THE INFLUENCE OF ISCHAEMIC INJURY AND MANIPULATION OF THE NITRIC OXIDE SYNTHESIS PATHWAY ON PULSATILE MACHINE PERFUSED PORCINE KIDNEYS

By

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September 2005

A thesis submitted to the University of Leicester
For the degree of Doctor of Medicine
STATEMENT OF ORIGINALITY

The experimental work on which this thesis is based was performed between February 1999 and September 2000 and is my own independent work except where acknowledged.

Miss Amanda J. Knight
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DEDICATION

To Paul, for his love and support over the last 14 years.

To Thomas and Edward, without whom this thesis would have been written much sooner.
ACKNOWLEDGEMENTS

The people to whom I owe a large amount of gratitude whilst undertaking this thesis include the following:

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PRESENTATIONS

Oral: Cold ischaemia further increases intrarenal resistance when non heart beating kidneys are pulsatile machine perfused.
AJ Knight, EA O'Leary, ML Nicholson.

AJ Knight, ML Nicholson.

Cold ischaemia increases intrarenal resistance with pulsatile perfusion.
AJ Knight, EA O’Leary, ML Nicholson.

PUBLICATIONS

In full: Methods of renal preservation.
AJ Knight, ML Nicholson.

Abstract: Cold ischaemia further increases intrarenal resistance when non heart beating kidneys are pulsatile machine perfused.
AJ Knight, EA O’Leary, ML Nicholson.

Measurement of intrarenal resistance during pulsatile machine perfusion accurately predicts warm ischaemic injury.
AJ Knight, ML Nicholson.

Cold ischaemia increases intrarenal resistance with pulsatile perfusion.
AJ Knight, EA O’Leary, ML Nicholson.
Transplant Proc 2001;33:893-894.
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<tr>
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<tr>
<td>ADMA</td>
<td>$N^\alpha, N^\beta$-dimethylarginine</td>
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<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>AG</td>
<td>Aminoguanide</td>
</tr>
<tr>
<td>ALG</td>
<td>Anti-Lymphocyte Globulin</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine Monophosphate</td>
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<td>Analysis of Variance</td>
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<td>ATG</td>
<td>Anti-Thymocyte Globulin</td>
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<td>Acute Tubular Necrosis</td>
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<td>Adenosine Triphosphate</td>
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<td>BCIP/NBT</td>
<td>5-Bromo-4-Chloro-3 Indolyl Phosphate/Nitroblue Tetrazolium</td>
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<td>BGG</td>
<td>Bovine Gamma Globulin</td>
</tr>
<tr>
<td>BH$_4$</td>
<td>Tetrahydrobiopterin</td>
</tr>
<tr>
<td>$\beta$ME</td>
<td>Beta Mercaptoethanol</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>Ca$^{++}$</td>
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<tr>
<td>CaM</td>
<td>Calmodulin</td>
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<tr>
<td>cGMP</td>
<td>Cyclic Guanylate Monophosphate</td>
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<tr>
<td>CIT</td>
<td>Cold Ischaemic Time</td>
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<tr>
<td>cNOS</td>
<td>Constitutive Nitric Oxide Synthase</td>
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<tr>
<td>CPP</td>
<td>Cryoprecipitated Plasma</td>
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<tr>
<td>CPR</td>
<td>Cardiopulmonary Resuscitation</td>
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<td>CS</td>
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<tr>
<td>CyA</td>
<td>Ciclosporin A</td>
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<tr>
<td>DBTL</td>
<td>Double Balloon Triple Lumen</td>
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<tr>
<td>DGF</td>
<td>Delayed Graft Function</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<tr>
<td>EDRF</td>
<td>Endothelium Derived Relaxation Factor</td>
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<tr>
<td>eNOS</td>
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<tr>
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<tr>
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<tr>
<td>GFR</td>
<td>Glomerular Filtration Rate</td>
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<tr>
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<tr>
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<td>Hydroxyethyl Starch</td>
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<tr>
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<td>Interleukin</td>
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<tr>
<td>iNOS</td>
<td>Inducible Nitric Oxide Synthase</td>
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<tr>
<td>IRR</td>
<td>Intrarenal Resistance</td>
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<tr>
<td>KW</td>
<td>Kruskal-Wallis</td>
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<td>LDH</td>
<td>Lactate Dehydrogenase</td>
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<tr>
<td>L-NAME</td>
<td>N(^G)-nitro-L-arginine methyl ester</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharides</td>
</tr>
<tr>
<td>MD</td>
<td>Macula Densa</td>
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<tr>
<td>metHb</td>
<td>Methaemoglobin</td>
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<tr>
<td>MP</td>
<td>Machine Perfusion</td>
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<tr>
<td>NADPH</td>
<td>Nicotinamide Adenine Dinucleotide Phosphate</td>
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<tr>
<td>Abbreviation</td>
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<tr>
<td>NHBD</td>
<td>Non Heart Beating Donor</td>
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<td>nNOS</td>
<td>Neuronal Nitric Oxide Synthase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>NO\textsuperscript{2−}</td>
<td>Nitrite</td>
</tr>
<tr>
<td>NO\textsuperscript{3−}</td>
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</tr>
<tr>
<td>NOS</td>
<td>Nitric Oxide Synthase</td>
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<tr>
<td>O\textsubscript{2}\textsuperscript{1}</td>
<td>Superoxide</td>
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<tr>
<td>OD</td>
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<tr>
<td>OFR</td>
<td>Oxygen Free Radical</td>
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<tr>
<td>O NO\textsuperscript{2−}</td>
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<tr>
<td>OH</td>
<td>Hydroxyl Radical</td>
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<tr>
<td>PAV</td>
<td>Percentage Adjusted Volume</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PMN</td>
<td>Polymorphonuclear</td>
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<tr>
<td>PNF</td>
<td>Primary Non Function</td>
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<td>RBF</td>
<td>Renal Blood Flow</td>
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<td>RNA</td>
<td>Ribonucleic Acid</td>
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<tr>
<td>SD</td>
<td>Standard Deviation</td>
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<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis</td>
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<td>SEM</td>
<td>Standard Error of Mean</td>
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<td>sGC</td>
<td>Soluble Guanylyl Cyclase</td>
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<td>SNP</td>
<td>Sodium Nitroprusside</td>
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<td>TBS</td>
<td>Tris Buffered Saline</td>
</tr>
<tr>
<td>TNF\textalpha</td>
<td>Tumour Necrosis Factor Alpha</td>
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<td>UCLA</td>
<td>University College of Los Angeles</td>
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<tr>
<td>UNOS</td>
<td>United Network for Organ Sharing</td>
</tr>
<tr>
<td>UW</td>
<td>University of Wisconsin</td>
</tr>
<tr>
<td>VLA</td>
<td>Very Late Antigen</td>
</tr>
<tr>
<td>WIT</td>
<td>Warm Ischaemic Time</td>
</tr>
<tr>
<td>WT</td>
<td>Warm Time</td>
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THE INFLUENCE OF ISCHAEMIC INJURY AND MANIPULATION OF THE NITRIC OXIDE SYNTHESIS PATHWAY ON PULSATILE MACHINE PERFUSED PORCINE KIDNEYS

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ABSTRACT

Background

Renal transplant programmes in the UK continue to be seriously limited by a shortage of suitable organ donors and this has lead to renewed interest in kidney transplantation from non-heart-beating donors (NHBD). Acceptable renal function and graft survival has been achieved using NHBD kidneys [1]. The main problem has been high incidences of both delayed graft function and permanent non-function [2] as a result of the warm ischaemic insult which NHBD kidneys inevitably suffer. A related problem is that the exact period of warm ischaemia is difficult to establish in situations where death is followed by a period of unsuccessful resuscitation. If NHBD kidney transplantation is to become more widely accepted, we need to develop a reliable pre-operative test to predict tissue viability in order to avoid the transplantation of kidneys that have been exposed to irreversible ischaemic damage.

Improved methods of renal preservation are also needed in an attempt to reduce the rate of delayed graft function following NHBD transplantation. Preservation by machine perfusion may have advantages over static ice storage in that it allows the study of renal flow dynamics and the measurement of metabolic products in the effluent perfusate prior to organ transplantation [3]. These parameters may prove to be useful predictors of renal viability and subsequent graft function. Also, the
kidney itself may be biopsied whilst being perfused and this may provide a further basis for developing a viability test.

The role of nitric oxide (NO), produced by nitric oxide synthase (NOS), in renal ischaemia-reperfusion injury is unclear. There is evidence that renal ischaemia leads to elevated NOS and the end products of NO, nitrites and nitrates [4]. Evaluation of NOS and/or nitrites and nitrates may therefore be useful for pre-transplant viability testing. Manipulation of NO production by either NO donors or NOS inhibitors has shown contradictory results, with improvement in post-transplant renal blood flow (RBF) following administration of a NO donor and decreased RBF with a NOS inhibitor in one study [5] and the opposite in another study [6] where the same NO donor lead to increased oxidative damage, which was ameliorated by the administration of a NOS inhibitor.

Aims of the study
1. To assess the use of machine perfusion in predicting the severity of injury in porcine kidneys subjected to warm ischaemic damage.
2. To assess the use of machine perfusion in predicting the severity of injury in porcine kidneys subjected to warm plus cold ischaemic damage.
3. To assess the potential therapeutic effect of nitric oxide donors and nitric oxide synthase inhibitors on porcine kidneys subjected to prolonged periods of warm plus cold ischaemia.

Experimental design and basic methodology

Study 1: Prediction of warm ischaemic damage by machine perfusion of porcine kidneys.

Landrace pigs weighing approximately 40Kg were stunned and then killed by lethal injection in the large animal facility at Sutton Bonington Agricultural College.
Groups of animals (n=6) were left in a state of circulatory arrest for periods of <10, 15, 30, 45, 60 and 90 minutes (the warm ischaemic time). At the end of the period of warm ischaemia the kidneys were then retrieved and on-table renal perfusion with 500mls of hyperosmolar citrate solution at 4°C was performed prior to the kidneys being packed in ice for the journey back to the Department of Surgery at the Leicester General Hospital. After 2 hours of static ice storage one kidney from each pig was attached to the RM3 Renal Preservation System for 6 hours (Waters Medical Systems, Rochester, MN, USA). Pressure and flow characteristics were measured continuously by the machine, allowing calculation of the intravascular renal resistance. Effluent perfusate was collected hourly for analysis of nitrite and nitrate by the Greiss reagent and biopsies of the kidney were also taken hourly for semi-quantitive analysis of NOS by Western blotting techniques.

**Study 2:** Prediction of cold ischaemic damage by machine perfusion of porcine kidneys.

Porcine kidneys were retrieved by the same method as study 1. Kidneys were subjected to either a short (15 minutes) or long (45 minutes) period of warm ischaemia prior to static ice storage of either 2, 12, 24, 36 or 48 hours (n=6 per group). At the end of the period of cold ischaemia the kidneys were machine perfused for 6 hours. Intrarenal resistance was calculated and two hourly samples of the perfusate and biopsies of the kidney for analysis were performed as previously described.

**Study 3:** Assessment of the effect of NO donors and NOS inhibitors on ischaemically damaged porcine kidneys.

Porcine kidneys were retrieved by the same method as study 1. All kidneys were subjected to prolonged warm ischaemia (45 minutes) and cold ischaemia (24 hours). The kidneys were then machine perfused with the addition to the perfusate of either a NO donor (sodium nitroprusside) or one of 3 NOS inhibitors ($N^2$-nitro-L-
arginine methyl ester, aminoguanide or \(N^G,N^G\)dimethylarginine) each of which have slightly different modes of action (n=6 per group). These were then compared to a control group where no additions to the perfusate were made. Intrarenal resistance was calculated and analysis of NOS by Western blotting was performed as before.

**Results**

In study 1, a significant relationship between the length of WIT and IRR was found. During pulsatile MP, the IRR reduced in all WIT groups. Nitrate concentration and eNOS expression tended to become higher as the WIT increased.

In study 2, the kidneys subjected to the shorter WIT demonstrated a significant linear relationship between the CIT and the IRR. This relationship was not found in the kidneys subjected to a longer WIT. As the CIT increased the nitrate concentration tended to also increase, however the eNOS expression decreased.

In study 3, manipulation of the NOS pathway with NO donors and NOS inhibitors did not significantly affect the IRR or eNOS expression.

**Conclusions**

Of the indices measured, IRR had the closest and most convincing relationship with WIT. It would seem possible that this could be used as a viability assessment measure. The fact that in all studies IRR decreased over the six hours of pulsatile MP provides positive evidence of the potential benefit of MP in minimising the deleterious effects of warm and cold ischaemia.

The relationship between CIT and IRR was not so clear, but certainly when the kidneys investigated were relatively ischaemically undamaged there was a linear relationship between the two.

This study did not find any evidence of an association between ischaemic damage and the NO pathway.
SECTION A

LITERATURE REVIEWS
CHAPTER ONE

NON HEART BEATING DONOR KIDNEYS

1.1 Introduction

1.2 The distinction between heart beating and non heart beating donors

1.3 Controlled and uncontrolled NHBDs

1.4 Warm ischaemic renal injury

1.5 Protocols for donor management

1.6 Kidney cooling techniques
1.6a Intra-vascular cooling
1.6b Intra-peritoneal cooling
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1.7 Immunosuppressive protocols
1.8 Results of NHBD kidney transplantation
1.8a Primary non-function rates
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1.8c Acute and chronic rejection rates
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1.9 Potential for NHBDs to solve the organ shortage problem

1.10 Legal and ethical issues
1.10a The diagnosis and definition of death
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1.11 Summary
1.1 Introduction

Renal transplant programs continue to be severely limited by the number of available organs and this has led to renewed interest in non-heart-beating donors (NHBD) as an additional source of kidneys for transplantation. A number of excellent reviews of NHBD kidney transplantation have been published [7-9] and rather than re-iterate the arguments already presented, this review is intended to augment them.

Analysis of the results of NHBD kidney transplantation clearly demonstrates that good results can be achieved. Nonetheless, the use of NHBD kidneys for transplantation remains controversial and there are a number of problems which continue to stimulate discussion. Only a few NHBD programs have been established in Great Britain and a significant number of transplant surgeons and physicians still believe that NHBD’s provide second class organs.

The principle difference between kidneys from NHBD and conventional heart-beating donor (HBD) organs is the length of the warm ischaemic time. This is minimal in the HB situation but often considerable for NHBD kidneys. The inevitable consequence of this has been higher rates of delayed graft function and sometimes primary non function in NHBD series. Current efforts are concentrating on minimising the deleterious effects of warm ischaemic damage in order to facilitate safe access to the NHBD pool, which is potentially a very large source of transplant kidneys.

1.2 The distinction between heart beating and non heart beating donors

The distinction between HBD and NHBD lies in the mode of death and the way in which it is diagnosed. To paraphrase Pallis[10], death can be defined as the irreversible loss of the capacity for consciousness and the irreversible loss of the
capacity to breathe, hence to maintain a spontaneous heart-beat. Although death most commonly results from cardiopulmonary arrest and is diagnosed using cardiac criteria, it may also arise from catastrophes within the head and be diagnosed using brainstem criteria. In heart beating donors, death has been diagnosed using brainstem criteria and in non heart beating donors, death has been diagnosed using cardiac criteria. In cases of brainstem death the patient has always been ventilated prior to their death and the heart remains beating at the time of organ retrieval. This along with carefully executed in situ organ cooling allows the virtual elimination of a warm ischaemic period. By definition the kidneys are removed from NHBD’s after a variable period of cardiac arrest and hence warm ischaemia.

1.3 Controlled and uncontrolled NHBD’s

An important distinction needs to be made between controlled and uncontrolled NHBD’s. Prior to the introduction of legislation defining brainstem death criteria, all cadaveric kidney transplants were performed using controlled NHBD’s. This involved withdrawal of ventilation leading to cardiac arrest and the subsequent retrieval of organs for transplantation. The important point which defines the controlled donor is that the cardiac arrest is planned. In appropriate circumstances this allows the ventilator to be switched off in the operating theatre in order to facilitate rapid organ cooling and removal. This may be achieved either by in situ cooling using an aortic catheter placed via a femoral artery cut-down or by performing a rapid laparotomy, removing the kidneys en bloc and then cooling them ex-vivo. This relatively controlled procedure allows kidneys to be removed with warm ischaemic times of less than 10 minutes. In the uncontrolled donor, cardiac arrest occurs suddenly and is unplanned. The commonest example would be a myocardial infarction complicated by an arrhythmia, but other causes of death such as
trauma may be included in this group. If all efforts to resuscitate the patient fail, then
death is pronounced and organ donation may be considered. In these uncontrolled
NHBD's, the warm ischaemic period is likely to be much longer when compared to
controlled NHBD's. These considerations need to be borne in mind when interpreting
the literature.

