting in significant injury to the transplanted organ. IRI is the most significant antigen-independent insult affecting organs destined for transplant. Ischemia during transplantation has been divided into three consecutive phases. The first event involves a transient episode of ischemia in the donor caused by instability of the circulation due to hypotension and vasoconstriction, parameters associated with brain death. Retrieval of organs from donors requires the cessation of the vascular blood supply, which concomitantly depletes the oxygen and nutrient supply, leading to varying degrees of anoxic tissue damage to the organs. The second phase involves an extended cold ischemic phase associated with preservation and storage, and the third phase a period of relatively insignificant warm ischemia that takes place during revascularization of the graft. The ischemia period is not the major cause of the IRI but rather leaves the organ more susceptible to damage in the reperfusion phase, i.e. the reflow paradox. Reperfusion produces rewarming, reoxygenation, and a return to aerobic metabolism. However, reactive oxygen species are also generated in high concentrations in ischemic tissue after reperfusion. A set of mechanisms, collectively referred to as a “generalized inflammatory response”, causes direct tissue damage by initiating a cascade of deleterious cellular responses.
Molecular and cellular events associated with IRI

Elevated production of vasospasm agents such as endothelin$^{95}$ and increased leukocyte–endothelial cell and neutrophil-neutrophil interactions culminate in cell death either by apoptosis or necrosis$^{6,53-59,96}$. Reperfusion amplifies the injury by initiating an inflammatory cascade including oxygen free radicals, endothelial factors and leukocytes. In the initial phase, activation of leukocytes and the interaction between leukocytes and the endothelium takes place. The subsequent inflammation triggers the innate immunity, leading to apoptosis and necrosis of tissue cells in the late phase of IRI$^{97,98}$. The triggered immune response interferes with the microcirculation, causing attraction, activation, adhesion and migration of neutrophils (Fig. 5).

Vascular endothelial cells form the interface between circulating leukocytes and proinflammatory cytokines involved in promoting neutrophil and monocyte adhesion to endothelial cells. Ischemia increases tissue levels of inflammatory mediators such as tumour necrosis factor (TNF-α), cytokines and complement factors.$^{97,99}$ Several other inflammatory mediators are endothelium-derived such as platelet-activating factor (PAF). These mediators increase vascular permeability and leukocyte adhesion to the microvasculature. Selectins are expressed on endothelial cells in the early phase of ischemia. Cytokines are elevated, and IL-1 and TNF-α facilitate the adhesion of neutrophils, monocytes and lymphocytes to the endothelium.$^{99}$ After selec-
tin-mediated leukocyte rolling, the circulating cells are immobilized via a series of integrins and members of the immunoglobulin superfamily-intercellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM)-1, CD4 and CD8- that facilitate leukocyte migration across the endothelium. Up-regulated adhesion molecules thus orchestrate the recruitment of leukocytes to damaged tissues.

Immunogenicity of the transplant organ
Free radicals activate endothelial cells and leukocytes. This activation increases the immunogenicity of the graft by up-regulating the major histocompatibility complex (MHC) on cell surfaces and by releasing cytokines and growth factors in the tissue. IRI is thought to increase the risk of acute rejection through the effects of innate and adaptive immunity. A general inflammatory state triggers host alloresponsiveness during IRI, and the reperfusion causes endothelial injury that may later contribute to immunological events and the development of chronic graft injury.

Oxygen free radicals
The catabolism of ATP during the ischemic phase leads to accumulation of intracellular hypoxanthine. The conversion of hypoxanthine to xanthine is the generator of the simultaneous production of hydrogen peroxide ($H_2O_2$), the superoxide anion ($O_2^-$) and uric acid. When $H_2O_2$ is catalyzed by iron, it generates a cytotoxic hydroxyl radical that initiates lipid peroxidation of the cell membrane. During reperfusion of an anoxic tissue, oxygen-derived free radicals are generated in high concentrations. The unpaired electrons on free radicals bind immediately to many molecules in the neighborhood, e.g. cell membrane components, and induce damage to structural proteins and DNA. The cytotoxic effect of reactive oxygen species results in cell dysfunction, cell death by apoptosis or necrosis, and irreversible tissue damage.

Acute kidney injury (AKI) and delayed graft function (DGF)
The initial delayed graft function is the best-known manifestation of IRI in the context of renal transplantation, and represents a major challenge to clinicians. DGF is defined as a temporary divergence between the functional
capacities of the engrafted kidney to support the physiological demands of the recipient. The severity of DGF depends on many variables, such as donor and recipient characteristics, tissue injury caused prior to retrieval and preservation. Delayed function leads to the necessity for dialysis post-transplantation and, most commonly, uncertainty regarding a simultaneous immunological event requiring invasive or non-invasive diagnostics, prolonged hospitalization, delayed rehabilitation and higher costs.\textsuperscript{78,104}

![Diagram of IRI triggers](image)

**Figure 6.** IRI triggers shedding of the tubular brush border and disruption of cell-matrix adhesion molecules and tight junctions.

It is clear that endothelial ischemia results in cell damage and swelling. The impaired blood flow resulting from increased renal vascular- and afferent arteriolar resistance reduces the glomerular filtration rate and reperfusion. Blood flow to the outer medulla is disproportionately reduced in relation to the reduction in total kidney perfusion in animal models of AKI\textsuperscript{105,106} and most likely also in human kidneys following ischemic injury. An overall decrease in renal blood flow (RBF) of approximately 40 to 50% has been observed in poorly functioning kidney transplants\textsuperscript{107}.

Renal tubular tissue is particularly vulnerable to ischemia-induced hypoxic injury, triggering the shedding of the tubular brush border.\textsuperscript{77} Cytokines induce disruption of cell-matrix adhesion molecules and tight junctions fol-
lowing ischemia (Fig. 6). The swelling of the tubular epithelial cells may also decrease blood flow through the vasa recta, ultimately causing further ischemia. These classic events known as acute tubular necrosis (ATN) are caused by back pressure from overloaded tubules that have been blocked by cell debris due to the ischemic insult.

### Endothelial glycocalyx (GCX)

Endothelial glycocalyx (GCX) covers the luminal surface of a healthy vascular endothelium. Its principal components are transmembrane and membrane-bound macromolecules such as syndecans and heparan sulfate. In vivo the GCX binds plasma proteins, forming a surface layer in microvessels with a thickness of 500 to 1000 nm. GCX orchestrates several endothelial cell functions, including modulation of vascular rheology, vascular permeability and regulation of inflammation. The combination of inflammation and intravascular oxidative stress lead to endothelial dysfunction and GCX degradation appears to be an important component. Oxidative degradation of GCX during IRI results in increased adhesion of circulating inflammatory cells to the endothelium, activation of the coagulation cascade and constriction of the microcirculation.
Aims

I To create a reproducible and clinically relevant model for brain death induction in pigs, and to improve overall understanding of the pathophysiological processes that occur during brain death.