At the first International Workshop on NHBD's in Maastricht, the
Netherlands, four categories of donor were identified [11, 12]. Category I (dead on
arrival) refers to patients who have died outside the hospital. The likely causes of
death would be severe head injuries or high cervical fractures which were
immediately fatal. In order to be able to retrieve viable organs in this situation, the
cadaver needs to be urgently transferred to a suitably experienced hospital and
consent obtained as a matter of urgency. This, however, may present great difficulties.
These obvious logistical problems present a serious barrier and experiences of this
category remain limited. Category II donors have died after an unsuccessful attempt at
resuscitation either in the emergency room or a general ward; these have been the
main source of uncontrolled NHBD's. The cause of death may be cardiac, traumatic
or an intracerebral haemorrhage. As the circumstances and place of death vary, there
is a considerable range of possible warm ischaemic times. Category III donors
( awaiting cardiac arrest) are derived from patients ventilated on an intensive care unit
in which a clinical decision is made to withdraw life-support measures. This category
defines the traditional controlled NHBD. As the ventilator switch-off procedure is
planned, the warm ischaemic time can be limited. Category IV defines intensive care
based donors who have been diagnosed as brainstem dead but in whom cardiac arrest
occurs unexpectedly. This is a relatively uncontrolled situation but in units with a
rapid response NHBD team the warm time is usually less than that associated with
category I or II donors. This classification is helpful in discussing some of the legal
and ethical issues surrounding NHB donation. It also highlights differences in the
potential for organ viability between the groups. In this regard, warm ischaemic time is the most important factor and it is simpler to refer to controlled and uncontrolled NHBD's.

1.4 Warm ischaemic renal injury

Warm ischaemia is known to be a major determinant of renal function after kidney transplantation. Nonetheless, the amount of reversible warm ischaemic injury that the human kidney can sustain is still not known for certain. Porcine kidneys, which are arguably the most relevant model of the human situation, have shown the capacity to recover from 120 minutes of warm ischaemia in a cyclosporine based transplant model [13]. Most human NHBD kidney transplant protocols would exclude kidneys with such prolonged warm times, the usual cut-off being in the region of 30-45 minutes [9, 14, 15]. Renal allografts from controlled NHBD’s may function immediately, suggesting that the human kidney tolerates short periods of warm ischaemia quite well [16]. In uncontrolled NHBD’s the warm time is not always accurately known. It may need to be determined by taking a history from relatives, ambulance staff and medical personnel and in some cases this will provide only a rough estimate.

The period of warm ischaemia is usually defined as the time between cardiac arrest and the start of cardiopulmonary resuscitation. This could more accurately be described as the absolute warm time as there may be other, less obvious, periods of warm ischaemia. The efficacy of external cardiac massage in achieving renal perfusion is not definitely known and will vary according to how well resuscitation is performed. It is likely that a degree of renal warm ischaemia is common during CPR and this could be described as relative (as distinct from absolute) warm time. On the other hand, cardiac massage and ventilation which is effective in oxygenating the
kidneys after a period of circulatory standstill may also be deleterious by initiating the reperfusion injury syndrome.

NHBD kidneys which have suffered prolonged periods of warm ischaemia have been transplanted with success. The series described by Koffmann et al [17] included some donors dying in a hospice remote from the retrieving hospital and 11 kidneys were transplanted after warm times of 46 to 65 minutes. One year creatinine was significantly worse in this prolonged warm ischaemia group but there was no difference in graft survival when compared to NHBD kidneys transplanted after shorter periods of warm ischaemia.

The warm ischaemic period is not the only factor responsible for damage to cadaveric kidneys. Rowinski et al [18] suggest that haemodynamic, metabolic and hormonal factors in the pre-agonal phase also influence the functional results of kidney transplants from marginal and NHBD’s. An example would be a prolonged period of hypotension leading to oliguria or even anuria. These influences are likely to be more important in controlled NHBD where the pre-agonal phase may be prolonged.

1.5 Protocols for donor management

Donor management varies between institutions and there has been a call for a standardised protocol [19]. Although a period of warm ischaemia is inevitable in all NHBD’s, this must be reduced as far as possible and the main requirement for this is a rapid response team who are equipped to perform rapid organ cooling and retrieval. Some groups recommend continuation of external cardiac massage and ventilation with 100% oxygen after death [15, 19]. This can be carried out manually or by using a mechanical resuscitator [15, 20]. It is difficult to be certain of the effectiveness of these manoeuvres in providing significant renal perfusion in a NHBD. Two pieces of
indirect evidence suggest they are worthwhile. First, analysis of blood samples taken from the intra-aortic catheter immediately prior to the start of the in situ perfusion clearly demonstrates that the aortic blood is well oxygenated (PaO₂ 20-25 kPa; personal communication from Professor ML Nicholson). Secondly, Szostek et al have reported successful transplantation of NHBD kidneys after over 4 hours external cardiac massage and ventilation [20].

In NHBD's prolonged cardiac massage and ventilation may be required and this is very labour intensive if it is carried out manually. Moreover, the medical and nursing staff involved must bear the psychological burden of performing cardiac massage and artificial ventilation on a dead body; because of these considerations, some groups have abandoned these procedures [21].

Heparin can be administered in an attempt to prevent intravascular thrombosis and alpha blockers, such as phentolamine, have also been given to try to reduce intrarenal vasospasm. These agents may be administered either by intracardiac injection or in the first bag of preservation fluid. Although the efficacy of these interventions has not been formally tested, they are simple to perform, based on sound principles, and at the very least are unlikely to have any adverse effects.

1.6 Kidney cooling techniques

Although kidneys are usually stored at very low temperatures in ice prior to transplantation, the first target is to reduce the core temperature to 10-15 °C as this is the temperature at which oxygen consumption is only 10% of normal [22, 23]. The sodium-potassium ATPase pump, which is responsible for 85% of renal tubular oxygen consumption, ceases to function at these low temperatures [24]. Three main methods of in-situ renal cooling have been described in the NHBD situation:
1.6a Intra-vascular cooling

This has been the simplest and most popular method to date. The technique uses a version of the double balloon-triple lumen (DBTL) catheter first described by Garcia-Rinaldi in 1975 [25]. The femoral artery and vein are exposed by a cut-down made in the groin. The DBTL catheter is then introduced into the aorta through an arteriotomy in the common femoral artery. Correct positioning is important and the catheter is designed so that when the lower balloon is jammed at the aortic bifurcation, the upper balloon is positioned above the renal arteries, thus isolating the renal circulation. The position of the balloons can be checked by inflating them with dilute radiographic contrast media and if the radiograph is made available quickly, it is possible to reposition an incorrectly placed catheter [26]. The kidneys are then flushed with preservation fluid held at 4 °C and the system is vented by placing a return catheter in the femoral vein or inferior vena cava. On occasion introduction of the intra-aortic catheter may prove difficult or even impossible as a result of narrow, tortuous or atheromatous iliac arteries. Palpation of the loin during in situ perfusion can give a guide to whether or not the aortic catheter is correctly positioned; the loin cools quickly in comparison to the surrounding tissues when the catheter is correctly positioned. A common error is to pull the lower aortic balloon too far caudally so that it sits in the common iliac system. Under these circumstances the contralateral leg will also be perfused with preservation fluid and this may be detected clinically by palpation of the temperature of the legs. The efficiency of organ perfusion can be assessed by inspection of the effluent preservation fluid. Initially the effluent is very blood stained but when the system is working efficiently this reduces quickly and may clear completely. Perfusion efficiency can also be judged to some extent by the rate of flow of perfusate. A flow rate of 100-200 ml per minute when the perfusate reservoir is held one metre above the kidneys suggests that the system is working well. The size
of the venous vent is an important rate limiting factor of perfusate flow and non-compressible large-bore (>24 gauge) cannulae should be used whenever possible.

A number of improvements to DBTL catheter design have been made in recent years. Catheters are now manufactured using harder plastics, which being less deformable, are easier to introduce. The distance between the balloons has been increased in order to reduce the chance of the upper balloon being positioned at or below the renal arteries and this modification has also allowed an increase in the number of side perforations between the balloons. The incorporation of secondary control balloons allows the operator to be certain that the main balloons are correctly inflated and have not ruptured. Finally, experimental studies have demonstrated that high flush pressures (70 mmHg) produce far more effective cortical perfusion than lower pressures of 30-50 mmHg [27]. In order to control perfusion pressure some units use roller pumps to control the rate of perfusate flow and a four-lumen catheter has been developed in order to allow continuous pressure monitoring [28, 29].

1.6b Intra-peritoneal cooling

This has been used alone or in combination with intra-vascular cooling. In the method described by Orloff et al [23] a 32 Fr chest drain tube is inserted deep into the pelvis via a 5 cm supraumbilical incision. A Foley catheter with a 30 ml balloon is introduced through the same wound to occlude the fascial defect and the skin is then closed to complete the seal. Surface cooling is achieved using Ringer's lactate solution at 4 °C infused through the chest drain and drained via the Foley catheter. An emergency laparotomy is then performed for organ retrieval. Rapid kidney parenchymal hypothermia is critical in limiting warm ischaemic injury and it is logical to suggest that more efficient cooling techniques might be associated with better results following NHBD kidney transplantation. A combination of intra-peritoneal and intra-vascular cooling has been shown to speed up the rate of kidney
cooling in the experimental situation but there was no apparent benefit to clinical transplantation [30, 31]. Light’s group in Washington have further improved the combined intra-vascular and intra-peritoneal method by the addition of a cooling coil to a closed recirculating system for intra-peritoneal cooling [14, 32]. The efficiency of the cooling coil has been increased by immersing it in an ice-alcohol mixture which reaches sub-zero temperatures. This allows an intra-peritoneal temperature of 10 °C to be achieved in only a few minutes. These thoughtful modifications have also yielded improved results in the clinical setting, with a reduction of delayed graft function rates in kidneys taken from uncontrolled NHBD.

1.6c Extra-corporeal total body cooling

This can be performed using a simple cardiopulmonary bypass circuit [33, 34]. Large bore cannulae are placed in the femoral artery and vein and connected to a simple bypass circuit consisting of a roller pump, oxygenator and heat exchanger. The system is primed with a combination of saline, gelatine hydrolisate (Haemaccel), mannitol, bicarbonate and heparin. Initially bypass is performed at a temperature of 37 °C and a pump flow of 2 litres per minute. These parameters are maintained for 15-20 minutes and then a rapid cooling stage is performed by reducing the heat exchanger temperature to 4 °C. The body core temperature is monitored using an oesophageal thermometer and once this has fallen to 15-18 °C, this temperature is maintained until the kidneys are retrieved. The relative complexity of this technique is a disadvantage. Although the machinery required is standard equipment in cardiothoracic units, it is expensive and does require some expertise and training to use effectively. The advantage of the method is that it is possible to maintain extra-corporeal circulation for many hours. This can be very helpful in a situation where consent for organ donation is delayed, for example when time is required to contact family members and to transport them to hospital.
1.7 **Immunosuppressive protocols**

The accumulated literature has paid relatively little attention to the importance of different immunosuppressive protocols in NHBD kidney transplantation. In general the reported NHBD series remain relatively small and it is common for several different immunosuppressive regimens to have been used in the same series [35, 36]. As with other forms of renal transplantation, immunosuppressive regimens have been based around the calcineurin inhibitors. Triple therapy with cyclosporine, prednisolone and azathioprine or quadruple therapy (with the addition of anti-lymphocyte globulin) has been the most commonly used protocols [17, 18, 35-45].

Induction therapy using either the monoclonal antibody OKT3 or polyclonal anti-lymphocyte preparations have been used in an attempt to avoid the double insult of warm ischaemia and early cyclosporine nephrotoxicity. A non-randomized comparison of high dose cyclosporin (14 mg/Kg/day) with oral prednisolone versus low dose cyclosporin (6 mg/Kg/day) with oral steroids covered by anti-lymphocyte globulin for 14 days has been reported in controlled NHBD kidney transplants [38]. Early allograft function and graft survival were better in the low dose cyclosporin/ALG group and this was particularly the case if the warm ischaemic time was 10 minutes or less. In a similar study of 108 controlled NHBD transplants, Asano et al [46] found that a low dose cyclosporin, steroid and azathioprine triple therapy regimen was associated with significantly better one year graft survival [47], than a high dose cyclosporin and steroid protocol [41] or a low dose cyclosporin, steroid and ALG group [35].

Kehinde et al used OKT3 induction with steroids but without cyclosporin in the first 17 uncontrolled NHBD transplants performed in Leicester [40]. Six patients developed neurological complications which were attributed to OKT3. The clinical syndrome included confusion, hallucinations, temporary blindness, irritability and
hyper-reflexia; three patients went into a coma and required mechanical ventilation. An increased risk of OKT3 related side-effects has been reported with delayed graft function [48] and Kehinde et al concluded that the neurological syndrome was due to poor clearance of undefined uraemic toxins and cytokines. The neurological syndrome was not seen after the OKT3 protocol was replaced by low cyclosporin, prednisolone and azathioprine triple therapy [41]. Schlumpf et al [39] used ATG induction therapy to avoid the early use of cyclosporin in a series of controlled NHBD transplants. Renal function and graft survival at one year were very good and neither neurological nor cytokine release side-effects were seen. The role of antibody induction therapy in NHBD transplantation is still unclear. Randomized comparisons with straight forward calcineurin inhibitor therapy do not appear to have been performed specifically for NHBD kidneys.

Experience with tacrolimus and the other newer immunosuppressive agents in NHBD kidney transplantation is more limited. Ohshima et al [45] found that graft survival was better in a series of 26 controlled NHBD transplants treated with tacrolimus 0.3 mg/Kg/day and oral steroids compared to low dose cyclosporin (4 mg/Kg/day), oral steroids and a 21 day course of anti-lymphocyte globulin. In the same paper the tacrolimus regimen was compared to a low dose cyclosporin, steroids and 14 day course of ALG followed by mizoribine. Although this was not a randomized comparison, these regimens were adopted at the same time in an effort to minimise the incidence of late acute rejections which had been seen with low dose cyclosporin and ALG regimens. Tacrolimus appeared to be the better option in this experience. Less late acute rejection was seen in the tacrolimus group (12 vs. 21) and graft survival at three years in the tacrolimus and mizoribine groups were 80 and 67 respectively. A group from Nagoya, Japan has compared cyclosporin and tacrolimus regimens in non-randomized studies of controlled NHBD kidneys transplants [47, 49]. The early results showed lower DGF and acute rejection rates in the tacrolimus group.
but long-term follow up did not show any statistically significant differences in graft survival rates and further data is needed to define the relative impact of these two calcineurin inhibitors.

There is considerable potential for trials of other newer immunosuppressive agents in NHBD transplantation. Mycophenolate mofetil and sirolimus are non-nephrotoxic and may prove to be useful as substitutes for ciclosporin and tacrolimus. Combination therapy with one of the IL-2 receptor monoclonal antibodies (Daclizumab or Basiliximab) may also prove beneficial in kidneys which have suffered a considerable degree of warm ischaemic damage. As the current rate of delayed graft function in NHBD kidneys is so high, it should be possible to demonstrate the effectiveness of a new regimen with quite small numbers of patients. Graft survival is a more important but more difficult end-point. In many NHBD series graft survival is very good despite high rates of delayed graft function. This has two consequences: first, very large numbers of patients will be required to show any differences between immunosuppressive drugs and second, there may be considerable reluctance to change established protocols which already yield excellent results.

1.8 Results of NHBD kidney transplantation

The literature relating to NHBD kidney transplantation has increased significantly over the last few years and many series are now available for analysis. Results must be interpreted carefully as most are single centre reports containing only small numbers of patients. As always it is important to compare like with like and particular attention must be paid to whether donors were controlled or uncontrolled.
1.8a Primary non-function rates

In the majority of NHBD series, the incidence of primary non-function (PNF) has been higher than the low single figure percentage rates expected, and achieved, in heart-beating cadaveric and live donor kidney transplants. PNF rates greater than or equal to 5 per cent have been recorded in a significant number of NHBD programs [1, 35, 36, 50-57]. Early primary allograft thrombosis is the principle cause of PNF in heart-beating cadaveric and live donor transplants but does not account for all the early failures in NHBD kidneys, many of which are due to ischaemic non-function as a result of renal cortical necrosis. On the other hand, a number of authors have reported a zero incidence of PNF in small series of NHBD kidney transplants [16, 20, 42, 58, 59]. These facts highlight the pressing need for effective pre-transplant tests of viability.

It is difficult to be sure of the relative rates of PNF in controlled and uncontrolled NHBD kidneys as few series include only uncontrolled donors and mixed studies tend to report pooled data for their controlled and uncontrolled groups. Nevertheless, it is clear that significant levels of PNF can occur when only controlled donors are being used, an example being a 9 per cent non-function rate in 239 controlled NHBD kidney transplants performed in Japan [53]. Only one report of uncontrolled donors has described a zero PNF rate and this consisted of only 8 transplants [20].

1.8b Delayed graft function

Relatively poor early graft function is to be expected following NHBD kidney transplantation and is reflected by the high proportion of patients requiring dialysis support in the early post-transplant period. This is a direct result of warm ischaemic injury leading to acute tubular necrosis and it is not surprising that kidneys from uncontrolled NHBD are invariably associated with delayed graft function (DGF) rates
of 50 to 100 per cent [1, 15, 52, 55-57, 59-61] compared with rates of 20-60 per cent in heart-beating donor kidneys [62-64]. Although NHBD kidneys from controlled donors also tend to have a high level of DGF, some controlled series have reported dialysis rates which are similar to those expected with HB cadaveric donor kidneys [23, 65].