II To study the potential protective effects of a newly developed organ preservation solution composed of histidine-tryptophan-ketoglutarate (HTK) solution and oxygen-precharged F6H8 on early IRI in kidneys from brain dead pigs.

III To assess the feasibility of coating the renal vessel walls with the macromolecular heparin conjugate CHC during HMP in the porcine brain death model.

IV To establish an ex vivo normothermic perfusion model for porcine kidneys, and to functionally evaluate the protective effect against IRI by coating the renal vessel walls with CHC.
Materials and Methods

Ethics (papers I-IV)

All studies (I-IV) were consistent with the guidelines for the use of laboratory animals published by the Swedish National Board for Laboratory Animals and European Convention of Animal Care. Qualified personnel handled the pigs. All of the studies were also approved by the regional ethics committee for animal experiments in Uppsala, Sweden.

Animals and anaesthesia (papers I-IV)

Male white landrace pigs (paper I n=6; paper II donors n=6 and recipients n=12; paper III n=6; and paper IV n=6) were included. The pigs were aged 10 to 12 weeks and with a weight of 24 to 42kg.

At arrival to the laboratory animals were premedicated with an intramuscular (im) injection of 2.2 mg/kg xylazine (Rompun; Bayer, Leverkusen, Germany) in combination with zolazepam 6 mg/kg (Zoletil; Virbac, Carros, France). A peripheral intravenous line was set up through an ear vein and used for induction and maintenance of anaesthesia. Anaesthesia was induced with 20 mg of morphine (Meda, Solna, Sweden) and 25 mg of ketamine (Ketaminol; Intervet AB, Sollentuna, Sweden) and then the pigs were intubated. Anaesthesia was maintained with a continuous intravenous (iv) infusion of pentobarbital (Apoteket, Uppsala, Sweden) 8 mg/kg/h, morphine 0.5 mg/kg/h and pancuronium bromide 0.25 mg/kg/h (Pavulon, Organon, Oss, The Netherlands). After endotracheal intubation and mechanical ventilation (Servo 900C; Siemens-Elema, Solna, Sweden), all animals were secured in the prone position. The ventilation was set to 30% oxygen in air and adapted to maintain a physiologic PCO$_2$ of between 4.5 and 5.5 kPa (34–41 mm Hg). The capnogram and peripheral oxygen saturation were displayed continuously (CO$_2$SMO Plus-8100; Novametrix, Wallingford, CT). The subclavian artery was catheterized and connected to pressure transducers for continuous measurements of arterial pressure. The jugular vein was catheterised for maintenance of anaesthesia, fluid support and blood sampling. Samples of
arterial and jugular blood were collected regularly for blood gas analysis (ABL 5; Radiometer, Copenhagen, Denmark). Electrocardiography was used for continuous heart rate and rhythm monitoring. All physiologic data were simultaneously collected and recorded on a personal computer with commercial data collection software (Biopac Systems, Goleta, CA). After sharp skin incision and bladder incision, the bladder was catheterized with a Foley catheter (8Fr; Willy Rusch AG, Kernen, Germany). All animals received fluid support consisted of intravenous administration of Ringer-acetate (Fresenius Kabi, Stockholm, Sweden) 30 mL/kg during the first hour and a continuous infusion of 10 mL/kg/h thereafter.

Induction of brain death (papers I-IV)

A sagittal incision was made in the midline of the skull and two drill holes placed about 0.2 and 1 cm on either side of the midline. An epidural balloon catheter (14Fr Foley catheter; Willy Rusch AG) was placed through the left drill hole. Via the right drill hole a multiparameter Neurovent-PTO probe (Raumedic, Munchberg, Germany) was introduced for measuring intracranial pressure (ICP) and brain temperature (Fig. 7).

Figure 7. Schematic axial view of the pig’s skull presenting the burr holes for placing the Foley balloon catheter and Neurovent (NV) probe (Papers I-IV). Anterior (A), posterior (P), cerebral perfusion pressure (CCP), mean arterial pressure (MAP), intracranial pressure (ICP). CPP was calculated according to CPP=MAP-ICP.
The experiment began with a 30-min hands-off period to set a baseline for ICP (ICP₀) and physiological data. The Foley catheter was then filled step-wise every 20 minutes with 1 mL saline in order to increase the ICP. Cerebral perfusion pressure (CPP) was considered to have ceased when the ICP exceeded the mean arterial pressure (MAP) calculated according to CPP=MAP–ICP. This procedure took approximately 200 minutes to complete (Papers I-IV).

Validation of the brain death model (paper I)

The cessation of the cerebral blood flow was confirmed by injecting 2x10⁶ yellow fluorescent nonradioactive microspheres (Dye-Trak; Triton Microspheres, San Diego, CA) with a diameter of 10 μm were injected through a catheter with its tip in the left atrium to achieve sufficient mixing with the arterial circulation. 30 mins after microsphere injection, the animals were euthanized by an overdose of potassium intravenously. Thereafter, in five of six pigs, small pieces (3–5 grams) of the cerebrum, brain stem, and kidneys were removed, blotted and fixed in 4% (vol/vol) formaldehyde for 2 days. The number of microspheres in the samples was then calculated manually in randomly chosen sections in a microscope equipped with both bright and fluorescent light illumination. Brain death (BD) was determined by absence or negligible amounts of microspheres in the cerebral vessels.

Surgery

Kidney recovery (papers II-IV)

All surgical procedures were conducted under aseptic conditions. A total of 5000 U Heparin was given intravenously and then the pig abdomen was opened through a midline incision and both kidneys were isolated. Hilum of both kidneys were mobilized during an adequate haemostasis. The renal arteries were ligated at aorta and the renal veins were divided at the junction of the vena cava inferior. Ureters were isolated and divided close to the bladder.
Kidney transplantation (paper II)

After performing median laparotomy, the porcine renal artery and renal vein were anastomosed to the recipient pig’s distal aorta and inferior vena cava, respectively. The ureter was cannulated with a plastic baby feeding tube for urine collection (Fig. 8).

Figure 8. Allograft kidney transplant in recipient pig.

Kidney Storage

Cold storage (paper II):

After removal, the porcine kidneys were flushed with 150-200 mL of either cold histidine-tryptophan-ketoglutarate (HTK) solution (control group) or an oxygen-precharged F6H8-emulsion composed of 75% HTK and 25% perfluorohexyloctane. The F6H8-emulsion was oxygenated for 30 min at a flow rate of 500 mL/min. Oxygenation of the emulsion resulted in a partial pressure of oxygen of approximately 600 mmHg, as measured using the optic fiber sensor technique (Precision Sensing, Regensburg, Germany). Subse-
sequently, the kidneys were stored for 18 h in 200 mL of the corresponding preservation solution at 4-6 °C.

**Hypothermic machine perfusion (HMP) (papers III-IV)**

Kidneys (paper III n=5+5; paper IV n=6+6) were placed on two separate perfusion systems (LifePort®; Organ Recovery systems, Chicago, IL, USA) and the chambers were prefilled with 1L of kidney perfusion solution (KPS-1; Organ Recovery Systems). Corline heparin conjugate (CHC; Corline Systems AB, Uppsala, Sweden) is a macromolecular heparin that consists of 70 heparin molecules that are linked by specific covalent bonds to a straight carrier chain composed of an inert aliphatic amine. All kidneys were preserved using a pulsatile flow of recirculating KPS-1®. 50 mg CHC (Paper III, including 25 mg biotinylated CHC) was added to one of the HMP systems in the experimental group while control kidneys were perfused with KPS-1 with added 50 mg of unfractionated heparin (UFH; Paper III) or KPS-1 alone (Paper IV). The perfusion was conducted with a mean arterial pressure of 15 mmHg at a temperature of 1-4°C for 18h (Paper IV) or 20h (Paper III).

**Sample collection**

*Paper I*

Samples were taken from kidneys, liver, heart, and pancreas brain and brain stem at the time of death morphologic examination and to determine the number of microspheres

*Paper II*

After 18h of cold ischemia the kidneys were transplanted into allogeneic recipients and assessed for tissue ATP content, morphology and the expression of genes related to hypoxia, environmental stress, inflammation and apoptosis.

Tissue samples were collected from kidneys at the following time points:

1. After verifying BD but before perfusion (baseline),
2. Immediately after back-table cold perfusion
3. After 18 h of CS
4. 0h, 2h and 4h after transplantation and reperfusion in the recipient
The tissue samples were snap-frozen in liquid nitrogen and stored at -70°C until additional processing.

*Paper III*

1. One ml samples of the perfusate were taken 0 h, 1 h, 6 h, 12 h and 20 h after the start of perfusion.
2. Wedge biopsies were taken along the same timeline.

*Paper IV*

Blood and urine samples were collected at hourly intervals (1 h, 2 h and 3 h) for analyses of serum and urine creatinine. A urine osmolality analysis was used as a tubular function marker. The total urine volume was recorded at hourly intervals.

**ATP (paper II)**

Fine needle snap frozen biopsies, were taken from renal cortex, in all time points assessed for ATP content using the ATPlite kit (Perkin Elmer, Upplands Väsby, Sweden) according to the manufacturer’s instructions.

**Quantitative real-time polymerase chain reaction (RT-PCR) (paper II)**

Total RNA extraction and complementary DNA (cDNA) synthesis were performed using the RNeasy Mini Kit (Qiagen, Holden, Germany) and Superscript II Reverse Transcriptase Kit (Invitrogen, Stockholm, Sweden), respectively, according to the manufacturer’s instructions. All cDNA sequences of porcine genes were obtained from Genbank and Invitrogen provided all primers. Gene-specific polymerase chain reaction products were measured continuously using the iCycler IQ real time detection system (Bio-Rad, Stockholm, Sweden) during 40 cycles with iQ SYBR green supermix (Bio-Rad). Target genes from each individual sample were normalized against β-actin expression. Gene expression of HIF-1a, VEGF, IL-1α, TNF-α, INF-γ, JNK-1, p38, cytochrome-c, Bax, Bel-2, caspase-8, and caspase-3 at all time points assessed using RT-PCR.
In vitro incubation of pig arteries (paper III)

Long femoral artery segments from pigs were cut in half, with one half used for CHC and the other half for UFH (control). The arteries were connected at both ends to 20-cm long pieces of polyethylene tubing (4 mm ID). After careful rinsing with Ringer’s acetate buffer, 5 mL of CHC or UFH was transferred to the segments and rocked back and forth across the endothelial lining several times over a few minutes. Both segments were dipped in a petri dish filled with the respective agents, and the experiments were performed at both room temperature and at a temperature of 4 °C at the same incubation times, 20 h, as described above. Immediately after the immobilization process, both samples were snap-frozen for later analysis.

CHC consumption in the perfusion fluid (paper III):

CHC consumption in the perfusion fluid was confirmed by two methods.

I: The presence of biotin linked to CHC (Corline Systems AB) was confirmed by allowing CHC or CHC-biotin to bind to a cationic matrix (96-well plates precoated with polyamine compound, PAV; Lab Site Heparin Coating Kit, Corline Systems AB) and then measuring the uptake of streptavidin conjugated with horseradish peroxidase. A linear standard curve was obtained by diluting the starting solution (1.0) to 0.8, 0.5 and 0.25, respectively. The read-outs obtained with samples from the perfusate at different time points were compared with the standard curve to obtain the relative change in CHC concentration.

II: A toluidine blue (TB) assay (Corline Systems AB) was used to measure the gradual loss of soluble TB at 630 nm with increasing concentrations of heparin (due to precipitation of a heparin-TB complex). The assay findings proved to be linear in the range of 10–50 mg/mL of heparin.

Immunofluorescence of the renal artery and tissues (paper III)

The porcine renal arteries and tissue samples were studied in 6-μm cryosections. Endogenous peroxidase was blocked using phosphate-buffered saline (PBS) (Life Technologies, stockholm, Sweden) containing 0.03% hydrogen peroxide. After thorough washing with PBS (pH 7.4), the tissue was incubated with streptavidin-Cy5 (Molecular Probes-Invitrogen, Life Technologies Europe BV, Stockholm, Sweden) at room temperature for 10 min and rabbit anti von Willebrand factor (A0082; Dako, Glostrup, Denmark) for 60
min. An Alexa 488-labelled goat anti-rabbit antibody was used as the secondary antibody for 30 min incubation. The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes-Invitrogen) 15 min.

Confocal microscopy (paper III)
The degree of coating of the renal artery and the renal tissue cryosections were visualized by confocal microscopy. Images were acquired using a Zeiss LSM 510 Meta confocal system (Carl Zeiss, Jena, Germany). All image pairs (CHC versus control) were acquired using the same instrument settings including laser power, detector voltage, offset, and image size and imaging depth.

Normothermic perfusion circuit (paper IV)
A normothermic perfusion (NP) circuit was developed to be able to evaluate the function of two separate kidneys (experimental and control), in parallel, after reperfusion (mol/L 10). The circuit consisted of a recirculating loop comprising a cardiopulmonary bypass component with a roller pump, heatexchanger unit (HLM: S3+ HCU; Stockert–Sorin Group, Italy), integrated with 2 parallel circuits with a pediatric membrane oxygenator and a venous reservoir (Dideco KIDS Physio, Sorin Group, Mirandola, Italy) in each circuit (Fig. 9).

Figure 9. Ex vivo normothermic perfusion system.
Blood gas parameters such as base excess (BE) and pH were adjusted within physiological levels. BE-analyzed values above and below -10 mmol/L were corrected by adding Tribonate (Fresenius Kabi, Uppsala, Sweden) in 6 mL and 3 mL blood reservoirs, respectively. Pressure flow was measured continuously via a transducer probe (3Fr; Argon Medical Device, Holte, Denmark) placed at the entrance of the renal artery and monitored throughout the experiment.