The influence of delayed graft function (DGF) on the outcome of renal transplantation has been controversial. Several studies have associated DGF with poorer graft survival rates [62, 64, 66-72]; however, a smaller number of studies have found no such relationship [73-75]. Prolonged DGF has been reported to be particularly deleterious, with a high incidence of graft failure being attributed to the development of accelerated chronic rejection [67]. It is perhaps surprising then that a number of series of NHBD kidneys have demonstrated long-term graft survival rates which are equivalent to those achieved with HBD [1, 2, 76]. In Leicester, DGF has been shown to adversely influence renal allograft survival from HBD [68] but not from uncontrolled NHBD [2]. Experimental data relating to the effects of brainstem death might be of importance in explaining this apparently contradictory finding.

Takada et al [77] have shown that explosive brainstem death leads to upregulation of the genes for a number of proinflammatory mediators in peripheral organs, including the kidneys. If these findings are reproducible in humans it is likely that organs from heart-beating brainstem dead donors will be more prone to early host inflammatory and immunological responses. The sudden death of uncontrolled NHBD’s would preclude such changes in gene expression and paradoxically it may be that NHBD kidneys are less prone to the effects of the ischaemia-reperfusion injury syndrome because of this.

Kidney transplants complicated by DGF have been shown to have a high financial cost when compared to allografts with initial function [78]. This is accounted for by a combination of longer in-patient stays, the requirement for dialysis...
until the resolution of ATN and higher drug, radiology and laboratory costs. Despite this, successful kidney transplants from NHBD are likely to be more cost effective than any form of dialysis.

1.8c Acute and chronic rejection rates

Only a minority of series have reported on the acute and chronic rejection rates in NHBD kidney transplants. The relationship between DGF and acute rejection rates has long been debated but as there is a significant amount of evidence to suggest that rejection rates are higher in kidneys with DGF [79] it might be expected that NHBD kidneys would also have higher rejection rates. In fact, the available data does not support this contention as rejection rates for NHBD and HBD kidneys are very similar [1, 2, 35, 36, 42, 56]. The study performed by Cho et al [76] is the only large comparative study which demonstrates more acute rejection in NHBD transplants; the rejection rates for NHBD and HBD transplants were 19 and 14 per cent respectively. Despite the fact that this represents a statistically significant difference it could be argued that such small differences are not of clinical significance. Moreover, these are low levels of rejection in comparison to European series which presumably can be explained by differences in HLA matching and immunosuppressive protocols. In contrast, graft loss due to acute rejection in the first 30 post-transplant days has been reported in 2 out of 21 NHBD kidneys transplanted under quadruple immunosuppressive therapy [42].

There is even less data relating to chronic renal allograft rejection in NHBD transplants. It may be hypothesised that NHBD kidneys are more susceptible to chronic rejection in view of the level of early warm ischaemic injury sustained.

Although the pathophysiology of chronic rejection is multifactorial and still not fully understood, the final common pathway to fibrosis appears to be a stereotyped response of the kidney to early injury. Despite these theoretical
considerations the reported chronic rejection rates after NHBD kidney transplantation range from 8 to 25 per cent, which does not seem unduly high [20, 35, 36, 56, 65]. In the comparative study described by Schlumpf et al [65] NHBD kidneys were compared to the same number of HBD control kidneys performed during the same time period and matched for sex, age, number of transplants and year of transplantation. During the follow up period of 4 – 95 months, 6 kidneys in both the NHBD and HBD groups failed as a result of chronic rejection. It is clear, however, that much more data is required in order to be certain about the relative acute and chronic rejection rates in NHBD and HBD kidney transplants.

1.8d Renal function

A number of studies have demonstrated that kidneys from NHBD can achieve early serum creatinine levels which are in the normal range [16, 53, 76, 80, 81]. Other series have recorded one-year or 'best ever' creatinine levels which are higher than expected for HBD transplants [20, 36, 52, 61]. Follow up data from some studies suggests that, in the longer term, average renal allograft function seems to be poorer in NHBD than in HBD kidneys. One year creatinines range from 152 µmol/1 (1.73 mg/dl) [64] to 248 µmol/1 (2.8 mg/dl) [20] and as expected kidneys from uncontrolled NHBD tend to have poorer renal function than those from controlled NHBD [61]. The Leicester experience mirrors these findings in that a small majority of NHBD kidney recipients achieve normal post-transplant renal function, but in the remainder serum creatinine levels never fall below 200-300 µmol/1 (2.3-3.4 mg/dl) at any time in the post-transplant period. Although this is disappointing, in many cases where the 3 month serum creatinine value is elevated there is not a dramatic decline in renal function with follow up over several years. The implication is that some NHBD kidneys, although viable have a limited number of functioning glomeruli as a result of
the initial ischaemic damage which may cause patchy ischaemic necrosis. Although there is literature to suggest that the quality of early HBD allograft function predicts long-term graft survival, this relationship has not always been found and may not be the case for NHBD kidney transplants.

1.8e Patient and graft survival data

One year patient survival ranges from 75 to 100% and one year graft survival from 40 to 100% [1, 35-37, 39, 42, 50-61, 80-91]. Such wide ranges are difficult to interpret and it is more useful to note that one year graft survival rates of 80 or over have been reported in a significant number of NHBD publications [2, 20, 36, 42, 55, 59, 61, 65, 76, 81]. These impressive graft survival figures have been achieved using both machine perfusion and cold storage. Although there is certainly a trend towards better graft survival when using only controlled donors, figures of greater than 80% can also be attained using uncontrolled donors [2, 55, 61].

The best series' make a comparison with the results of a parallel group of HBD transplants and the papers from Maastricht [1] and UCLA [76] are especially notable. In the Maastricht series 57 NHB kidneys were compared to 114 HB control kidneys matched for risk factors including immunosuppressive protocol, panel reactive status, donor age and type of preservation fluid. The UCLA series used the UNOS kidney transplant registry to compare the outcome of 229 NHBD transplants with 8718 heart-beating grafts performed in the same centres during the same time period. Both series included data from controlled and uncontrolled NHBD. The most important finding of these carefully conducted studies was that graft survival for HB and NHBD kidneys was very similar. In the UCLA study one year graft survival in the HB and NHBD groups was 86 and 83 per cent respectively. The Maastricht study had longer follow up data and reported 5 year graft survivals of 55 and 54 per cent for the HB and NHB groups respectively.
1.9 Potential for NHBD’s to solve the organ shortage problem

The size of the conventional brainstem dead heart beating donor pool seems to be contracting in many countries. Powerful and probably irreversible demographic changes underlie these trends. To use the UK and Europe as examples, there have been dramatic falls in the death rates from head injury and intra-cranial haemorrhage over the last 20 years. Although these falling death rates are welcome in themselves, they have had serious consequences for transplant programs. This has been a major impetus to re-examine the role of NHBD kidney transplants. Interested and motivated transplant units have shown that NHBD kidneys can make a very significant contribution to the overall transplant rate in single centres. During an experience lasting a decade, 21 per cent of kidney transplants in Maastricht were from NHBD sources [52]. Whilst no-one would argue against such impressive levels of activity, the proportion of NHBD kidneys in individual programs does not necessarily represent a genuine increase in transplant activity as the supply of HBD kidneys may be decreasing at the same time. A later publication from Kootstra's group showed that 40% of kidney transplants performed in a one year period were from NHBDs, but this did not increase the transplant rate compared to other years [54]. The Leicester experience has been similar. Over the last 8 years approximately 20 per cent of the kidney transplants performed in Leicester have come from NHBD, but during this period the overall transplant rate has remained steady. Although it might well be that the transplant rate would have fallen by 20 per cent without the NHBD program, another explanation is that the concentration of effort and resources on the NHBD kidneys may have resulted in a fall in the transplant rate from other sources such as live donors. In short, the professionals involved in organ procurement have only a finite amount of effort that can be concentrated on improving the transplant rate.
The potential pool of NHB kidneys donors has been estimated by retrospective review of hospital deaths [92]. This study suggested that a total of 603 in-hospital deaths in a single year could have yielded between 27 and 56 NHBD and that this could increase the kidney transplant rate by a factor of 2 to 4.5 fold. The same authors cite a similar study from the United States which used a computer algorithm of hospital deaths to predict possible NHBD numbers. The estimated potential number of NHBD was 123 per million population, which was twice as high as the projected pool of heart-beating donors.

Terasaki has proposed a strategy for the elimination of the kidney transplant waiting list in the United States [93]. The linchpin of this model is the use of NHBD to bridge the current considerable gap between the supply and demand for renal transplantation. Terasaki's calculations show that an increase in NHBD to 700 per annum over a 14 year period will yield approximately 10,000 extra kidneys. At this rate NHBD could replace the use of unrelated and related live donor kidneys and eventually remove the need to use older heart-beating cadaveric donor organs. If this plan was successful, 60 per cent of donors would be NHBD, 40 per cent would be HBD and there would be no need to use living donors at all. This model, whilst commendably optimistic, is dependent on a number of assumptions which may not hold true and the proposed outcomes may well not be attainable. Many transplant physicians and surgeons remain to be convinced of the benefits of NHBD kidneys, regarding them as second class organs. This would certainly appear to be the predominant attitude in Great Britain where only a few units have established a NHBD program. A further disincentive is the considerable extra work-load created by NHBD transplantation [60] and many units may not feel that they have the resources to meet these requirements.
1.10 Legal and ethical issues

Some of the most interesting and difficult issues surrounding the use of NHBD kidneys are legal and ethical ones. There are two major ethical issues: The first of these relates to the diagnosis of death using cardiac rather than brain criteria and must include some discussion of the definition of unsuccessful resuscitation. The second issue involves the place of in situ organ perfusion before consent.

1.10a The diagnosis and definition of death

Issues relating to the pronouncement of death depend upon whether death is sudden, resulting for example from a sudden cardiac arrest or planned, as in the case of a decision being made to switch off a ventilator. In both situations decisions must be made by physicians who are completely independent from the transplant team; although this may be regarded as a fundamental principle, in practice it cannot always be arranged [21]. In the case of an unplanned cardiac arrest, the medical team will perform cardio-pulmonary resuscitation measures until the point when it is decided that further efforts will be futile. CPR will then be abandoned and the patient pronounced dead. Only at that time should the issue of organ donation be raised. The problem is that the resuscitation efforts made and the timing of these probably vary quite considerably according to the clinical situation and the particular doctors involved. This potentially exposes the physicians involved to a conflict of interest. The only way to eradicate this is to develop written resuscitation protocols which specifically define the procedures to be followed in all cases of cardiac arrest before resuscitation can be pronounced unsuccessful and so discontinued. Unfortunately most hospitals have not put such protocols in place. This can only be addressed by the medical groups involved in emergency resuscitation with the advice of their cardiological colleagues and ethical committees. The current consensus view is that
30 minutes of efficient cardiac massage (which produces a palpable femoral pulse) and ventilation with 100% oxygen via an endotracheal tube, with the use of appropriate intravenous or intracardiac drugs and with the observation of unresponsive, dilated pupils is enough to be certain that cardiac arrest is irreversible.

The 'dead donor rule' [94] is another fundamental requirement in organ donation. This states that no one should be killed by the act of organ donation and that a donor has to be dead when the organs are removed. This becomes relevant when a decision is taken to discontinue life support measures in a patient who does not meet the criteria for brainstem death and raises the question of when should death be pronounced after cardiac arrest. The Pittsburgh protocol [95] stated that the dead donor rule can be applied after 2 minutes of asystole defined as the absence of a femoral pulse and electrical activity. This definition poses a difficult issue. It is known that two minutes cardiac standstill does not necessarily lead to complete loss of brain function. This leaves the anomalous situation where death can be made on cardiac criteria at a point when brain criteria are not yet fulfilled. This cannot be acceptable in a Western society which widely accepts that death should be defined using brainstem criteria. To quote Pallis, all death is brain death, whether it results from a primary intracranial catastrophe or from the intracranial consequences of circulatory arrest [10]. The latter only proves lethal if it lasts long enough for the brainstem to die. It is much sounder to insist that cardiac arrest is prolonged enough not only to make resuscitation impossible but also to assure that there is irreversible loss of brain function. These considerations were taken into account at the Maastricht workshop in 1995. The conclusion of these discussions was that it was safer to apply the dead donor rule only after a 10 minute period of asystole [11, 96]. There can be little doubt that after normothermic ischaemia for this period it would be impossible to restore myocardial function and there will be irreversible loss of all neurological function. In short, the criteria for cardiac and brain death will have been satisfied.
simultaneously. Unfortunately, although the kidneys may recover from this degree of warm ischaemic damage the same is not true for other transplantable organs such as the heart and liver and this has limited clinical transplantation of solid organs from NHBD to kidneys only. In Leicester the 10 minute rule is obeyed in all NHBD retrievals, whether from uncontrolled or controlled donors. As well as ensuring that the dead donor rule is met, this period is useful for all the medical and nursing staff involved as it clearly marks the transition between an individual being considered as a patient to that of a potential donor.

1.10b Organ perfusion before consent

The question of whether or not it is acceptable to begin organ preservation before the permission of the family is obtained has stimulated considerable debate [96]. In order to reduce the warm ischaemic time it is necessary to cool the kidneys in situ as soon as possible after irreversible cardiac arrest. This may pose problems in the uncontrolled situation as there is very little time in which to approach the relatives to ask permission for organ donation. As already pointed out, one way of bridging the time between death and organ perfusion is to continue external cardiac massage and ventilation after the ten minute period of asystole. The ideal solution is to perform in situ cooling immediately after the ten minute asystolic period, the problem being that in many countries this requires consent from the next of kin. An exception to this rule can be made when the potential donor is carrying a valid donor card. In this case organ perfusion can be performed legally without the relatives consent. Although the majority of states in the USA prohibit perfusion prior to family consent, a number (e.g. Washington DC) have enacted legislation allowing the introduction of cooling devices before consent. In these cases organ procurement can only proceed if the family can be contacted and give permission to donation. In the Netherlands, it has been legal to introduce a cooling catheter prior to family consent since 1998. The
position in Great Britain is similar to the United States in that there is no national legislation covering this issue. Many cases of sudden death which occur within 24 hours of admission to hospital come under the jurisdiction of the local Coroner who may authorize a post-mortem examination without needing the permission of the family. In such cases the transplant team may also seek the Coroner's permission for in situ organ cooling to be performed prior to family consent. This judgment is currently made at the discretion of individual Coroners.

In Spain the existence of presumed consent legislation makes it much easier to justify in situ organ cooling prior to family consent. Alvarez-Rodriguez et al. do, however, make the important point that devices and drugs are currently used for the maintenance of organs after brainstem death has been diagnosed and it is only consistent to consider that devices and drugs in the form of intra-aortic catheter cooling techniques should be allowed for the maintenance of organs from NHBDs [56].

1.11 Summary

The continuing shortage of transplant organs has stimulated renewed interest in NHBD's. Despite a high incidence of delayed graft function, the results of kidney transplantation from NHBD are good. Long-term renal function is acceptable and one year graft survival rates of over 80 per cent have been achieved in many units. The available evidence suggests that acute rejection rates are equivalent for NHBD and HBD kidneys, but much more data is required. There is also a paucity of information relating to the development of chronic allograft nephropathy in NHBD kidneys.

NHBD's have provided a substantial proportion of the kidneys transplanted in some programs. What is less clear is whether NHBD's have actually been responsible for a significant increase in the transplant rate, rather than being a useful measure to
hold a diminishing transplant rate steady. The potential pool of NHBD’s is certainly very large and if this can be safely accessed then projections suggest that a very significant improvement in transplant numbers is possible.

NHBD’s raise their own ethical problems related to the diagnosis of death by cardiac rather than brain criteria and the acceptability of simple organ preservation procedures before the consent of the family has been given. NHBD organ preservation techniques are simple but require a rapid response team and generate a considerable increase in work-load for the transplant team and a number of related specialties.

The development of NHBD kidney transplantation has been generated by a relatively small number of enthusiasts and it will be interesting to see if the admirable results achieved can be generalised to the rest of the transplant community. It will be important in the future to separately identify NHBD kidney transplants in national databases so that the overall results will be open to scrutiny.
CHAPTER TWO

METHODS OF RENAL PRESERVATION

2.1 Introduction

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ii. Delayed graft function

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i. Primary non-function

ii. Delayed graft function

iii. Rejection rates

iv. Renal allograft function

v. Patient and graft survival

2.6 Viability testing

2.7 Summary
2.1 Introduction

Since the inception of kidney transplantation short and long term results have steadily improved. This has been due to a number of factors, including the increasing experience of transplant centres and transplant surgeons, better selection of donors and recipients, improvements in immunosuppressive regimens and kidney preservation. However, primary non-function (PNF) occurs with reported rates between zero [16, 20, 42, 58, 59] and 20% [1, 35, 36, 50-57, 83, 97] of all renal transplants. Of those that do work anything from 20 to 100% only function after a delay (delayed graft function) of anything from a few days to two months, with the majority starting to function after about two weeks [1, 15, 52, 55-57, 59-64].

The causes of primary failure and delayed allograft function are multifactorial, including donor and recipient factors, but probably the most important reason is the ischaemic damage suffered by the kidney prior to transplantation. This has both a cold and a warm ischaemic component. Organs from non-heart beating (NHB) donors are procured after final and irreversible cardiac arrest and therefore a period of warm ischaemia is inevitable. Warm ischaemia can also occur prior to retrieving conventional cadaveric kidneys if the donor is haemodynamically unstable. Cold ischaemia is also unavoidable with cadaveric donors, as time is needed to perform the tissue typing and cytological crossmatch, to transport the kidneys to their final destination, and to prepare the recipient.

Attempts can be made to ameliorate warm ischaemic damage in the cadaveric situation by pharmacological interventions by the intensive care team prior to retrieval. Limitation of warm ischaemic damage in the NHB donor can be accomplished by cooling the recipient kidneys whilst they are still in situ via various methods such as a triple lumen double balloon femoral catheter [25, 26], intra-
peritoneal cooling [23] or even extra-corporeal total body cooling [33, 34]. However, these kidneys will always sustain some warm ischaemic damage and the exact length of warm ischaemia is often unknown. It is known that warm ischaemia is an important factor in governing post transplant outcome; therefore, it would be beneficial to be able to assess these kidneys prior to transplantation. As kidneys from NHB donors and other so-called “marginal” donors become more prevalent there has been renewed interest in the further improvement and optimisation of preservation of all donor kidneys, but of these in particular.

The two major techniques available for preservation are cold storage and machine preservation. The cold storage method is founded on the premise that hypothermia causes minimisation of cell metabolism and a hyperosmolar preservation solution helps prevent oedema. The principle behind machine perfusion is that even when metabolism is very low, as with hypothermia, the kidney must be provided with nutrients and waste products should be removed. A substantial amount of work has also been done on the use of recorded perfusion parameters and the use of substances washed out of the kidney on perfusion as viability markers.

Preservation is an important, but often overlooked aspect of renal transplantation where controversy still prevails. For example, there is still debate about what is the best method of preservation for the various types of kidney in regards to both initial and long term function. Also, although an assortment of methods for pre-transplant viability testing have been reported no one test has become universally accepted and doubts still exist as to the reliability of such tests in predicting functional graft outcome.

The aim of this chapter is to give a general overview of the different types of preservation techniques available. This includes a brief history of preservation, the mechanisms of renal injury and how different types of preservation, and preservation
fluids, ameliorate damage, the advantages and disadvantages of each type of preservation with their short and long term results, and viability testing.

2.2 Preservation Methods

Carrel and Lindberg first developed kidney perfusion in 1938 [98], however it was almost two decades later before any further work was published in the area of kidney preservation. This was in 1956 by Bogardus and Schlosser who cooled canine kidneys to 23-26°C and showed that this resulted in marked protection from the functional damage produced by two hours of renal pedicle occlusion [99]. In 1963 Calne and colleagues reported that canine kidneys could be preserved for 8-12 hours by immersing them in ice water [100]. This work was taken a step further by Keller et al in 1966 who used an “intracellular” type preservation solution that was high in potassium and magnesium. This was to prevent the loss of potassium and magnesium ions that occurred in kidneys preserved with isotonic saline [101]. Humphries et al were the first to describe preservation by pulsatile machine perfusion in 1963 [55], followed by Belzer et al reporting the preservation of canine kidneys for up to 72 hours with continuous hypothermic perfusion in 1967 [102]. This was followed by reports in 1970 of successful clinical work with the same procedure [103]. Collins and colleagues subsequently reported successful preservation of canine kidneys for transplantation by initial flushing with a hypothermic “intracellular” type solution, followed by cold storage for up to 30 hours [104].

In subsequent years, the aim of the majority of studies has been to prove one preservation method is better than the other, rather than studies trying to improve either method. However, there have been studies where attempts have been made to
modify these preservation procedures to both optimise allograft survival and to prolong preservation time beyond 72 hours.

In the 1970’s pulsatile machine perfusion was the standard preservation method for procured renal allografts, although few written protocols existed [105]. A variety of studies comparing pulsatile machine perfusion with cold storage were then undertaken and these showed little benefit of pulsatile machine perfusion over cold storage [106] and therefore by the early 1980’s cold storage was almost universally adopted as the preservation method of choice for procured renal allografts. However, renewed interest was shown in pulsatile machine perfusion in the early 1990’s for two main reasons. Firstly, new preservation solutions were developed which seemed to improve results with pulsatile machine perfusion [107]. Secondly, the continuing and worsening donor shortage led practitioners to seek out new sources of kidneys for donation. As well as focusing attention on the use of living donation this led to interest being shown in kidneys which had previously not been considered for donation. These kidneys had not been deemed suitable because of a variety of reasons, including the advanced age of the donor, adverse donor medical history or because the donor was classified as “non-heart-beating” (NHB) or “asystolic”. In the case of NHB donors, death resulted in irreversible cardiac arrest before the organs were procured, rather than the commoner picture with conventional cadaveric donors where the donor is certified dead on the grounds of brainstem death despite the fact that the heart is still beating. Therefore, kidneys procured from NHB donors suffer an ischaemic insult, which is often of uncertain duration and severity. The length of warm ischaemic time is defined as the period from circulatory standstill until the start of organ cooling and does not include the period of time where attempted resuscitation is taking place.
At the beginning of the 21st century we are now at the stage where the use of pulsatile machine perfusion as opposed to cold storage is a widely debated and controversial issue. Centres world-wide take differing stances as to whether one method is superior to the other, and this stance often changes depending on the origin of the kidney. This will be looked at later, with comparison of the results of pulsatile machine perfusion with cold storage for conventional cadaveric and NHB donors, which are reported in various studies and papers.

Cold storage and pulsatile machine perfusion each have their own spectrum of advantages and disadvantages when the use of either is considered. On the positive side for cold storage is the ease and simplicity of the system. This means that cold storage does not require any specially trained personnel and leads to the easy transport of procured organs from centre to centre which therefore facilitates organ sharing. In these times of economic rationalisation the relative low cost of the procedure is also a benefit.

The main disadvantage of cold storage is the significantly higher rates of delayed graft function (DGF) reported in various papers [108, 109]. This leads to further costs: initially in monetary terms to support the patient over this period, as there is often an increased length of hospital stay and a period of dialysis before the kidney starts to function. The costs in the wider sense also include psychological costs to the patient and long-term costs to the kidney as some studies have suggested that initial DGF leads to poorer long-term allograft function [66, 110, 111].

The potential advantages of pulsatile machine perfusion are numerous, including:

i. Ability to provide metabolic support to the kidney i.e. the supply of oxygen and nutrients and the elimination of potentially toxic waste products.
ii. Ability to manipulate the flow through the kidney by pharmacological means. Various substances have been tried including calcium channel blockers, prostaglandin E1, mannitol, protease inhibitors and ATP [112-117].

iii. Allows for assessment of the kidney status during preservation. The flow or the pressure can be standardised, depending on the specific machine, then the other parameter can be measured and intrarenal resistance can be calculated. Renal tissue and perfusate effluent are also available for metabolic analyses.

iv. Some data exists showing that machine perfusion allows longer preservation times, especially when organs are being stored for greater than 24 hours [118, 119].

v. Lower rates of DGF have been reported which may improve long-term graft outcome.

Pulsatile machine perfusion does have drawbacks as well. It is technically more demanding and requires highly trained personnel. The procedure is certainly more complicated than cold storage and as such is vulnerable to equipment failure and human error. With the sterile, non reusable equipment which is often necessary it is also substantially more expensive than cold storage, although if pulsatile machine perfusion does result in lower rates of DGF as has been reported then this expense may be offset by the savings made due to shorter hospital stays and reduced dialysis post transplant. Another worry is the possibility of endothelial cell damage that has been reported [120].
2.3 Pathophysiology of Renal Injury

To understand the mechanisms by which preservation techniques and preservation solutions lead to kidneys being available for transplantation in as favourable a condition as possible it is essential to have some knowledge of the underlying changes in a kidney associated with ischaemia and hypothermia. This involves consideration of metabolic pathways within the cell and of the changes that take place inside the kidney as a whole.

2.3a Intracellular metabolic pathways: Normal

Before considering the changes associated with ischaemia and hypothermia, it is important to firstly look at the metabolism and ion flux pathways in the kidney cell under normothermic aerobic conditions. In the normal cell, multiple processes are occurring at the same time to maintain an ionic equilibrium. Firstly, the passive non-energy dependent processes include [121]:

i. Influx of sodium into the cell and efflux of potassium from the cell due to the high concentration of sodium within the extracellular space and the high concentration of potassium within the intracellular space.

ii. Sodium ions together with chloride ions also enter the cell, bringing water with them.

iii. Ionised calcium diffuses into the cell due to the large gradient of free calcium across the cell plasma membrane. This is from a concentration of approximately 1 mM in the extracellular fluid to 0.0001 mM in the intracellular space.
iv. Donnan effect (cause of intracellular oncotic pressure) draws water into the cell.

Active processes are dependent on the aerobic production of high-energy adenosine triphosphate (ATP) from its precursors within the mitochondria. ATP is then used to drive various ATPase pumps including:

i. Sodium-potassium-activated ATPase pumps out three sodium ions for every two potassium ions. This sets up a membrane potential that is negative on the inside of the cell.

ii. Sodium-calcium-dependent ATPase pulls in one sodium ion in exchange for extrusion of one calcium ion.

2.3b Intracellular metabolic pathways: Following ischaemic injury

Ischaemia by definition is due to an inadequate flow of blood to a part of the body (in this case the kidney). This therefore causes hypoxia i.e. a deficiency of oxygen within the tissues. When the supply of oxygen to the kidney, which functions primarily by aerobic metabolism, is interrupted the level of ATP within the cell falls rapidly. This is due to the cessation of ATP production by the oxygen dependent processes within the mitochondria. Depletion of ATP means that the cellular processes dependent on ATP that are described above cease to work. The active exchange of sodium for potassium stops and the membrane depolarises. The membrane potential was the only thing impeding the influx of chloride ions and these can now freely cross the plasma cell membrane, bringing sodium with them. The sodium-potassium-activated ATPase system also controls cell volume and once it stops the cell takes up water and swells. This cell swelling then causes obstruction of
the tubular lumen and further compromise of the regional blood supply [122]. I will discuss this in more detail later.

The membrane depolarisation also causes voltage-dependent calcium gates to open, allowing an influx of calcium. In addition, the sodium-calcium-dependent ATPase system ceases functioning, so calcium cannot continue to be extruded. The mitochondrial membrane also loses its potential allowing further increases in the cytosolic calcium level as calcium is also released from these intracellular stores. This accumulation of cytosolic calcium has been cited by some as the most detrimental effect of hypoxia leading to cell death, although others are unsure whether the changes caused by this elevation in intracellular calcium actually occur prior to irreversible cell death or as a consequence [24, 122, 123]. The effects of high levels of intracellular calcium are believed to include:

i. Activation of phospholipases A1, A2 and C, which leads to increased membrane phospholipid hydrolysis and the accumulation of free fatty acids. These further enhance the permeability of the cell and mitochondrial membranes leading to further cell damage and ultimately cell death.

ii. Alteration to the cell cytoskeleton. This is thought to interfere with volume regulation, causing further cell swelling.

iii. Activation of protease's in lysosomes and the cytosol, which could potentially contribute to tissue damage during ischaemia. There is, however, little information about their role during renal ischaemic injury.

iv. Binding of calcium to membrane proteins causing cell membrane rigidness. This is less important in the renal cell, which is static and more important in the erythrocyte where a degree of flexibility is required to transverse the smaller blood vessels. Therefore, ischaemia may result in these rigid
erythrocytes becoming trapped within capillaries and causing obstruction [124].

2.3c Intracellular metabolic pathways: Under hypothermic conditions

It was shown in the 1970’s that the sodium-potassium-activated ATPase system in both the canine and human kidney is still active in hypothermia, but reduced [125]. Therefore, the kidney can still exchange sodium for potassium, but at a much-reduced rate. The reasons for this reduced rate include the fact that the sodium-potassium-activated ATPase activity within the cell plasma membrane is itself decreased by hypothermia. Also, production of ATP from ADP occurs in the mitochondria by the action of the enzyme ATP synthetase. This is only slightly impeded by hypothermia, so ATP can still be produced. However, the enzyme adenine nucleotide translocase is necessary for the transport of ATP through the mitochondrial membrane into the cytosol and also for the transport of ADP back into the mitochondria. Southard et al in 1980 showed that the enzyme adenine nucleotide translocase is cold sensitive and therefore limits the rate that the precursor ADP enters the mitochondria [126]. This leads to a high level of ADP within the cytosol, which triggers the conversion of ADP by the enzyme adenylate kinase to ATP and AMP. AMP then further breaks down to adenosine which is permeable to the plasma cell membrane and therefore moves from the cytosol to the extracellular space. Outside the cell, adenosine is further broken down to inosine and then hypoxanthine. By this process, the precursors of ATP are lost from the intracellular environment. This results in a loss of ATP during cold preservation, which may be exacerbated by the method of pulsatile machine perfusion [121]. Consequentially, this limits the production of ATP at the time of transplantation when the kidney is reperfused.
therefore leaving the cell energy-depleted when normothermic conditions are resumed.

Hypothermia does lead to a loss of ATP and its precursors from the cell; however, hypothermia does have beneficial effects. It is known that hypothermia inhibits enzymatic processes, resulting in a two to threefold decrease in enzymatic reaction velocity for every 10°C of temperature reduction [127-130]. This is beneficial because not only does it inhibit degenerative processes that consume ATP such as gluconeogenesis [126], but it also inhibits processes such as phospholipid hydrolysis (which is increased by hypoxia as described previously) that would require ATP to reverse their effect [122].

2.3d Renal tubular cell and epithelial cell changes: Ischaemia and hypothermia

With both ischaemia and hypothermia, the vascular endothelial cell membrane can also be damaged as described above. The endothelium is an important component of the renal microvasculature, which is necessary for adequate vascular perfusion. As well as being an integral structural component of the renal microvasculature, the endothelium also plays a dominant role in the regulation of vascular tone by the release from it of vasoconstrictive factors such as endothelin and vasodilatory factors such as nitrous oxide. Any alteration to the renal endothelium may result in the important balance between these released substances from the endothelium being shifted so that the ratio of vasoconstrictive factors present is higher than the vasodilatory factors, leading to vasoconstriction and a further decrease in blood flow within the kidney.

As can be inferred from the above, another effect of ischaemia is an increase in vascular intrarenal resistance. The causes of this are multifactorial. They include
factors already mentioned such as swelling of the endothelial cells [131], loss of the ability of the endothelium to release endothelium-derived relaxing factor [132] and release of other local vasoconstrictive factors as well as other factors such as nervous stimulation.

As mentioned previously, disruption of the sodium-potassium-activated ATPase system also causes swelling of the renal tubular cells. Swelling of these cells within the outer medulla can impede blood flow through the vasa recta causing congestion and further tubule cell ischaemic damage. Both tubule cell swelling and the trapping of erythrocytes mentioned previously lead to the circumstance called the “no-reflow” phenomenon where there is failure of the circulation to return to a tissue upon reperfusion [133-135]. This is thought to be an important causal factor of reperfusion injury [136, 137].

The tubular cells themselves are also damaged in ischaemia. When they are damaged they form “blebs” that get released from the injured cell and lie within the lumen where they contribute to cast formation which in turn leads to tubular lumen obstruction, higher tubular pressures and reduction in single-nephron glomerular filtration rate [122].

2.4 Preservation Techniques and Solutions

To counteract the damage sustained by the kidney prior to retrieval and upon preservation, various methods of preservation and various preservation solutions have been considered. This work was pioneered by two people in the late 1960’s: Dr Folkert Belzer and Dr Geoff Collins [102, 104].
Dr Belzer et al, as previously described, reported three-day preservation of canine kidneys by continuous pulsatile machine perfusion. This was performed at between 6 to 8°C using a plasma-derived perfusate. The plasma that was used contained lipid material such as lipoproteins that aggregated during the hypothermic perfusion, therefore causing obstruction of the renal microvasculature. This problem was overcome by freezing, thawing and then ultrafiltrating the plasma to remove the lipoproteins. This new perfusate, called cryoprecipitated plasma (CPP), became the standard perfusate solution for human kidneys as the accounts from Belzer et al of initially canine and then human studies showed good results [102]. However, the plasma from which this perfusate was derived was obviously human in nature and this led to several disadvantages, namely batch-to-batch variability, complex preparation techniques and the potential risk of disease transmission [120]. All of this resulted in CPP falling into disfavour. Other solutions were tried such as a plasma based solution where the lipoproteins were removed by silica gel filtration [138], a Ringer’s-type solution which used human serum albumin for the colloidal osmotic support [139] and a solution using plasma protein fraction [120]. However, none of these solutions demonstrated results any better than those achieved with CPP and it wasn’t until the early 1980s that advances were made when Belzer’s group developed a synthetic perfusion solution that contained hydroxyethyl starch (HES) for the colloidal osmotic support instead of albumin. This HES-based perfusate is advantageous not only because of its shelf stability and the obliteration of any risk of infection [107] but also because subsequent studies have shown increases in preservation time of kidneys of up to seven days using this perfusate [118, 140, 141] and lower incidences of DGF as compared to the CPP [142].