The NP circuit was primed with 250 mL of whole autologous blood containing 2500 Units (U) of heparin (Leo Pharma, Malmö, Sweden) 200 mL of Ringer-acetate (Fresenius Kabi, Uppsala, Sweden), 18 mL of Mannitol (Baxter, Kista, Sweden), 6 mL of Tribonate (Fresenius Kabi AB), 375 mg of Cefuroxime (Pfizer AB, Sollentuna, Sweden) and 100U of Humilin (Eli Lilly AB, Solna, Sweden), resulting in a total volume of 500 mL. Nutrition was provided by an infusion of essential amino acids (Nutriflex Lipid Plus; Braun, Danderyd, Sweden) at 10 mL/h and glucose 50 mg/mL (Braun) 10 mL/h. The urine output was replaced by Ringer-acetate (Fresenius Kabi) at hourly intervals. Due to the absence of endogenous creatinine production, each circuit was loaded with 1000 µmol/L creatinine (Sigma-Aldrich, Steinheim, Germany) to resemble uremia levels in blood. The same arterial route that was used for HMP was also used for NP. The renal vein was cannulated on ice and the kidneys were flushed with 100 mL ice-cold (4 °C) saline through the renal artery before being connected to the NP circuit. NP of both kidneys was started simultaneously. Both ureters were subsequently cannulated for urine collection shortly after NP.

Histology (papers I-IV)

Kidney tissue samples were fixed in formaldehyde and embedded in paraffin. Sections (5 µm) were cut and stained with haematoxylin and eosin and Periodic acid-Schiff (PAS) and evaluated for morphology and overall structure using light microscopy. Histopathological changes were assessed semi-quantitatively by using changes in three structural compartments: glomeruli, vessels and tubuli. In the glomeruli, the focus was on the presence of hyperemia, congestion, necrosis and inflammatory cells. Vessels were analyzed with respect to endothelial activation and cell damage. The tubular system was assessed for the presence of tubular dilatation, debris, necrosis, nuclear shrinkage, swelling, vacuolization and infiltration of inflammatory cells. All slides were blinded for the assessor and each variable scored using a scale of 0 - 3 according to the level of damage, where: 0 represents normal cells; 1 mild damage; 2 moderate damage; and 3 severe morphological changes.
Statistical analyses

All statistical analyses were performed using Prism software (Version 5.0 for Paper II and Version 6.0b for Papers III and IV; GraphPad Software, San Diego, CA, USA). All data are expressed as the mean ± standard error of the mean (SEM). Comparisons between the experimental groups were performed using the Mann-Whitney U-test, and comparison of repeated continuous variables over time were analyzed using a paired two-way analysis of variance (ANOVA). P<0.05 was considered statistically significant.
Results

Paper I

**Intracranial pressure**
In all cases a volume-dependent gradual exponential elevation of the ICP was observed between 0 and 5 mL, starting from baseline ICP values of 13 ± 4.8 mmHg to 60 ± 11.9 mmHg. After increasing the volume by 2 mL (6-7 mL in total), the ICP did not change, instead it declined slightly. Subsequently, at a total volume of 8 mL, ICP severely increased again to 89.8 ± 9.7.
This course of events was observed in all experiments (Papers I-IV).

**Cerebral perfusion pressure**
All pigs showed a CPP ≤0 mmHg after 7-10 mL inflation of epidural balloon (7 mL in 2 pigs; 8 mL in 3 pigs; 10 mL in 1 pig). Baseline CPP was 72.3 ± 15 mmHg, and it declined as the volume of the epidural balloon increased. After 7 mL inflation in the epidural balloon volume, the calculated CPP was below 0 mmHg. At a volume of 10 mL, the CPP was -28.6 ± 26.2 mmHg.

**Changes in haemodynamics**
No remarkable changes were observed in MAP or heart rate (HR) during the initial part (0-5 mL) of inflation of the epidural balloon. These parameters remained close to the baseline levels of 85.0 ± 10.4 mmHg and 98.5 ± 15.4 per min, respectively. MAP decreased and HR increased significantly with an additional increase in balloon volume between 5 and 6 mL. At the end of the experiment, MAP was 59.0 ± 7.5 mmHg and HR was 114 ± 12.7 per min.

**Confirmation of brain death using iv microsphere injection**
The presence of microspheres was analyzed in five cases after reaching physiological brain death criteria (negative CPP for more than 60 min). A negligible amount of microspheres was found in the cerebrum 34.66 ± 9.95 per gram tissue, and in the brainstem 60.41 ± 36.28 per gram tissue, indicat-
ing an absence in cerebral flow. In comparison, 17,979 ± 5086 microspheres per gram were detected in the kidney tissue.

**Morphology**

The majority of the cross-sections of the kidneys, liver, pancreas and heart tissue showed well-preserved histology, but smaller foci with organ damage were seen in all organs in all animals. In the kidneys (Fig. 14A), these features presented as intraluminal cell debris and tubular dilation.

**Paper II**

**Hypoxia-related gene expression**

Expression of mRNA for HIF-1α, VEGF and GRP-94 changed only marginally immediately after CS (0 h) compared to the baseline samples. The kidneys preserved with F6H8 emulsion were characterized by significantly lower mRNA expression of HIF-1α (Fig. 10A) and VEGF at any point during the experiment compared to the HTK-perfused kidneys.

![Figure 10. Expression of mRNA (mean ± SEM) of (A) human inducible factor (HIF)-1α (hypoxia-related gene), (B) tumour necrosis factor (TNF)-α (cytokine gene expression) and (C-D) caspase-3 and Bax/Bcl-2 ratio (apoptosis related gene expression). Kidneys preserved with histidine-tryptophan-ketoglutarate (HTK) solution (gray bars) or preoxygenated perfluorohexylcetane (F6H8) (white bars). *P < 0.05 for F6H8 emulsion versus HTK at the corresponding time points.](image)
Cytokine gene expression
A steady and discrete reduction of gene expression was noted at all time points after reperfusion and transplantation, regardless of the preservation solution used. However, the F6H8 kidneys were characterized by significantly lower cytokine mRNA expression at all time points in the experiment (Fig. 10B).

Apoptosis related gene expression
Kidneys preserved with F6H8 emulsion were characterized by lower mRNA expression of Bax and significantly greater expression of Bcl-2, resulting in a Bax/Bcl-2 ratio that was significantly reduced at any time point of the experiment compared with HTK (Fig. 10D). The time-related expression of the initiator and terminal effector elements of the caspase cascade, such as caspase-8 and caspase-3 (Fig. 10C), did not change compared to the tissue before perfusion (baseline) when the kidneys were preserved with F6H8 emulsion. In contrast, a significantly enhanced up-regulation was noted in HTK-perfused kidneys for both caspase-8 and caspase-3 compared to the F6H8-preserved kidneys.