Meanwhile, Dr Collins et al developed a method of cold storage to preserve kidneys, which does not require the use of a machine. This simply requires the
flushing out of the kidney with a solution and then storage in ice at 4°C. The eponymously named solution originally developed by Collins is called an “intracellular-type” preservation solution because it contains a high concentration of potassium and a low concentration of sodium. This is to help maintain the intracellular concentration of potassium despite the reduction in sodium-potassium-activated ATPase pump activity previously described. Collins solution also contains a high concentration of glucose (approximately 140 mM) to make it isosmotic. Collins reported successful preservation of canine kidneys for up to 30 hours using this solution. Since the development of Collins’ solution various modifications have been made. Originally, Collins’ solution contained magnesium at a concentration of 30 mM and phosphate at a concentration of 57.5 mM; however it was found that these concentrations exceeded the magnesium phosphate solubility product, thereby causing a precipitate and the formation of crystals around and within the kidney. These crystals were never shown to cause injury to the kidney, but were obviously visually disturbing. The original solution was therefore modified by the omission of magnesium, resulting in the solution known as EuroCollins (EC) solution [143].

Another cold storage perfusate solution was developed in Australia. This is called hyperosmolar citrate (HOC), or Marshalls, solution and is based on the use of citric acid, to neutralise alkaline sodium and potassium hydroxides, as the impermeant anion [144]. This solution has become popular in Australia itself and here in the United Kingdom.

Belzer et al also developed a cold storage perfusate solution that is called the University of Wisconsin (UW) solution. The perfusate additives used were chosen systematically to stimulate metabolism in hypothermically preserved kidneys [121]. Firstly, the addition of high adenosine and high phosphate concentrations in the perfusate prevents the loss of high-energy phosphate bonds during preservation [145],
thereby ensuring the kidney is not energy depleted at the time of transplantation and on reperfusion, as described before [146]. Calcium efflux is detrimental, and may be a cause of cell death (see previously) hence calcium is not added to the perfusate. The impermeable anion used in UW solution was initially gluconate. This has a molecular weight of 180 kDa (c.f. chloride with molecular weight of 37 kDa) which means that gluconate is significantly less permeable when the cell membrane potential dissipates with hypothermia. In later formulations of UW solution, lactobionate was substituted for gluconate as this has an even larger molecular weight (358 kDa) which subsequent studies on liver preservation have suggested is the single most important aspect of UW solution [147]. Glutathione is added as a reducing agent, for its potential antioxidant role in the cell. It has been shown to be beneficial in both liver [148] and kidney preservation [149]. Other substances have been added for their theoretical benefit, for instance steroids as a membrane stabiliser and Hepes to maintain normal extra- and intra-cellular pH [121].

2.5 Comparison of Machine Perfusion and Cold Storage

A range of studies comparing continuous pulsatile machine perfusion and cold storage have been performed over the years with very divergent conclusions being made at various times by different centres. This section reviews the literature looking at several parameters. Firstly, it examines the effect of donor type as regard the beneficial effects of machine perfusion or cold storage i.e. whether the results of these procedures are the same regardless of whether the kidney comes from a conventional “heart-beating” cadaveric donor or whether the kidney is from a non-heart-beating cadaveric donor. Secondly, it looks at several end points including:
i. Primary non-function (PNF) rates

ii. Delayed graft function (DGF) rates

iii. Rejection rates

iv. Renal allograft function

v. Patient and graft survival data.

2.5a Heart-beating cadaveric donors

i. Primary Non-function

PNF rates in cadaver kidneys which have been preserved by pulsatile machine perfusion are reported at rates between zero and 3% for preservation times of less than 48 hours [142, 150-152], rising to 9% for preservation times above 48 hours [119]. However, studies comparing PNF rates between the two preservation methods are few in number and the studies have been small. Merion et al in 1990 reported on a prospective controlled study comparing cold storage and machine perfusion in 51 paired kidneys. Those kidneys that were preserved by machine perfusion had a PNF rate of 2% compared to a rate of 6% for those kidneys in the cold storage group, however, this difference was not statistically significant [153]. Kumar et al in 1991 compared machine perfusion with cold storage using either EuroCollins or University of Wisconsin (UW) solution in cadaveric kidneys that had been preserved for longer than 48 hours. They reported PNF rates of 9% in the machine perfusion group, 16% in the EuroCollins group and 14% in the UW group. Again, although these results favoured machine perfusion they were not significant due to the small number of kidneys in the machine perfusion group (11 kidneys) [119].
ii. **Delayed Graft Function**

In renal transplantation the kidney often does not regain normal function immediately. This "delayed graft function" is usually defined as the need for dialysis in the first postoperative week following the renal transplant. DGF is multifactorial in nature and although hypothermic preservation undoubtedly causes some of the damage it is difficult in the clinical situation to separate the various elements, which must include donor, recipient, preservation and immunological factors. Vastly different rates of DGF have been reported from the use of heart-beating cadaveric kidneys, ranging from zero to over 80%. This section examines the studies that have been undertaken to try and establish which method of preservation is superior.

A small amount of animal work has been performed to compare the effect of cold storage and machine pulsatile perfusion on DGF. Gregg et al in 1986 used a total of 16 adult male dogs, 9 of which had their kidneys stored on ice and 7 of which had their kidneys preserved by machine perfusion for 24 hours prior to unilateral nephrectomy and autotransplantation. Of those kidneys preserved by cold storage 3/9 (33%) produced less than 1 ml of urine in the first two hours, compared to the machine perfused group where all 7 kidneys functioned immediately. Function in the machine perfusion group remained better to the end of week two. However, after one month of recovery there were no longer any significant differences in function between the two group's [154]. Further animal work was performed by Baron et al in 1990 where canine kidneys were preserved for a total of 96 hours by either machine perfusion or cold storage. The seven dogs in the cold storage group were all dead by day 4, whereas six of the eight dogs in the machine perfusion group survived with creatinines reaching normal levels by day 12 post-transplant [118]. The results of this study suggest that machine perfusion is definitely superior to cold storage for longer preservation times as regards immediate function; however, in these dog transplant
models the kidneys can be preserved for substantially longer than in the clinical situation with little evidence of the development of DGF [155] and so we need to look at human studies to understand better the clinical significance of these modes of preservation.

Only a handful of prospective randomised controlled trials have been performed comparing cold storage and pulsatile machine perfusion as the preservation method. These are all matched pair studies i.e. from each donor one kidney was machine perfused and the other was cold stored. All of the studies were small with the number of recipients in each group ranging from 10 to 51. Alijani et al in 1985 and Kwiatkowski in 1996 both showed significantly lower DGF rates with pulsatile machine perfusion [108, 109]. Alijani showed a drop in DGF from 63% to 17% with the use of machine perfusion and Kwiatkowski showed a similar drop from 80% to 40%, albeit in a study of only 20 recipients. Several other small prospective trials have also been performed showing DGF rates of between 28 and 41% after cold storage and of between 33 and 52% after preservation with machine perfusion; none of which were significantly different [111, 153, 156]. Nevertheless, a study by Heil et al in 1987 of 27 matched pair kidneys showed that whilst the DGF rates were not significantly different (41% after cold storage, 52% after machine perfusion), when DGF did occur the kidneys that had been preserved by cold storage had a significantly longer DGF time (14.9 ± 2.2 days vs. 9.9 ± 2.0 days) and poorer 1-year survival (74% vs. 89%) than those that had been preserved by pulsatile machine perfusion [111].

Much larger, non-randomised trials have been performed, the majority of which favour machine perfusion [119, 157-159], however there are also further trials maintaining that there is no difference between DGF rates for the two groups [152, 160]. In the USA, a very large retrospective study was performed looking at the entire United Network for Organ Sharing (UNOS) data from 1988 to 1995. This comprised
a total of 60,827 cadaveric kidney transplants. Multivariate logistic regression analyses were used and showed a 2.13-fold increase in the odds of requiring dialysis in the first week if the kidney was cold stored as opposed to machine perfused. If the donor was ≥55 years old then this odds ratio rose to 2.33 [161].

Although reducing the rate of DGF certainly reduces economic cost and length of hospital stay [108, 152, 162, 163], its long-term significance is disputed and I will discuss this later in the sections on renal function and graft/patient survival.

**iii. Rejection Rates**

A substantial body of work has been performed looking at the mechanisms behind the process of acute rejection and the role of endothelial cells is well-recognised [164]. This has led to theoretical concerns about the potential adverse effect of pulsatile machine perfusion on acute rejection rates. During machine perfusion, kidney endothelium is continuously exposed to the pulsatile flow and the hypothesis behind these concerns is based on the fact that mechanical injury to endothelial cells results in exposure of interstitial collagen and impairs barrier function, which promotes leakage of intravascular fluid with macromolecules such as fibronectin and fibrinogen. Fibronectin and collagen can then be recognised by circulating leucocytes through integrins pathway (VLA-5, VLA-1). Therefore, a site of inflammation is formed that may increase the risk of acute graft rejection [165].

The study by Kosieradzi in 1999 is the only one to have investigated this theoretical risk. Kidneys from 38 haemodynamically unstable donors were procured and preserved by either cold storage or machine perfusion. Standard triple immunosuppression therapy was used in both groups (cyclosporine, azathioprine and prednisolone) and rabbit antithymocyte globulin (ATG) was used as induction therapy for recipients of a second (or more) transplant. Patients were followed-up for between
7 and 37 months, with a median time of 22 months. 13 patients in the machine perfusion group had at least one episode of acute rejection with a mean rate of 0.57 episodes per patient, this compares favourably with the cold storage group where 19 patients experienced acute rejection, with a rate of 1.06 episodes per patient. This was not, however, statistically significant. Rates of steroid-resistant rejection were also similar, occurring in 15% of the machine perfusion group and 15% of the cold storage group, with 0.45 and 0.85 doses of ATG/OKT3 per patient respectively [165].

Although the number of patients in this study was small, this paper helps alleviate concerns that machine perfusion induces acute rejection.

iv. Renal Allograft Function

Very few of the studies comparing cold storage and pulsatile machine perfusion have focused on renal function as an end-point. There are contradictory reports from Baron and Gregg who have both performed animal experiments in this area. Baron, as mentioned previously, preserved 30 canine kidneys for 96 hours with either machine perfusion or cold storage. Creatinine levels in the machine perfusion group decreased rapidly from the highest levels on day 4 and reached normal levels by day 12. Those dogs in the cold storage group had significantly higher serum creatinines on day 4 than the machine perfusion group (14.7 ± 0.9 mg/dL vs. 6.9 ± 1.1 mg/dL) and died rapidly thereafter, however these results are merely a reflection of the fact that those in the cold storage group had DGF and therefore without any treatment died: these results do not tell us anything about the longer term outcome of preservation with either cold storage or machine perfusion [118]. Gregg performed similar work using kidneys from 16 male dogs that were preserved for 24 hours before contralateral nephrectomy and autotransplantation. As already discussed,
the rate of DGF was higher in the cold storage group and renal function recovered more rapidly in the machine perfusion group. However, by one month there was no longer any difference in creatinine clearance or glomerular filtration rate (GFR) between the two groups [154]. In 1976, Kraemer had performed a very similar study to Gregg; bilateral cervical autotransplantation of canine kidneys was performed, then preservation techniques were compared. Kraemer also found GFR measurements to be initially better in the machine perfusion group, but no difference was demonstrated after the one to two month period [166].

The study by Kwiatkowski [109] of twenty consecutive cadaver kidneys also shows a significantly higher creatinine in the cold storage group at day 7 post-transplant; however this can likewise be attributed to the fact that 70% of the kidneys in this group were still anuric or oliguric. No longer-term results were reported in this paper. The only report of better renal allograft function after one or two years following machine perfusion preservation is by Kosieradzki et al in their 1999 paper comparing the outcome of 38 kidneys procured from haemodynamically unstable cadaveric donors which were then preserved by either cold storage or machine preservation [165]. There were 37 kidneys in each group with one-year follow-up. Of these, 33 of the kidneys in the cold storage group had "good" renal function with a mean creatinine of 1.59 mg% compared to 35 kidneys in the machine perfusion group with "good" function and a mean creatinine of 1.31 mg% (statistically significant with a \( p \)-value of <0.05). After two years there was follow-up on 17 kidneys in each group. Again, those in the machine perfusion group had better renal function than those in the cold storage group with a mean creatinine of 1.05 mg% (compared to 1.77 mg%, \( p<0.01 \)).

The paper by Merion [153] reporting the results of a prospective controlled trial of 60 consecutive cadaveric renal donors comparing cold storage to machine
perfusion had contradictory results. Merion measured creatinine on day 1, 7 and 30 post-transplant and at no point was there a significant difference between the two groups.

\[ \textit{v. Patient and Graft Survival} \]

Of the small number of studies looking at patient survival after preservation of kidneys by machine perfusion or cold storage, no differences have been found [108, 167]. More studies have been performed investigating the relationship between initial organ preservation method and long-term graft survival. Several studies have shown a clear benefit in long term allograft survival after preservation by pulsatile machine perfusion [111, 168, 169]. One such trial was performed by Heil et al in 1987 [111].

Heil’s trial compared the effects of cold storage and machine perfusion on 27 kidney pairs. As mentioned previously, no difference in DGF was found between the two groups; however Heil did discover that when DGF did occur, 1-year graft survival was significantly poorer in the cold storage group than in the machine perfusion group (74% and 89% respectively, \( p < 0.05 \)).

Several papers have been published by Opelz and Terasaki between 1973 and 1982 which all report significantly lower 1-year allograft survival rates in those patients who received a kidney which had been preserved by machine perfusion than for those with a cold stored kidney [170-172]. The report in 1976 reviewed over 1000 transplants performed in 90 transplant centres between 1974 and 1975. The 1-year graft survival rate for cold stored kidneys was 59% whilst those preserved with Belzers perfusion method had only a 41% survival rate (106).

Most reports, however, have not shown any difference in allograft survival after preservation with either method [106, 108, 119, 156, 160, 162, 167, 173, 174].
The study by Scott in 1974 reported the results of cold storage and machine perfusion in two series, each of 100 cadaveric kidney transplants. From April 1969 until January 1972, 100 consecutive kidneys were preserved by cold storage and then between February 1972 and December 1973 a further 100 consecutive kidneys were preserved by machine perfusion. 1-year graft survival was very similar with rates of 63% and 65% respectively [160]. A retrospective analysis of the data from the UNOS and UCLA Transplant Registry between 1980 and 1991 was reported by Zhou and Cecka in 1992. They found no difference in 1-year graft survival between renal allografts preserved by machine perfusion versus cold storage [175]. A prospective study of 18 paired kidneys was performed by Veller et al in 1994 which again showed similar 1-year graft survival in both groups: Those preserved by cold storage had a survival rate of 82% whilst those machine perfused had a rate of 83% [156].

Several papers have shown a relationship between initial graft dysfunction and poorer long term renal allograft survival [66, 110], however, in these studies comparing the different preservation modalities, even those studies that have shown less DGF with machine perfusion do not show this benefit to have led to better long term allograft survival, which is surprising [108, 153, 167].

2.5b Non-heart-beating donors

There has been less published work looking into the effects of preservation method in kidneys from NHB donors. Often the assumption is made that whatever is found to be true for heart-beating cadaveric donors can also be applied to NHB donors. Nevertheless, this section presents the results of whatever work there has been.
i. Primary Non-function

PNF rates in NHB donor kidneys have been reported at anything from zero [176, 177] to 19% [97]. Matsuno et al performed a small prospective study of 13 paired kidneys from NHB donors. One kidney from each donor was preserved by machine perfusion and the other was preserved by cold storage. The PNF rate for those that were cold stored was 8% and for those that were machine perfused the PNF rate was zero. However, when we look at these figures more closely, we can see that 8% of 13 kidneys equates to PNF in one kidney in the cold storage group and these results obviously do not show any statistically significant difference [177].

ii. Delayed Graft Function

One animal study has looked at the effect of preservation method after the animal model had sustained a period of warm ischaemic damage. This was reported by Booster et al in 1993. They removed one kidney from a female beagle dog (n=12) following 30 minutes of warm ischaemia which was induced by cross clamping the appropriate renal pedicle. Six of the kidneys were then cold stored for 24 hours and the other six were preserved by pulsatile machine perfusion. In the cold storage group only one dog survived, with the other five dying by day 5. In the machine perfusion group one dog died due to aspiration pneumonia, which was classified as a technical error and excluded from the analyses. The remaining five dogs survived, suggesting that machine perfusion is superior to cold storage for the preservation of NHB donor kidneys [55].