Renal ATP content
A higher ATP content was found in kidneys after 18 hours of CS with F6H8 compared to HTK (4.4 ± 0.2 ng/µg versus 2.6 ± 0.2 ng/µg; P<0.05). Even greater ATP replenishment was observed 2 and 4 hours post reperfusion in the kidneys preserved in F6H8 preserved kidneys (P<0.05).

Histological evaluation
Semiquantitative assessment of kidney samples 4h after reperfusion revealed focal tubular necrosis, ischemic injury and dense glomeruli in the HTK-perfused kidneys compared to F6H8-perfused kidneys, which showed minimal tubular dilatation and no ischemic necrosis (P<0.05).

Paper III
Consumption of CHC during HMP
The toluidine blue assay showed no loss of heparin in the perfusion solution at any time period when the kidneys were perfused with UFH. The TB assay indicated a loss of more than 50% of CHC for all time points when the kidneys were perfused with CHC. This was confirmed by measuring the binding of CHC-biotin to the cationic matrix. In five consecutive experiments it was shown that the CHC concentration was reduced to 25% or less at all
time points (1 h, 6 h, 12 h and 20 h). Analysis of CHC in the perfusate of the kidney not connected to the device showed no consumption after ongoing perfusion.

**Vascular renal resistance**

The analysis of prospectively collected vascular renal resistance (RR) values of kidneys stored by HMP showed that RR was higher at the start and decreased to sustained consistent low levels in all cases when CHC or UFH was added to the perfusion solution.

**Confocal microscopy**

The confocal microscopy analysis of the renal artery (Fig. 11C) and the renal tissue consistently demonstrated uniformly distributed fluorescence, indicating that CHC covered almost all renal artery walls and tissues with distinct fluorescence, following the endothelial cell lining in the kidneys treated with CHC. No fluorescence was detected in UFH-perfused kidneys (control).

*Figure 11. Immunofluorescent staining of cryosections from biopsies of porcine femoral arteries (A and B) incubated for 20h at 4°C in vitro with CHC-biotin (A) or unfractionated heparin (UFH) (B). Renal arteries (C and D) after 20 h hypothermic machine perfusion (HMP). CHC or UFH was added into perfusion solution respectively, CHC (C) and UFH (D). Blue: DAPI; Green: von Willebrand factor (vWF); Red: Streptavidin-Cy5 (to detect biotinylated CHC).*
The semi-quantitative estimation of the fluorescence by software imaging revealed a significant level of biotinylated CHC in the CHC-perfused kidneys compared to the kidneys perfused with UFH (control; P<0.05).

Paper IV

Renal function

Serum creatinine

The serum creatinine levels after 3 h of NP were lower in the CHC-treated kidneys (302 ± 82 μmol/L) compared to the control group (446 ± 50 μmol/L; P=0.023; mol/L 12) (Fig. 12).

Figure 12. Creatinine levels during NP. One kidney was first perfused with KPS-1 and 50mg of CHC (Corline Systems AB, Uppsala, Sweden) during HMP while the other kidney was perfused with KPS-1 alone before NP.

Urine osmolality

Urine osmolality in four pigs (n=4) per group and in the CHC-treated group (290-340 mosmol/kg) did not differ from the control group (290-339 mosmol/kg).

Total urine volume

The total urine volume after 3 h of NP was higher in the CHC-treated group (239 ± 71 mL) than in the control group (172 ± 42 mL; P=0.031)
Figure 13. (A) Porcine kidney during NP. (B) Urine production from porcine kidney during NP. One kidney was perfused with KPS-1 and 50mg of CHC (Corline Systems AB, Uppsala, Sweden) during HMP while the other kidney was perfused with KPS-1 alone during HMP.

Acid–base

The serum lactate levels in the CHC-treated group were lower (5.4 ± 0.7 mmol/L) than in the control group (6.1 ± 1.2 mmol/L; P=0.03). The pH and BE in both groups remained stable over 3 h. No difference was seen between the groups.

Graft morphology and histology

Paper III

No severe morphological changes were observed in the different compartments for any of the samples of the CHC- and UFH-perfused kidneys after 1 h, 6 h or 20 h of HMP. After 20 h of HMP, all of the samples displayed minor to (in a few cases) moderate morphologic changes (Fig. 14B).

Paper IV

More severe tubular injuries were seen in the control group compared to the CHC-treated group (p=0.045; Fig. 14C and D). No differences with respect to glomeruli or vascular changes could be seen between groups.

Acid–base

The serum lactate levels in the CHC-treated group were lower (5.4 ± 0.7 mmol/L) than in the control group (6.1 ± 1.2 mmol/L; P=0.03). The pH and BE in both groups remained stable over 3 h. No difference was seen between the groups.
Figure 14 (A) Porcine kidneys after induction of brain (death paper I), intraluminal cell debris and tubular dilation (black arrows). (B) Porcine kidneys after brain death and 20 h of HMP treated with 50 mg of CHC (Corline Systems AB, Uppsala, Sweden)(paper III). (C-D) Porcine kidneys after brain death and 20 h of HMP, and 3 h of ex vivo NP (paper IV). Kidneys treated with 50 mg of CHC during HMP (C) and kidney used as control(D). Black arrows indicate tubular swelling and debris.
Discussion

Transplantation has evolved as the treatment of choice for many patients with irreversible organs disease and deceased donors still remain the primary source of organs. The current understanding of the pathophysiology of renal ischemia and reperfusion injury has resulted in the development of strategies, focusing on donor and recipient management, organ procurement and preservation techniques and pharmacological treatment to prevent or reduce IRI. Despite functional one-year graft survival rates of over 90%, deterioration of the transplanted organs over time has not changed significantly over time. This failure to raise the slope and prevent organ deterioration might be explained in part by the expansion of the criteria for acceptable donors and the more frequent use of marginal and older donors.

It has been hypothesised that the events surrounding brain death, occurring before organ removal, may be essential. In addition to nonspecific events relating to events surrounding the donor, the perfusion and storage of organs, may initiate an inflammatory response. Brain death is associated with the deterioration of allograft quality and accelerated immunogenicity, which are clinically linked to reduced patient and graft survival\cite{16,120}. As a result, organs from brain-dead donors seem to experience increased episodes of acute rejection after transplantation. This concept would explain the obvious relationship noted clinically between the effects of initial delayed graft function and acute rejection episodes, as well as the different outcome of organs originating from brain-dead in comparison to living donors\cite{101,121}.

Significant progress has been made in describing the functional and morphological changes of different organs during BD and IRI, but the complex molecular and cellular interactions between endothelial cells, inflammatory cells and the injured epithelium are not fully understood. It is clear that aspects of organ quality and additional nonspecific injuries, at least in part, seem to predict transplantation outcomes. The consideration that injuries resulting around the time of organ transplantation, become risk factors for late allograft failure suggests that the long-term changes may be programmed early in the process.