Contrary to the mixed evidence relating to the beneficial effect of machine perfusion on initial function in cadaveric kidneys, the studies involving NHB donor kidneys universally report decreases in the rate of DGF when ischaemically damaged
kidneys are preserved by machine perfusion rather than by cold storage [97, 163, 177-179].

**iii. Rejection Rates**

Several reports have been published showing acute rejection rates in NHB donor kidneys that have been preserved by pulsatile machine perfusion to be as low, if not lower, than those kidneys preserved by cold storage.

In 1994, Matsuno’s work showed an early rejection rate causing ATN and necessitating post-transplant dialysis in 15.4% of those in the cold storage group versus zero percent in the machine perfusion group. However, there were only 13 patients in each group, so this difference equates only to 2 out of 13 patients and is not significant [177].

Light et al in 1996 compared 39 “ideal” cadaveric donors that were preserved by cold storage, with 35 “marginal” donors (including NHB donors) that were preserved by machine perfusion. Fewer rejection rates were reported in the machine perfusion group at 1, 3 and 6 months post transplant (6 vs. 13, 5 vs. 11 and 2 vs. 7 respectively), giving an overall rejection rate at the end of 6 months of 13 out of 35 (37%) in the machine perfused group and 31 out of 39 (79%) in the cold storage group, with no graft losses due to rejection in the machine perfusion group and 2 graft losses in the cold storage group [163]. This difference in acute rejection rates in the two groups is impressive, however most centres would find a rejection rate of 79% in an “ideal” donor to be somewhat high.

Lastly, in 1997 Daemen et al published the results of a study comparing 37 NHB donor kidneys that were preserved by machine perfusion with 74 matched pair
heart-beating cadaveric kidneys preserved by cold storage. No difference was found between the two groups as regards the acute rejection rate at three months [97].

iv. Renal Allograft Function

The only work reporting the renal function in kidneys from NHB donors following either cold storage or machine perfusion is by Matsuno. Firstly reporting on the results of 26 paired kidneys in 1994, the mean “best” serum creatinine in the cold storage group was 1.70 mg/dl and in the machine perfusion group it was essentially the same at 1.69 mg/dl [177]. Matsuno then reported again in 1996 on the results of 32 paired kidneys [180]. Creatinine was lower in the machine perfused group than in the cold storage group in 10 out of 16 pairs, however no values are reported and the significance of this is dubious: probably the most that can be said is that renal function is no worse in those kidneys that have been machine perfused.

v. Patient and Graft Survival

The only study of NHB donor kidney recipients that comments on patient and graft survival is Matsuno’s 1994 paper [177]. In the 26 paired kidneys preserved by either cold storage or machine perfusion, patient survival was 100% in each group, whilst graft survival was better (but not significantly so) in the machine perfused group, which had a survival rate of 100% compared with a rate of 76.9% (10/13) in the cold stored group.

These results are encouraging, but this is obviously an area where more work would be beneficial.
2.6 Viability testing

One of the potential advantages of pulsatile machine perfusion as a preservation technique lies in the prospect it allows for viability testing. Interest in viability testing has occurred in recent years due to the falling donor numbers and the increasing renal transplant waiting list. This has led to a search for new sources of donor kidneys. Marginal donors of various types, including NHB donors are now being investigated and used more fully to try and make up the short-fall in donation that is needed. However, these marginal donor kidneys are at a higher risk of graft dysfunction, including delayed graft function and primary non-function, than the more conventional cadaveric kidneys. Therefore, techniques have been investigated to differentiate those kidneys that are likely to function well from those that are going to either never function at all, function poorly, or are only going to function after a long, expensive and potentially damaging delay.

Pulsatile machine perfusion provides a system for measuring functional parameters whilst perfusing. These include the measurement, or setting, of perfusion pressure and flow. From these the intrarenal vascular resistance (IRR) can be calculated (mean perfusion pressure/flow). As well as these functional parameters, renal tissue and perfusate effluent are readily available for metabolic analysis. Both functional parameters and the results of the metabolic analyses have been assessed to see if they provide an accurate assessment of the quality of the kidney, especially in differentiating viable from non-viable kidneys and, to a lesser extent, in discriminating kidneys that will function immediately from those that will experience initial graft dysfunction.

Functional perfusion characteristics have been studied in both NHB donor and other marginal donor situations. Several studies have shown a low perfusion rate
and/or a high intra-renal vascular resistance (IRR) to be associated with DGF [120, 178, 181]. The paper by Polyak in 1997 showed a 17% decreased flow in those kidneys that experienced DGF compared to those with immediate function and a 27% increase in IRR [181]. Several other papers have maintained a relationship between low flow and/or high resistance and primary non-function [150, 177, 182]. A common fault with these papers is that often the kidney with unfavourable perfusion characteristics is discarded, therefore not demonstrating beyond doubt that these kidneys are non-viable. This is an understandable defect as the consequences of transplanting a potentially non-viable kidney are immense. However, these findings repudiate the results of two earlier papers by Sampson and Newman.

Sampson, in 1978, transplanted 100 cadaveric kidneys, which had been machine perfused irrespective of their recorded flow rates. The kidneys were divided into 3 groups: flow <80 ml/min, flow between 80-100 ml/min and flow >100 ml/min. He found no differences in flow rates between those kidneys that had immediate function, delayed function and primary non-function. Allograft function at three and twelve months was also not significantly different between the three groups [183].

Newman analysed 23 cadaver kidneys, which were preserved by pulsatile machine perfusion in 1981. Lactate, lactate dehydrogenase (LDH), alterations in pH and flow rate were all measured and then compared to graft function following transplantation. LDH concentration was significantly higher in those kidneys that did not have immediate function, but was unable to differentiate those kidneys that would have merely DGF from those, which would never function. None of the other parameters correlated to eventual function at all [184].

Danielewicz et al machine perfused 86 ischaemically damaged kidneys between 1994 and 1997, investigating various parameters. From their results they developed reference values after 4 hours of perfusion for flow, lactate level and LDH
index. They asserted that these values allowed accurate prediction of initial graft function in 60% of grafts [178]. Daemen et al machine perfused 67 NHB donor kidneys between 1993 and 1995. 21 of these kidneys were not transplanted, as they were deemed non-viable due to adverse donor data, macroscopic appearance, perfusion characteristics or histological examination. Of those that were transplanted 9 never functioned. Daemen found that IRR was higher in those kidneys that never functioned as was the alpha glutathione-S-transferase (α GST) level, LDH was not different between the two groups’ [182]. Further work into the usefulness of α GST by the same group was not able to demonstrate a level of α GST that could accurately discriminate between viable and non-viable organs [185].

The interesting, but sometimes contradictory, results of these studies have not resulted in a single, universally accepted viability test and this may be an unreasonable aim. Further studies are ongoing and it may be that by combination of various functional parameters and metabolic assays that an index of probability of potential graft function could evolve.

2.7 Summary

Renal preservation remains an important and interesting aspect of renal transplantation. Mechanisms of injury to the kidney following ischaemia and hypothermia have been extensively researched and are now accurately understood, resulting in the availability of an extensive range of preservation solutions. Cold storage and pulsatile machine preservation remain, at the moment, the only endorsed methods of preservation. However, results have improved greatly in the 30 years since Dr Belzer and Dr Collins pioneered machine perfusion and cold storage respectively.
Controversy still exists over which method of preservation is best, with contradictory reports continuing to be published. Certainly, the preponderance of studies suggests that early graft function is improved with pulsatile machine perfusion.

Long-term results do not seem to be influenced by this improvement in immediate graft function and the available literature does not demonstrate any great advantage of one preservation method over the other. The adverse effect of DGF in terms of long-term function previously reported does not seem to be borne out by the results of the studies here.

A reduction in the rate of early graft dysfunction is in itself beneficial on several levels. Firstly, economic savings are made due to the shorter hospital stay and the reduction in episodes of post-transplant dialysis. Secondly, post-operative patient management is easier, both in terms of fluid management and the detection of the presence of acute rejection. Lastly, patient moral is improved by visible proof of a working transplant.

Pulsatile machine perfusion is also advantageous because of its role in viability testing. Although no single test has yet to be discovered to differentiate the viable from the non-viable kidney- and perhaps never will be- substantial work continues in this area.

As DGF and potential kidney non-viability are both more prevalent in grafts from NHB, and other marginal, donors it is not surprising that machine preservation has become more widely used in this area than for the preservation of the more conventional heart-beating cadaveric donors where PNF and DGF are less of a problem.
Where prolonged preservation is necessary machine perfusion again has been shown to be beneficial, especially in term of reducing DGF, in both NHB and heart-beating cadaveric donors.

Therefore, it is quite possible that pulsatile machine perfusion will find its niche as the preservation method of choice in the future for kidneys from sub-optimal donors such as “marginal” donors, NHB donors and those kidneys preserved for prolonged periods of time, whilst cold storage will be reserved for the kidneys from the “ideal” donor.
CHAPTER THREE

PATHOPHYSIOLOGICAL ROLE OF NITRIC OXIDE IN RENAL ISCHAEMIA/REPERFUSION INJURY

3.1 Introduction to nitric oxide

3.2 Biochemistry and biosynthesis of nitric oxide

3.3 Function of nitric oxide
   3.3a Overview
   3.3b Role of nitric oxide in renal physiology

3.4 Pathological role of nitric oxide in the kidney
   3.4a Inflammation
   3.4b Ischaemia
3.1 Introduction to nitric oxide

Interest in the clinical role of nitric oxide was first elicited in 1980 by Furchgott and Zawadski [186]. They demonstrated that acetylcholine-dependent – i.e. receptor dependent – vasodilatation was caused by a non-prostanoid, endothelium-dependent factor, which they termed endothelium-derived relaxation factor (EDRF). R.F. Furchgott went on to share the Nobel Prize for Medicine in 1998 with L.J. Ignarro and F. Murad "for their discoveries concerning nitric oxide as a signalling molecule in the cardiovascular system"[187]. This factor was identified in 1987 when Palmer et al demonstrated that the biological activity of EDRF could be explained by the release of nitric oxide (NO) [188] from the metabolism of L-arginine [189].

NO has been implicated in a wide variety of biological processes. These include vasodilatation, non-specific immunity, neurotransmission (both central and peripheral), modulation of platelet function [190], inhibition of neutrophil chemotaxis, adherence and activation [191] and immunomodulation following an allograft [192].

This chapter is going to give an overview of the biochemistry and biosynthesis of NO, then concentrate on the role of nitric oxide in renal physiology and pathology with special interest in its role in renal ischaemia reperfusion injury.

3.2 Biochemistry and biosynthesis of nitric oxide

NO is a small inorganic, but biologically active, messenger molecule which is found in many species including humans [193]. It is a gaseous free radical which has the ability to diffuse quickly in both lipid and aqueous environments. Therefore, NO is able to disperse rapidly between tissues regardless of any intervening biological membranes. In fact, in physiological conditions NO has a cell diffusion co-efficient
greater than that of oxygen [194]. This property may be one of the reasons why NO has been implicated in the wide variety of biological processes outlined previously.

Synthesis of NO occurs under the control of a group of bidomain apoenzymes called nitric oxide synthases (NOSs): The N-terminal oxygenase domain which contains binding sites for haem, tetrahydrobiopterin (BH$_4$) and arginine is linked by a calmodulin (CaM) recognition site to a C-terminal reductase domain that contains binding sites for flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and the reduced form of nicotinamide-adenine dinucleotide phosphate (NADPH)[195].

Electrons (e') are donated by NADPH to the reductase domain of the enzyme and proceed via FAD and FMN redox carriers to the oxygenase domain. There they interact with the haem iron and BH$_4$ at the active site to catalyse the reaction of oxygen with L-arginine, generating citrulline and NO as products. Electron flow through the reductase domain requires the presence of bound Ca$^{++}$/calmodulin (Figure 1).

![Figure 1. Overall reaction catalysed and cofactors of NOS (adapted from 191)](image)
L-citrulline passes into the urea cycle to regenerate L-arginine. However, NO is a labile molecule with a short half-life (<4 seconds) in biological solutions. This is principally due to:

i. Oxidation of NO to nitrate (NO$_3^-$) and nitrite (NO$_2^-$) [196].

ii. Quenching with the superoxide radical [197].

iii. Binding to haem-containing proteins with Fe-S sites, such as oxyhaemoglobin (Hb-O$_2$) to form methaemoglobin (metHb) [198](Figure 2).

![Figure 2. Metabolism of NO.](image)

When oxidation of the terminal guanidino nitrogen of L-arginine occurs under the control of NOS there is a subsequent elimination of equimolar amounts of NO and superoxide (O$_2^-$) and generation of L-citrulline [199]. NO reacts rapidly and almost
irreversibly with $O_2^-$ to produce peroxynitrite (ONO$_2^-$). Such quenching would be
cytoprotective as the product (peroxynitrite) is less toxic than the constituents are.
However, peroxynitrite is very short lived and is quickly protonated before its decay
to generate the highly injurious hydroxyl radical (OH).

There are three principal NOS isoforms:

- Neuronal NOS (nNOS/ NOS I)
- Inducible NOS (iNOS/ NOSII)
- Endothelial NOS (eNOS/ NOS III)

These were initially named according to the tissue in which they were discovered.
However, it is now known that each isoform is much more widely expressed than was
initially thought [194](Table 1). Often nNOS and eNOS are banded together and
referred to as constitutive NOSs (cNOS).

Table 1. Main NOS isoform distribution under physiological conditions

<table>
<thead>
<tr>
<th>Isozyme</th>
<th>Main tissue distribution</th>
</tr>
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<tbody>
<tr>
<td>eNOS</td>
<td>endothelial cells</td>
</tr>
<tr>
<td></td>
<td>hippocampal neurones</td>
</tr>
<tr>
<td></td>
<td>cardiac myocytes</td>
</tr>
<tr>
<td></td>
<td>uterus</td>
</tr>
<tr>
<td>iNOS</td>
<td>normal kidney</td>
</tr>
<tr>
<td></td>
<td>bronchial epithelium</td>
</tr>
<tr>
<td></td>
<td>alveolar macrophages</td>
</tr>
<tr>
<td></td>
<td>ileum</td>
</tr>
<tr>
<td></td>
<td>uterine epithelium</td>
</tr>
<tr>
<td></td>
<td>platelets</td>
</tr>
<tr>
<td></td>
<td>retina</td>
</tr>
<tr>
<td></td>
<td>skeletal muscle</td>
</tr>
<tr>
<td></td>
<td>cerebellum</td>
</tr>
<tr>
<td>nNOS</td>
<td>central nervous system</td>
</tr>
<tr>
<td></td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td></td>
<td>penis</td>
</tr>
<tr>
<td></td>
<td>pancreatic B cells</td>
</tr>
<tr>
<td></td>
<td>myocardium</td>
</tr>
<tr>
<td></td>
<td>uterus</td>
</tr>
<tr>
<td></td>
<td>macula densa</td>
</tr>
<tr>
<td></td>
<td>adrenal medulla</td>
</tr>
</tbody>
</table>
Three distinct genes for the human nNOS, iNOS and eNOS isoforms exist, with a single copy of each in the haploid human genome. The NOS genes have a similar genomic structure [195] and the NOS isoforms share 55% to 60% amino acid homology [200], suggesting a common ancestral NOS gene.

Although constitutive forms of NOS (eNOS and nNOS) are responsible for a continuous, basal release of NO this production can be upregulated – for example, in pregnancy and secondary to the administration of oestradiol. Although the exact reasons for this remain unclear, it is thought to be involved in the vasodilation of uterine vessels during pregnancy [194]. The constitutive forms of NOS are membrane bound and possibly linked to the cytoskeleton [201]. They are dependent on calcium and calmodulin for their activation: they remain inactive until intracellular calcium levels increase sufficiently to promote calmodulin binding. This explains why they only produce low concentrations of NO and has led to the assumption that cNOS forms a low output NO pathway that is involved in physiological cell-to-cell communication [193]. In contrast, iNOS binds calmodulin at resting intracellular calcium concentrations. Therefore, the activity of iNOS has been seen as Ca^{++}/calmodulin independent [200, 202]. Inducible NOSs are usually free cytosolic enzymes, although research has shown that iNOS can be membrane associated in 50-80 nM vesicles in certain cells [194]. Although iNOS was initially identified by the fact that it was quiescent until immune stimuli transcriptionally activated the gene, it subsequently became clear that iNOS accounts for a continual low release of NO in some tissues [194]. These tissues include:

- Cells of the normal kidney
- Retina
- Respiratory epithelium
- Skeletal muscle
- Ileum
- Various foetal tissues

With the exception of the tissues mentioned above, iNOS is expressed at barely
detectable levels and has only been identified by reverse transcriptase polymerase
chain reaction techniques as the quantities are otherwise too small to be recognized by
immunohistochemistry.

Once induced, iNOS is permanently active for the life of the enzyme (i.e. up to
24 hours) [203] and produces NO in amounts 1000-fold greater than cNOS
(nanomolar rather than picomolar concentrations) [204].