A better understanding of the molecular, cellular and genetic processes underlying kidney injury could potentially lead to targeted therapies to prevent
injury and promote repair, and possibly increase the survival of transplanted organs. This thesis aimed to create a reproducible standardized and clinically relevant large animal BD model, simulating the clinical situation in which a more gradual and prolonged increase of intracranial volume and ICP was applied. Injury of the renal microvascular endothelium alters barrier function after ischemia. It becomes increasingly evident that damage to the glycocalyx represents the earliest stage of the endothelium disruption after IRI. Definition and understanding of these potential changes may suggest therapeutic approaches that could be initiated even before the transplantation procedure. We hypothesized therapeutic strategies targeting the endothelial glycocalyx by application of CHC directly to endothelium surface mimics the biological protection activity, which exerted by heparansulfate at the endothelial glycocalyx, can ameliorate IRI and hence functional capacity.

**Paper I**

Pigs were used as a large animal model to create reproducible standardized brain-death model. In order to mimic a clinical situation, a stepwise increase of intracranial pressure progressed until BD occurred. Brain death mainly occurs after trauma or a non-traumatic cause such as massive infarction or sudden intracranial hemorrhage. The latter group (brain death of non-trauma causes) is becoming more common in clinical transplantation in line with the change in demographics of organ donors, entailing more elderly or marginal donors. Two types of models of BD have previously been described, namely the sudden onset or gradual onset models. In the sudden onset BD model, intracranial pressure increases within 30 to 60 s, simulating acute and significant cerebral trauma. In gradual onset BD, intracranial pressure is increased commencing with an instantaneous large volume injection and increasing gradually for up to 65 min. In the clinical situation, intracranial hypertension develops frequently slowly secondary to neurologic damage but not instantly or within minutes.

Porcine brain-death models have been widely used in transplant surgery studies. Ryan et al., Lyons et al. and McLean et al. all used sudden inflation of subdural balloons to induce BD. Barklin et al. performed a study that lengthened the induction phase to 60 min with gradual epidural balloon inflation up to 15 mL to establish a more clinically relevant model. Furthermore, the latter also suggested that a prolonged BD process might yield the necessary preconditions to trigger a systemic inflammatory response that might contribute to organ dysfunction. In contrast to other
experimental models, the experiments in our studies were not finished until CPP was <0 mmHg for at least 1 h, so the organs were exposed to processes both before and after complete cessation of the cerebral blood flow. The pig was selected because of the similarities in its brain structure and size to the human brain, allowing the use of the same neuromonitoring equipment as used in human neurointensive care. We therefore wanted to prolong the BD induction phase up to 200 min by a stepwise increase in intracranial volume, followed by a 60 (Paper I, II, IV) and 120 min (Paper III) observation period. We did not find it feasible to extend the experiment further for logistic reasons, even though that would have better mimicked the clinical situation. Rather, it was considered desirable to capture the pathophysiological process in more detail using neuromonitoring devices measuring ICP, CPP and B\textsubscript{i}PO\textsubscript{2}, and to confirm BD using microsphere injections.

Moreover, sudden onset BD models often require the use of inotropic medical support to maintain a normal blood pressure. Inotropic support could potentially bias the results in studies concerning organ damage caused by brain death, since it has been shown to have positive effects on the peripheral organ blood flow\textsuperscript{132}. In this study, the monitoring results showed a classical intracranial pressure-volume relationship\textsuperscript{103,133}. Furthermore, IC decreased gradually as ICP increased, which reflected a decreased ability to compensate. At the end of the experiment, B\textsubscript{i}PO\textsubscript{2} reached close to 0 mmHg in all cases (1.1 ± 0.23 mmHg), suggesting a total absence of oxygen in the brain tissue, i.e. brain death, even though the final B\textsubscript{i}PO\textsubscript{2} was not exactly 0 mmHg. Microspheres were injected into the arterial bloodstream after BD and a negligible number of microspheres were detected in the cerebral tissue in comparison to the extensive amount found in the perfused extracranial control tissues.

Morphologic examination of different organs showed systemic effects, such as small tissue necrosis and edema in parenchymatous organs, which may influence organ function after transplantation. Kidneys, livers, pancreases and hearts examined after induction of BD presented mainly with well-preserved morphology. Smaller foci in all of the organs did however show distinct signs of damage. In sham-operated animals (n=4, data not shown), the same procedure was performed without the volumetric expansion of the epidural balloon. This was consistent with the absence of the intracranial pressure-volume relationship, histological changes and accumulation of microspheres in all organs including the brain and brainstem.

Paper II
The study described in Paper II was designed to provide an initial proof of concept of the efficacy of F6H8 emulsion for cold storage to reduce IRI.
HTK was originally developed as a cardioplegic solution but, because of its low viscosity, was quickly adopted for clinical organ transplants. HTK is an extracellular solution containing Mannitol as an impermeant and histidine as a buffer. It also contains tryptophan to stabilize cell membranes and prevent oxidant damage, and ketoglutarate, a substrate to support anaerobic metabolism. It has been observed that oxygen-precharged HTK-F6H8 emulsion protected the kidney tissue from severe ATP depletion during cold storage and improved restoration of ATP levels after reperfusion. Clinical studies demonstrated that the gene expression profile in kidneys from brain-dead donors was different than grafts obtained from living donors by increased apoptosis. Most of the up-regulated genes in organs recovered from diseased donors related to inflammation, redox state, metabolism and protein modification, and cytokine profiles are different in diseased grafts compared with grafts from living donors. An up-regulated gene expression of pro-inflammatory cytokines involved in acute inflammation, TNF-α and others, was seen in response to renal ischemic injury, and this gene expression was reduced in kidneys preserved with F6H8. IRI leads to activation of a cascade of intracellular caspase proteins. The degree of activation correlates to anoxia and DNA damage, which is reflected in the release of caspase activation factors such as cytochrome c. Regulation of this activity is controlled by a balance of pro- and anti-apoptotic proteins (Bax and Bcl-2, respectively). These apoptosis markers were promoted in kidneys preserved with F6H8. Hypoxia- or environmental stress-related genes were also assessed. In kidneys perfused with the F6H8 emulsion, a significant reduction in protein expression was found for HIF-1α and VEGF. A similar observation was made for the expression of stress-related proteins such as GRP-78 and HSP-27. Significantly less vascular, tubular and glomerular damage was furthermore seen in kidneys perfused with oxygen-charged F6H8 emulsion. In following with the increased gene expression of pro-inflammatory mediators, we observed an enhanced number of polymorphonuclear neutrophils in the HTK-perfused kidney grafts.

In this study, we were able to demonstrate that the detrimental effects of early IRI be ameliorated using the oxygen-binding perfluorohexyloctane F6H8 as an additive to the HTK preservation solution.

**Paper III**

Based on the finding that a heparin conjugate, CHC, irreversibly binds to collagen structures and thereby expresses functional heparin, a feature that cannot be accomplished by ordinary unfractionated heparin, the present study demonstrated binding of CHC to the renal artery and renal tissue, whereas no binding of UFH (control) was detected with an identical ap-
proach. CHC typically consists of 70 heparin molecules linked by specific covalent bonds to a straight carrier chain composed of an aliphatic amine without compromising the antithrombin binding sites of the heparin. This synthesis is carried out as a separate step, and the resulting CHC is extensively purified to ascertain that no harmful residual substances are present. CHC has been used to improve blood compatibility of biomaterials. It inhibits coagulation and complement activity, reduces platelet adhesion and stimulates endothelial infiltration. Heparan sulfate (HS) is the most common glucosaminoglycan on the endothelial surface and makes up 50–90% of all endothelial proteoglycans. HS plays an important role in the physiopathology of vascular homeostasis and inflammatory reactions in the regulation of leukocyte extravasation. The degradation of HS induced by IRI has profound implications on tissue damage and the restoration of the glycocalyx on the endothelial surface is consequently an attractive target for reducing IRI. Circulating fragments of HS act as mediators of inflammation by modulating the immune system through Toll-like receptor 4 (TLR-4) on the surface of antigen-presenting cells (APC) such as macrophages and dendritic cells. Several significant anticoagulant mediators, such as antithrombin III, heparin cofactor II, and tissue factor pathway inhibitor (TFPI), connected to the HS. Both ischemia and hypoxia initiate glycocalyx degradation, shown through direct targeting of the glycocalyx in experimental and clinical settings. This phenomenon is not only seen in experimental models, but also in patients undergoing major vascular surgery with global and regional ischemia. We presented a strategy to restore the endothelial GCX using functional immobilized heparin, which is very similar in molecular structure to HS. Kidneys from brain-dead pigs were used, thus the organs are affected by damage mechanisms that occur in connection with brain death and organ retrieval also seen in human organ transplantation. It was evident that the binding of CHC occurs not only under warm conditions but also under the cold conditions. We showed that the binding to the kidney tissues occurred relatively quickly, that is within an hour after start of the perfusion. In particular, it was demonstrated that CHC was not trapped in the device components during HMP. Confocal microscopy revealed consistently uniformly distributed fluorescence following the endothelial lining covering the surface of vessels in kidney arteries and tissues. CHC may thus be employed in the coating of the vascular wall and to act as a vessel-modifying agent during cold preservation.
Paper IV

Restoration of the glycocalyx represents an attractive way to reduce IRI during organ transplantation. In Paper III it was shown that it was possible to coat the surface of the artery and tissue of porcine kidney grafts with a macromolecular heparin conjugate during HMP. We hypothesized that this modification of the organ could improve the early functional capacity of the organ and reduce IRI after brain death and reperfusion. It was demonstrated that perfusion of porcine kidneys with CHC increased the early functional capacity of the kidneys as indicated by increased urine production and a faster reduction in creatinine levels compared to the control group. This favorable effect was also reflected in improved morphology, mainly seen in tubuli. This novel model is easy to implement practically in a clinical setting. Translation of the findings of this preclinical experiment may potentially lead to the use of this treatment concept in the clinical setting of organ preservation and kidney transplantation. Treatment with CHC not only improved post-ischemic renal function but also promoted the ongoing post-ischemic repair processes reflected in lower serum lactate levels in the perfusate.

Ex vivo NP for 3 h showed remarkable regression of ischemic characteristics such as leukocyte infiltration, tubular necrosis and glomeruli activation compared to HMP alone (Paper III), irrespective of group. This comparison confirmed the favorable effect of NP rather than a CHC effect alone.

The 3-hour time period for NP was chosen based on previous studies showing that there is a breaking point whereafter the kidney begins to deteriorate. This time point was naturally also practical for logistic reasons. Successful extracorporeal porcine liver perfusion for 72 hours, and 6 hours for kidneys, has been reported\textsuperscript{156-158}. It has also been reported that leukocyte depletion from blood promotes acidosis during NP\textsuperscript{158}. In our study, we used whole blood, including leukocytes, in NP to preserve the circulating leukocytes, which are an important factor in IRI\textsuperscript{92,97,99}. Interestingly, CHC was not associated with an increased risk of hemorrhage compared to the control group based on TEG analysis.

Future expansion of organ transplantation likely requires modulation of the organ before transplantation to protect it from IRI and subsequent DGF and chronic deterioration. This becomes even more profound considering today’s more frequent use of ECDs, since ECD organs are more susceptible to IRI than organs from regular donors. The restoration of endothelial glycocalyx during HMP appears to ameliorate IRI. In this study we demonstrated that perfusion of porcine kidneys with CHC during HMP reduces early IRI and improves graft function. This approach could become useful to increase the long-term outcome of kidney transplants from deceased donors.
Conclusions

This thesis focused on brain death-associated injuries and IRI in the context of renal transplantation in experimental models in the pig. Further, two separate approaches, namely an oxygen-precharged perfusion solution and HMP with macromolecular heparin, were tested in an aim to reduce IRI and to improve organ function.

The growing utilization of organs from ECDs, with a limited functional reserve and an increased susceptibility to ischemia, demands more advanced donor management and modifying methods to improve the graft’s resistance to IRI. An experimental model of brain death was developed in pigs to resemble the clinical situation as closely as possible, enabling brain-death-associated injuries in different organs to be characterized (Paper I). The influence of BD on initial graft injury confirmed that this is a major area of importance in improving organ quality. Progressive ischemic tissue damage, potentially triggered by brain death, is followed by inevitable cessation of blood flow that further increases anoxia during cold storage\textsuperscript{101}. Cellular ATP depletion triggers inflammatory and pro-apoptotic cascades that culminate in cell death through apoptosis or necrosis after reperfusion\textsuperscript{159}. Perfusion of porcine kidneys with oxygen-precharged F6H8 perfusion solution reduced IRI both at the cellular and molecular level (Paper II).

Endothelial glycocalyx plays a major role in maintaining vascular homeostasis and initiating an intracellular inflammatory signaling cascade\textsuperscript{119}. The degradation of GCX during ischemia is a process that further increases the injury during reperfusion. Paper III showed that it is possible to coat the inner surface of the renal vessels with a macromolecular heparin molecule (CHC) during HMP, an artificial approach that could potentially be used to restore disrupted GCX. Perfusion with CHC increased organ function and decreased tubular injury after reperfusion in an ex vivo NP model (Paper IV).