3.3 Function of nitric oxide

As previously outlined, this section is going to mainly review the role of NO
in the kidney. However, before concentrating on this area it will be useful to mention
the multiple functions within the body that NO has been linked with.

3.3a An overview

In the broadest term, NO is an intercellular messenger. It acts primarily as a
neurotransmitter and vasodilator [190]. However, it also has a role in inflammation,
tissue injury and host defence.

As a neurotransmitter, it is involved both centrally and peripherally. It acts on
the pre-synaptic neurones to modify synaptic transmission. Centrally, this long-term
potentiation forms the basis of memory formation, whilst peripherally NO mediates
muscular relaxation and is an important factor for organised gut motility [193].

NO plays a key role within the vascular system. Vascular tone is regulated
through pulsatile flow and laminar shear stress acting on the endothelium [193]. Both
iNOS and eNOS are expressed in endothelial cells. In addition, iNOS is also synthesised by vascular smooth muscle cells when there is large scale cytokine release such as sepsis or the systemic inflammatory response [201]. Vasodilation is subsequently mediated by soluble guanylyl cyclase (sGC), which is activated by haem group nitrosylation and produces cyclic guanylate monophosphate (cGMP). Kinases dependent on this secondary messenger phosphorylate smooth muscle cell proteins leading to relaxation [205]. The NO produced also has the ability to inhibit platelet function [190] and neutrophil cytotoxicity, aggregation and interaction with the endothelium [191, 206]. This is partially achieved by decreasing the expression of adhesion molecules on both neutrophils and the endothelium [207]. Therefore, not only is NO important in regulation of blood flow and pressure, but it also protects against vascular damage. This is highlighted by the fact that decreased production or availability of NO has been implicated in almost every disease associated with increased vascular tone, vasospasm or enhanced platelet aggregation. This includes many cardiovascular conditions, such as hypertension, diabetes mellitus, hypercholesterolaemia and atherosclerosis, as well as disorders induced by hypoxia and ischaemia [194].

A diverse number of cells, including macrophages, are able to express iNOS in response to injurious stimuli (Table 2) [208]. This then provides a primary defence mechanism against pathogenic invading organisms and tumour cells. The iNOS within the cells becomes activated by specific cytokines, including tumour necrosis factor (TNFα) and interleukin-1β (IL-1β), bacterial endotoxins or lipopolysaccharides (LPS) [207] resulting in the production of large, unregulated quantities of NO for the life of the enzyme. The level of iNOS activity is directly related to the cell type in which it is induced [194]. In the concentration generated by iNOS, NO is both cytostatic and cytotoxic to a wide range of pathogens. These include the organisms
responsible for Leishmania, tuberculosis and malaria, as well as some fungi and tumour cells. NO is therefore considered to be a mediator of non-specific immunity in these circumstances [209]. It is able to perform this by quickly diffusing into the target cell where it inactivates enzymes essential to the mitochondrial respiratory chain [200], oxidative metabolism [210] and DNA/RNA synthesis [211].

Table 2. Some of the cells capable of iNOS expression in response to injurious stimuli

- Macrophages and monocytes
- Vascular smooth muscle and endothelial cells
- Renal tubular epithelial cells
- Mesangial cells
- Cardiac myocytes
- Megakaryocytes
- Liver parenchymal cells
- Fibroblasts
- Astrocytes

However, these elevated concentrations of NO can be sustained for hours, if not days, with little or no regulatory mechanism for the activity of iNOS once it has been initiated. Such prolonged over production can also result in host cell damage as NO can not distinguish between them and the target, pathogenic, cells. This non-specificity of action makes NO a primitive defence mechanism and leads to its involvement in many pathological as well as physiological processes [193].

These elevated levels of NO are thought to contribute to the vasodilatation, vascular leakage and tissue damage which are characteristic of several conditions, especially septic shock. Induction of iNOS has been also reported in the joint fluid of
patients with rheumatoid arthritis, in the gut mucosa of patients with ulcerative colitis and in the ventricles of patients with cardiomyopathy [209].

3.3b Role of nitric oxide in renal physiology

A major role for NO in normal renal physiology has been established with both eNOS and iNOS being expressed. However, their relative distributions vary within the kidney, with eNOS mainly localised in the preglomerular vasculature, macula densa (MD) and collecting ducts. Inducible NOS has a more general distribution and is only absent from the preglomerular vessels and the MD [212](Table 3).

Table 3. Renal distribution and semi quantification (using PCR) of cNOS and iNOS

<table>
<thead>
<tr>
<th>Site</th>
<th>cNOS</th>
<th>iNOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preglomerular vessels</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Glomerulus</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Macula densa</td>
<td>++++</td>
<td>-</td>
</tr>
<tr>
<td>Proximal tubule</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Loop of Henle</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Distal tubule</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Cortical collecting duct</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Medullary collecting duct</td>
<td>++++</td>
<td>++</td>
</tr>
</tbody>
</table>

NO has multiple functions within the kidney. By the use of NOS blockers and micropuncture techniques, it has been possible to determine the role of NO in normal renal homeostasis [202]. Its main role is as a vasodilator and signalling molecule [5]. NO helps to regulate the glomerular filtration rate, extracellular fluid volumes, renal
blood flow and medullary oxygenation [194, 213-215]. As well as directly affecting afferent tubuloglomerular feedback by acting as a signalling molecule, NO also works indirectly via regulation of sodium homeostasis [5, 216]. The interaction between NO and the rennin-angiotensin system is a complex, and unclear, one. It has been established that NO antagonises the vasoconstrictive effect of angiotensin II on both the afferent and efferent arterioles [194, 217]. However, whilst one study using intravenously anaesthetised rats suggested that NO may act in a paracrine way within the glomerulus to stimulate the release of renin from juxtaglomerular cells [218], another study [216] found that plasma renin activity could be increased by blocking NOS in conscious dogs. The effect of certain anaesthetic agents on NOS may be one cause for such discrepancies [219], although inhalational anaesthesia has been demonstrated not to affect NOS activity [220].

3.4 Pathological role of nitric oxide in the kidney

In pathological circumstances, NO is mainly generated in a wide number of cells expressing iNOS in response to inflammation or ischaemia [221]. As mentioned previously in other tissues, this is in sufficient quantities to render tissues cytostatic or cytotoxic. As well as having the ability to be harmful in its own right, NO also leads to the generation of more harmful substances such as peroxynitrite. This has led to speculation about its involvement in certain renal diseases such as immune-mediated glomerulonephritis, post-ischaemic renal failure, obstructive nephropathy and acute and chronic renal allograft nephropathy [194].

3.4a Inflammation

As mentioned previously, the inflammatory effects of NO include its ability to increase the vascular permeability of inflamed tissues, vasodilation [209], the
induction of the inflammatory cytokines TNFα and IL-1β [207] and the subsequent formation of highly reactive molecules such as peroxynitrite [222].

NO does seem to have a duality of beneficial and detrimental effects, for whilst there is evidence if it contributing to the development of some renal diseases as mentioned above [194], there is also evidence of its protective properties. For example, Blantz and Munger [222] showed that NO inhibits cell proliferation via inhibition of polyamine synthesis and cell uptake, acting as a “brake” on the proliferation response following cytokine exposure. Trachtman et al [223] demonstrated that NO regulates the synthesis of extracellular matrix by mesangial cells. Their results indicated that renal production of NO in glomerular diseases may attenuate the production and accumulation of matrix proteins and therefore limit the severity of glomerulosclerosis.

Furusu et al [221] demonstrated further that the expression of NOSs in the human kidney is a complex issue. They examined the expression of three isoforms of NOS – iNOS, eNOS and brain NOS (bNOS) – in the renal tissue of patients with various renal diseases. These were: IgA nephropathy, lupus nephritis, membranous nephropathy and minimal change nephritic syndrome. Normal portions of surgically resected kidneys served as a control. Sections were immunostained and the correlation between the expression of each NOS and the degree of glomerular injury was examined. They found that the expression pattern of eNOS in each glomerulus was the reverse of that of iNOS. Indeed, the extent of staining for eNOS correlated negatively for the degree of injury, whereas the extent of staining for iNOS correlated positively with the degree of glomerular injury. This led them to suggest that each NOS may play an important, but different, role in the human inflamed glomerulus.

The expression of iNOS has probably created the most interest in this area as it is the major source of NO in many pathological states affecting the kidney [194, 221,
Peak NO production has been shown to coincide with the main macrophage infiltration in glomerulonephritic disorders and macrophages are known to be involved in the pathogenesis of tissue injury in glomerulonephritis, particularly causing interstitial and glomerular hypercellularity and tissue damage [224]. Immunochemistry has demonstrated that in biopsies of diseased kidneys, the predominant cellular sources of iNOS are infiltrating monocytes and macrophages. Decreased renal function and tubulointerstitial damage have also been found to be greater in patients whose kidneys expressed iNOS. No detectable iNOS has been found in normal human renal tissue [221, 224]. All this evidence therefore suggests that iNOS expression is disease related in the kidney [224].

### 3.4b Ischaemia

Renal ischaemia reperfusion injury (IRI) involves a complex inter-related sequence of events [225]. Whilst in most organs ischaemia primes the tissue for the injury incurred during reperfusion, in the kidney the situation is more complex as the ischaemia itself causes early, irreversible damage. In the majority of organs the injury is principally mediated by oxygen-derived free radicals (OFR) and polymorphonuclear (PMN) leucocytes, whereas in the kidney the role of PMN remains unclear [202]. Studies on isolated proximal tubules have demonstrated that early in the ischaemic phase NOS activity is increased, with NO generation maximal by 10 minutes [226]. The equilibrium between NO and the OFRs derived during reperfusion may be critical in determining the degree of postischaemic damage. The most important interaction is probably between NO and superoxide (O$_2^-$). These react rapidly to produce peroxynitrite (ONOO$^-$). As mentioned previously, such quenching would be cytoprotective as the product (peroxynitrite) is less toxic than the constituents. However, peroxynitrite is very short lived and is quickly protonated to HOONO which then decays to generate the highly injurious hydroxyl radical (OH$^-$).
During the ischaemic phase, only NO is generated. This may damage the actin cytoskeleton through ATP depletion, as well as directly altering DNA and inhibiting the subsequent repair.

Therefore, it would be easy to conclude that postischaemic NO is cytotoxic. However, in certain circumstances NO-mediated nitrosylation has been shown to have a cytoprotective effect. One target for HOONO is the cell membrane where nitrosylation helps to maintain cellular integrity. Further evidence of the cytoprotective effect of NO has been seen in its interaction with leucocytes. A number of post-ischaemic mediators, including xanthine oxidase, platelet activating factor, thromboxane and leukotrienes, are involved in leucocyte sequestration. These neutrophils then adhere, activate and migrate under the control of adhesion molecules, most notably the B-integrins. Both these (the CD11/CD18 complex in particular) and the matrix proteins are integrally involved in the subsequent respiratory burst. The involvement of NO in the proteolysis of B-integrin has been demonstrated, as has the effect of NO on the extracellular matrix. Thus, by interfering with the neutrophil respiratory burst NO should help ameliorate postischaemic renal damage. This has been confirmed experimentally.

It is therefore too simplistic to suggest NO has either a sole cytotoxic or cytoprotective role. Furthermore, the balance of free radicals, especially NO and O₂⁻, during reperfusion may be critical in determining the degree of eventual damage. An excess of NO over O₂⁻ in the postischaemic tissue may have an overall cytoprotective effect to counteract the ONOO⁻ toxicity.

Weight et al demonstrated a biphasic role for NO. They used rat kidneys which underwent a 45 minute warm ischaemic insult, followed by reperfusion and unilateral nephrectomy. An increase in NO level was seen early in reperfusion. This was prevented by inhibiting cNOS but not iNOS – suggesting a predominant role for cNOS early in reperfusion. Compared with the control, early oxidative protein
damage was significantly increased by the addition of NO donors and reduced following NOS blockage, suggesting that NO is injurious at an early phase of reperfusion. To assess the intermediate phase (day 2) of reperfusion, renal function was used as a marker. It was found that NO donors and iNOS inhibitors improved the GFR whilst joint cNOS/iNOS inhibitors impaired it. Weight suggested that this inferred that induction of NOS was injurious whilst the upregulation of cNOS ameliorated the injury, possibly by maintaining renal blood flow. By day 7, NO levels remained high, possibly maximally [236], in the control and iNOS inhibitor group. However, in both the NO donor and cNOS/iNOS inhibitor groups, levels were significantly reduced. Weight proposed that this was for opposite reasons as compared to control values, NO donation reduced the injury, presumably resulting in more normal (lower) levels of NO. However, following cNOS/iNOS inhibition, more severe injury was seen in association with lower NO concentrations, postulated to be due to cNOS dysfunction.

Weight goes on to hypothesis that the early rise in NO ameliorated late injury due to the interaction of NO with both the vascular endothelium and neutrophils: NO helps maintain renal blood flow and prevent post ischaemic vasospasm [237] and microvascular dysfunction [233] with a attendant reduction in endothelium-neutrophil interaction which should limit postischaemic renal failure [238]. NO also inhibits neutrophil chemotaxis, production of oxygen and enzyme release [239].

Weight made two conclusions from his experimental findings. Firstly, that the early rise in NO during reperfusion appeared to be cytotoxic. Secondly, that inhibiting this rise led to more severe, late damage suggesting that a prolonged increase in NO concentration was cytoprotective. His finding also supported the idea that cNOS rather than iNOS was fundamental to this prolonged rise. Weight went on to hypothesis the reason for this duality. As mentioned previously, early in reperfusion NO and superoxide quench to from peroxynitrite which decays to the more cytotoxic
hydroxyl radical. This, therefore, can account for the early NO cytotoxicity. The late
cytoprotection may stem from the interaction of NO and leucocytes with the
secondary damage caused by the sequestration of activated neutrophils [238] being
inhibited by NO [239]. Lastly, Weight's results suggested that iNOS and cNOS have
contrasting functions in the postischaemic kidney, with cNOS being cytoprotective
and iNOS injurious.
Statement of Intentions

As has been described previously, the continuing shortage of organs for transplantation has stimulated renewed interest in organs from NHBDs. Although several centres have published acceptable results [16, 20, 23, 42, 58, 59, 65] with the use of kidneys from these donors, concerns still remain. Notably, the rates of DGF [1, 15, 52, 55-57, 59-61] and PNF [1, 35, 36, 50-57] in some series have been unacceptably high.

These factors have led people to look at ways in which “good” kidneys can be differentiated from “bad” kidneys by the use of viability testing. This is one area where machine perfusion has the advantage over static cold storage as it allows easy testing of the kidneys. Several studies have shown an association with either low perfusion rate or high IRR and DGF [120, 178, 181] or PNF [150, 177, 182].

Therefore, the first aim of this thesis was to look at the effect of warm ischaemia on a porcine kidney model to ascertain the following:

• Is there a relationship between WIT and IRR during pulsatile MP?
• Is there a relationship between WIT and NO production, as measured by nitrate/nitrite concentration within the effluent perfusate?
• Is there a relationship between WIT and the expression of eNOS within the kidney, as measured by tissue biopsies during perfusion?
• If a relationship is found with any of the above, how is this relationship affected by machine perfusion over six hours? And is there a correlation between IRR, NO production and eNOS expression?
• Can any of these be used for viability testing of the kidneys?

Secondly, this thesis looked at the effect of cold ischaemia on the same porcine kidney model and asked the following:
- Is there a relationship between the CIT and IRR during pulsatile MP?
- Is there a relationship between CIT and NO production, as measured by nitrate/nitrite concentration within the effluent perfusate?
- Is there a relationship between CIT and the expression of eNOS within the kidney, as measured by tissue biopsies during perfusion?
- If a relationship is found with any of the above, how is this relationship affected by machine perfusion over six hours? And is there a correlation between IRR, NO production and eNOS expression?
- Are any of the above relationships changed when the kidneys are subjected to a longer period of warm ischaemia (45 minutes) as compared to a short WIT (15 minutes)?

Lastly, this thesis looked at the effect of adding NO donors or NOS inhibitors to the perfusate to determine the following:

- Does the addition of an NO donor such as sodium nitroprusside (SNP) change the IRR or eNOS expression during pulsatile MP as compared to a control group where there were no additions to the perfusate?
- Does the addition of an NOS inhibitor change the IRR or eNOS expression during pulsatile MP as compared to a control group where there were no additions to the perfusate?
- Do different NOS inhibitors affect the IRR and eNOS expression differently? This was achieved by adding three different NOS inhibitors to the perfusate. These were:
  - L-NAME ($N^G$-nitro-L-arginine methyl ester) an iNOS and cNOS inhibitor.
  - ADMA ($N^G$, $N^G$-dimethylarginine) a cNOS inhibitor.
  - Aminoguanide an iNOS inhibitor.
SECTION B

METHODS
CHAPTER FOUR

KIDNEY SELECTION AND MACHINE PERFUSION

4.1 Selection of pigs

4.2 Mode of death

4.3 Kidney retrieval

4.4 Timing of warm and cold ischaemia
4.4a Experiment 1 – the influence of warm ischaemic injury
4.4b Experiment 2 – the influence of cold ischaemic injury
4.4c Experiment 3 – the influence of NO donors and NOS inhibitors

4.5 Machine perfusion
4.5a RM3 control unit
4.5b The cassette
4.5c Measuring flow
4.5d The circulation pump and reservoir
4.5e Transfer of data
4.5f RM3 checklist and initial set up
4.1 Selection of pigs

All the pigs used for the experiments in this study were Landrace pigs from the agricultural college at the Sutton Bonington campus, University of Nottingham. The pigs were all between 35 – 45 kg in weight and were aged between 3 – 4 months. Pigs of this type and age were used as landrace pigs are noted for their early, rapid growth: their weight at weaning being higher than that of other breeds [240]. They are also reaching sexual maturity [241], consequently the kidneys retrieved were suitable for the experiments contained within this study and allowed comparisons to be made to human adult kidneys.