By creating a standardized brain-death model and an ex vivo NP model, it was possible to evaluate two different approaches for reducing IRI in porcine kidneys, thus showing that both models are valuable for future studies in this field. Perfusions of porcine kidneys with an oxygen-precharged solution or macromolecular heparin were both shown to reduce IRI in experi-
mental transplantation and reperfusion. These approaches might also prove to be useful in humans and may, alone or in combination, repair and protect human organs before transplantation and improve organ function in the long run. Further, they might possibly lead to an increase in the donor pool by facilitating a more frequent use of organs from ECDs.
Future perspective

During the last decades, our understanding of the mechanisms mediating IRI in kidney transplantation, has improved significantly. Enormous efforts of recent researches have focused on ameliorating these injuries, although the results in experimental models have not yet been successfully translated to the clinic. Graft quality determines, at least in part, the success of organ transplantation. It is therefore of utmost importance to control and potentially also reduce IRI in organ transplantation.

A single therapy is unlikely to offer complete protection or prevention of injury due to the complexity of IRI. The goal of multi approaches or targeting multiple signals will be the next step to attempt to reduce post-transplantation injury. Although the focus of this thesis was on studies on novel managements against ischemia-associated injuries in renal transplantation, but a future direction of these findings will likely be able to implement in extra renal organ transplantation.

The new normothermic perfusion setup can offer a versatile assessment tool for further therapeutic interventions before transplantation that may include drug therapies, immunomodulation or other interventional graft treatment. However, these data are based on studies in porcine model and proof in the clinical setting is awaited.
Sammanfattning

Organtransplantation har på senare tid visat sig vara mycket framgångsrikt och det har blivit det främsta behandlingsalternativ för patienter med sviktande organ. Tyvärr kan fortsatta framgångar komma att förhindras på grund av den växande skillnaden mellan tillgång och efterfrågan på organ. Resultaten efter organtransplantation beror främst på kvaliteten hos de donerade organen, vilka i sin tur påverkas av en mängd olika faktorer såsom givarens ålder, det kliniska ohändertagandet av donatorn, metoden för att förvara organet under transport samt förvaringstiden.

För närvarande används huvudsakligen organ från avlidna donatorer för transplantation av organ och celler. Hjärndöd förknippas dock ofta med fysioologisk instabilitet såsom sviktande blodtryck och massiv urinproduktion, som kan leda till försämring av organkvaliteten före donatoroperationen. Den kliniska innebörden av hjärndöd utmärks på sikt av sämre organfunktion och organöverlevnad än hos organ från levande givare. Bristen på organ har resulterat i ett ökat tillvaratagande av organ från äldre donatorer och från medelålders donatorer med sjukdomar som exempelvis diabetes eller högt blodtryck.

välkänt problem som ökar i omfattning och som påverkar transplantationsresultatet på längre sikt.

Syftet med denna avhandling var att undersöka hur man kan förbättra de skador som uppstår i organ efter hjärndöd och av ischemi/reperfusionsskador. Alla experiment har utförts i grismodell eftersom grisens fysiologi och immunsystem är väldigt lika människans.

Acknowledgments

I want to convey my deepest gratitude to many people who supported me in many ways during the work on this thesis, but I would especially like to express my gratitude to the following persons:

Foremost, I would like to thank my supervisor and a great friend Tomas Lorant, for your constant dedication, sense of fairness and support, and for sharing your great intellect and brilliant talent for sorting things out. This thesis would not have been possible without your concern care, encouragement, guidance and enthusiasm.

Professor Gunnar Tufveson, my co-supervisor and appreciated colleague. I cannot express how grateful I am for your encouragement and insightful examination of the research material and your support in keeping the research brief and focused.

Professor Olle Korsgren, my co-supervisor, for your support and sharing your comprehensive knowledge in transplantation immunology and enthusiasm for research.

This research could not have been completed without the support of my colleagues and friends Lars Bäckman, Alireza Biglarnia, Vivan Hellström, Helene Malm, Shinji Yamamoto, David Berglund and Bengt von Zur-Mühlen at the Department of Transplantation Surgery. Thank you for always being very positive and supportive regarding my project and for challenging and inspirational teamwork.

Professor Rolf Larsson, my co-author, most especially for his enthusiasm and generosity and timeless commitment to sharing his knowledge.

Professor Erik Larsson for his patience and humbleness and persistence in examining histological materials.
Anders Nordgren and Monica Hall, for excellent technical assistance during the animal experiments.

Bo Norlin, for support and maintenance of the experimental application of normothermic perfusion, and Fredrik Lennmyr, for excellent collaboration, both from the Department of Thorax Surgery.

Karlis Purins, Anders Lewén, and Professor Per Enblad, my co-authors at the Department of Neurosurgery, for excellent collaboration.

Peetra Magnusson, Sofia Nordling and Johan Brännström, my co-authors and laboratory mentors at the Department of Immunology, and in particular Maria Karoutsou, for her great contribution and extremely instructive work in the laboratory.

All of the staff at the Department of Transfusion Medicine involved in the work and Norbert Lübenow in particular, who helped with the thromboelastography analysis.

Special gratitude also goes out to Sana Asif and all my co-authors for outstanding collaboration – Johan Nordström, Christian Molnar, Professor Leif Jansson, Heide Brandhorst, Carl Jörns, Greg Nowak, Sonja Theisinger, Simone Hoeger, Lars Wennberg, and Daniel Brandhorst.

Åsa Aringskog and Malin Dackborn, for your greatly appreciated assistance during the last experiment.

Fredrik Carlsson for our inspirational discussions concerning this work.

Elisabeth Bergqvist, secretary at the Department of Surgery, and Karin Johansson at the Institution of Surgical Sciences, Uppsala University, for kind secretarial assistance.

Claes Juhlin, head of the Department of Surgery at Uppsala University Hospital, Professor Lars Wiklund, former chair of the Department of Surgical Sciences, and current Professor Olle Nilsson, for support in this research.
I would like to thank Uppsala University for their support and commitment to renal transplantation in Uppsala. Without their financial support this research would quite simply not have been possible.

This study was supported by grants from the Professor Lars-Erik Gelin Memorial Foundation, the Bergholm’s Foundation, the Edvin Eriksson Foundation, and the Medical Faculty at Uppsala University.

My parents, for your enormous support in any situation, especially during the entire journey to my new life and my extraordinarily fortunate destiny come to Sweden, and for always being there for me and cheering me up from afar when I needed it most.

Lastly, I must thank my family, Sophie, Michael, Emma and my beloved wife Cecilia, for bringing me great joy and much love, for your unfailing understanding in every sense, and for inspiring me in my commitment.
References


118. Jacob M, Bruegger D, Rehm M, Welsch U, Conzen P, Becker BF. Contrasting effects of colloid and crystalloid resuscitation fluids on


121. <Both alloantigen-dependent and -independent factors influence chronic allograft rejection.pdf>.


126. <The gradual onset brain death model- a relevant model to study organ donation and its consequences on the outcome after transplantation.pdf>.


Acta Universitatis Upsaliensis

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 993

Editor: The Dean of the Faculty of Medicine

A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)