4.2 Mode of Death

The pigs were stunned by an electric shock of between 1.3 and 1.5 amperes to render them senseless. They were then killed by an injection of between 20 and 40 ml of 20% concentration pentobarbitone. This was injected directly into either the heart or one of the large chest vessels. Death was pronounced when several criteria were met. These were:

i) Absence of heart sounds

ii) Absence of breath sounds

iii) Absence of corneal reflexes.

Once asystole had occurred and death was pronounced, the stopwatch was started. This recorded the period of warm ischaemia. The warm ischaemic time (WIT) was defined as the interval from asystole until the start of cold preservation when the kidneys were flushed with hyperosmolar citrate (HOC) solution. Whilst the WIT took place, the kidneys were left in situ within the intact pig until approximately 5 minutes before the end of the WIT. This model was used to represent the situation that occurs
when kidneys are retrieved from a human NHBD. In the human situation, following unsuccessful resuscitation the kidneys are left in situ for varying periods of warm ischaemia, gradually cooling down until cold preservation is commenced. In human NHBD, this is often achieved with the use of a double balloon triple lumen catheter [26], however for simplicity in this pig model the kidneys were removed from the pig prior to cold preservation.

4.3 Kidney retrieval

At 5 minutes prior to the pre-decided WIT, the abdominal and thoracic contents of the pig were removed en bloc by the abattoir attendant, leaving the retroperitoneal structures. The kidneys were then removed sequentially (right followed by left), with capsule left intact and renal vein and ureter divided short. The renal artery was dissected to the aorta and taken with a small patch of aorta. Each kidney was then placed into a non-sterile receiver and the obliquely cut end of an intravenous (IV) giving set was inserted into the renal artery and secured into position with small artery forceps. The IV giving set was connected to a 1 litre bag of HOC (Soltran Kidney Perfusion Solution, Baxters Healthcare Ltd, Thetford, Norfolk, England). This was at 4°C and set at a fixed height of 1 metre above the table upon which the kidney was placed. At the pre-defined WIT, as timed by the stopwatch, the IV giving set was switched on and the kidney was flushed with the hypothermic HOC solution. Approximately 500mls of the HOC solution was flushed through the kidney until the effluent perfusate was no longer blood stained and flowed clear. Once flushing of the kidney was completed the kidney was placed in a labelled plastic bag with a small quantity of the HOC solution. The kidney was then packed in crushed ice and transferred to the University Department of Surgery at the Leicester General Hospital. Depending upon the cold time for each individual kidney, the kidney was
either stored for longer packed in ice in a 4°C walk – in refrigerator, or was placed immediately onto the Renal Preservation System (Waters Medical Systems, Rochester, MN, USA) for pulsatile machine perfusion.

4.4 Timing of warm and cold ischaemia

This thesis is based on the results of three experiments; each experiment used porcine kidneys which had been subjected to different lengths of warm and cold ischaemia. However, the method of pig selection, culling and retrieval of kidneys remained constant for each kidney regardless of which experiment the kidney was to be used for. The duration of warm and cold ischaemic times for each experiment are outlined below.

4.4a Experiment 1 – the influence of warm ischaemic injury

The kidneys in this experiment were all subjected to a constant period of 2 hours cold ischaemia. Therefore, following retrieval and transfer back to the Department of Surgery these kidneys were placed immediately onto the Renal Preservation System for pulsatile machine perfusion. There were 6 different WIT (n=6 per group) used in this experiment. These were:

- <10 minutes
- 15 minutes
- 30 minutes
- 45 minutes
- 60 minutes
- 90 minutes

In the <10 minute group the kidneys were retrieved and then flushed with hypothermic HOC solution as quickly as possible. This group therefore is the nearest
to a zero WIT that could be achieved with this animal model. The other time periods were chosen to include – and with the 90 minutes WIT group to extend – the range of WIT that are commonly encountered in clinical practice.

4.4b Experiment 2 – the influence of cold ischaemic injury

In this experiment the WIT was limited to a short (15 minute) or long (45 minute) duration. Kidneys were then preserved for varying periods of cold ischaemia prior to machine perfusion. The cold ischaemic times (CIT) studied were:

- 2 hours
- 12 hours
- 24 hours
- 36 hours
- 48 hours

There were 6 kidneys in each group.

4.4c Experiment 3 – the influence of NO donors and NOS inhibitors

The aim of this experiment was to observe the effect of intervening during machine perfusion on ischaemically damaged kidneys. Therefore all of the kidneys used were subjected to a long period (45 minutes) of WIT and cold ischaemia (24 hours) to maximise ischaemic damage. During machine perfusion one of several pharmacological compounds were added to the HOC used for machine perfusion. These were all nitric oxide (NO) related compounds. Their names, doses used and presumed mode of action are listed below:

- Sodium nitroprusside:
  - This is a NO donor
  - 50mg was added to 1 litre of HOC solution
L-NAME ($N^\omega$-nitro-L-arginine methyl ester):
This is believed to inhibit both inducible nitric oxide synthase (iNOS) and constitutive NOS (cNOS)
3g was added to 1 litre of HOC solution

ADMA ($N^\omega$, $N^\omega$-dimethylarginine):
This is believed to inhibit cNOS
50mg was added to 1 litre of HOC solution

Aminoguanide:
This is believed to inhibit iNOS
3g was added to 1 litre of HOC solution

These were then compared to a control group subjected to the same length of WIT and CIT where no compounds were added to the HOC solution. Again, n=6 per group.

4.5 Machine perfusion

The RM3 Renal Preservation System (Waters Medical Systems, Rochester, MN, USA) was used for pulsatile machine perfusion of all the kidneys used for the experiments within this thesis. This is a two – part kidney preservation system which consists of (i.) a control unit (the RM3) for the pulsatile perfusion and monitoring of the kidneys and (ii.) a sterile, disposable cassette designed to circulate the perfusate through the kidney (Figure 3).
Figure 3. RM3 Renal Preservation System

4.5a RM3 control unit (Figure 4)

Figure 4. RM3 Control Unit
The function of the RM3 control unit is to monitor the kidneys. It also controls the cassette functions and regulates the pulsatile perfusion of the perfusate through the kidneys. It has a variable stroke volume control that allows the operator to mechanically adjust the occlusion of the pulsatile pump arm. If the stroke volume control is rotated clockwise, then the volume of perfusate from the pump head to the kidney is increased. This results in a corresponding increase in pressure. Alternatively, if the stroke volume control is rotated anti-clockwise then the pressure will fall.

The control unit is able to monitor real-time pressure, flow and temperature of the perfusate. From the pressure and flow it is thus able to calculate the intravascular renal resistance (IRR). This is calculated as mean perfusion pressure divided by the flow. These monitored, and calculated, parameters are then displayed for visual confirmation of perfusion activity (Figure 5). The trend of these parameters over time can also be displayed. This allows for objective evaluation of the kidney being preserved. If any of the parameters deviates either above or below set values then an alarm sounds, allowing for suitable adjustments to be made.

Figure 5. Main Menu Display with Pressure Waveform
4.5b The cassette (Figure 6)

Figure 6. DCM 100 Cassette

The cassette (MOX-100 DCM) is a gravity flow system that allows perfusate to circulate through the kidneys. The cassette is mounted onto the RM3 control unit, with the pulsatile pump head positioned within the pump arms of the control unit. The pressure transducer line, temperature probe cable and flow transducers are attached to the control unit. To complete the apparatus the heat exchange lines are connected from the ice water bath. For the most efficient operation, the inlet connection is located on the top and the outflow connection on the bottom. The temperature probe is located in the bubble trap. This controls the circulation pump that is located in the ice water bath and thermostatically maintains the temperature of the perfusate between pre-arranged values. For this work, the temperature was maintained between 4 and 8°C. The bubble trap separates any trapped air bubbles from the perfusate to
prevent air bubbles reaching the kidney. It also provides mounting positions for the perfusate temperature probe and the pressure transducer line. Since the bubble trap is operated at the perfusate pressure, it is critical to ensure that there are no air leaks in the pressure system. Sample ports are also located on the bubble trap. The syringe access pressure port allows the operator to regulate the proper perfusate level at all times. By inserting a needle and syringe into the pressure port and either adding or withdrawing air, the perfusate level can be moved up or down. To ensure accurate pressure measurement the perfusate level should be maintained at the perfusate indicator level line on the front of the bubble trap. The sample port below the perfusate level indicator line was used to remove a sample of perfusate at hourly intervals by a needle and syringe. Approximately 600 ml of perfusate can be emptied into the arterial reservoir. From the arterial reservoir the perfusate is pumped through the heat exchanger to the bubble trap from where it is delivered to the cannulated kidney. Pulsatile flow is provided by the pulse pump arm of the RM3 control unit and the pump head in the cassette. The pump head includes a pair of occlusive valves placed in series and is alternately compressed and released by the cam-driven arm of the pump arm. It is possible to perfuse two kidneys at the same time with this machine, but in the experiments contained within this thesis only one kidney was pumped at any one time to allow for sampling of effluent perfusate from individual kidneys. When only one kidney is attached, one limb of arterial tubing is clamped to prevent flow down that limb. After the perfusate has flowed through the kidney it returns to the arterial reservoir by gravity through the right or left venous reservoir.

4.5c Measuring flow

Flow can be measured by two methods. Firstly, real-time flows are measured using Transonic ultrasonic clamp-on flow probes on each inlet tube from the bubble trap into the organ chamber. The Transonic flow probes use the transit time
ultrasound method to accurately measure perfusate flow. Each flow probe incorporates two transducers that generate ultrasonic beams which alternately intersect the perfusate. The difference between the upstream and downstream ultrasonic beam transit times is a measure of volume of flow. The flow probes are precalibrated for use with the RM3 and perfusate (Figure 7). The second technique is the direct timer method. By selecting either “L Flow Timer” (left flow timer) or “R Flow Timer” (right flow timer) in the system menu of the RM3 control unit, the rate of flow from a kidney in either organ chamber can be measured by using the cassettes venous reservoirs. These venous reservoirs collect the perfusate effluent after it has flowed through the kidney. The flow from the right half of the cassette flows into the left venous reservoir and flow from the left kidney chamber into the right reservoir. These reservoirs are calibrated in 20ml increments ranging from 0 to 80mls. Once the left or right timer is selected, the LEVEL key will determine the volume of perfusate measured. The levels that can be selected are 20mls, 40mls, 60mls and 80mls. The volumes used in this thesis were either 20mls or 40mls, depending on the flow recorded concurrently by the flow probes. The flow is measured by occluding the tube exiting from the respective section of the venous reservoir chamber. For example:

Figure 7. Transonic Flowprobe Transducer
To measure the left flow:

1. From the MAIN MENU select the SYSTEM MENU.
2. In the SYSTEM MENU select L Flow Timer.
3. Adjust the timer for level 40cc.
4. Occlude the tube exiting the right venous reservoir.
5. When the perfusate reaches the 0 mark, press the START key. This starts a digital counter at the top of the display.
6. When the perfusate reaches the 40cc mark in the reservoir, press the STOP key.

The RM3 will now compute and hence display the flow on the digital display.

For this thesis, the flows that were used for statistical analysis were always measured using the Transonic ultrasonic flow probes. However, to ensure the flows calculated by the flow probes were accurate, direct measurement of the flow was performed periodically and compared to the flow measured by the flow probes. These proved the Transonic flow probes to be accurate as shown in appendix A. The greatest difference in flow was 5 mls/min (92 mls/min c.f. 87 mls/min, 5.4% difference), or in percentage terms, an 11.1% difference (27 mls/min c.f. 24 mls/min). Therefore, it is sufficient to measure flow using the Transonic flow probes alone as these correlate well with the direct flow measurement.
The circulation pump is a submersible pump located in the 5 litre coolant reservoir. It is able to circulate approximately 3.5 litres per minute through the cassettes heat exchanger to cool the perfusate. The reservoir is primed by the addition of 1.5 litres of cold water. The reservoir is then filled with crushed ice. As mentioned previously, the circulation pump is controlled by feedback from the temperature probe to maintain temperature between 4 and 8°C. If the temperature exceeds 8°C, then the pump switches on. Conversely, if the temperature drops too low then the pump will switch itself off automatically. To maintain satisfactory hypothermic conditions, most of the water was drained out every two hours and the reservoir was refilled with crushed ice.
4.5e Transfer of data

The RM3 control unit records the following data minute by minute:

- Time that perfusion has occurred for
- Temperature
- Flow
- Diastolic, mean and systolic pressure
- Intravascular renal resistance.

This data can then be transmitted to a host laptop computer. This is done by attaching a serial modem cable from the RS232 port on the RM3 control unit to a RS232 port on the laptop. The communication software Windows Terminal.exe (Microsoft Corp.) was then used. The communications protocol was set to 9600 baud, no parity, 8 data bits, 1 stop bit, no flow control and COM 1 serial port. Once this is set up it allows transfer of data from the RM3 control unit to the laptop by choosing “Receive Text File” from the hosts transfer menu in Terminal.exe and choosing a file name. In the RM3 system menu “RS232 Transfer” was selected. The data was then sent in an ASCII quote and comma delimited format. This can then be opened as an Excel spreadsheet using a “Text Import Wizard” for further reference and analysis.

4.5f RM3 checklist and initial set up

A summary of the steps involved in the initial set-up and operation of the Renal Perfusion System is shown in appendix B.
CHAPTER FIVE

MEASUREMENT OF NITRIC OXIDE

5.1 Measurement of Nitrate and Nitrite

5.1a Introduction

5.1b Pre-assay preparation

5.1c Measurement of nitrate
   i. Preparation of the nitrate standard curve
   ii. Preparation of the samples for total nitrate + nitrite measurement
   iii. Performing the assay

5.1d Measurement of nitrite
   i. Preparation of the nitrite standard curve
   ii. Measurement of the sample nitrite
   iii. Performing the assay

5.1e Calculations
   i. Subtract the blanks
   ii. Plotting the standard curves
5.2 Western Blotting

5.2a Introduction

5.2b Protein electrophoresis
   i. Sample preparation
   ii. Making the running buffer
   iii. Preparing the Ready Gels
   iv. Assembling the inner buffer chamber
   v. Sample loading
   vi. Gel electrophoresis

5.2c Protein transfer
   i. Making the transfer buffer
   ii. Assembling the transfer gel sandwich
   iii. Electrophoretic transfer

5.2d Protein visualisation
   i. Washes
   ii. Quenching
   iii. Primary antibody solution
   iv. Secondary antibody solution
   v. Detection system

5.2e Densitometry

5.2f Total protein quantification
   i. Reagent preparation
   ii. Sample preparation
   iii. Assay procedure
5.1 Measurement of Nitrate and Nitrite

5.1a Introduction

Nitric Oxide (NO) is synthesised in biological systems by the enzyme Nitric Oxide Synthase (NOS). NOS produces NO and citrulline from molecular oxygen and arginine. Once NO is produced, often in response to homeostatic stimuli [242, 243] it is scavenged very rapidly and has a half-life of only 4 seconds. Therefore, NO itself can not be measured in biological systems. NO undergoes a series of reactions with several molecules present in biological fluids. These include:

\[
\begin{align*}
\text{NO} + \text{O}_2^- & \rightarrow \text{ONO}_2^- + \text{H}^+ \rightarrow \text{NO}_3^- + \text{H}^+ \\
2\text{NO} + \text{O}_2 & \rightarrow \text{N}_2\text{O}_4 + \text{H}_2\text{O} \rightarrow \text{NO}_2^- + \text{NO}_3^- + 2\text{H}^+ \\
\text{NO} + \text{NO}_2 & \rightarrow \text{N}_2\text{O}_3 + \text{H}_2\text{O} \rightarrow 2\text{NO}_2^- + 2\text{H}^+
\end{align*}
\]

Consequently, the final products of NO \textit{in vivo} are nitrite (NO$_2^-$) and nitrate (NO$_3^-$). These can be measured and used as an indicator of the presence of NO. The relative proportions of NO$_2^-$ and NO$_3^-$ are variable and cannot be predicted with certainty. Therefore, the best index of total NO production is the sum of both NO$_2^-$ and NO$_3^-$. The Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical Company, Alexis Corporation (UK) Ltd, Nottingham, UK) used for the samples within this thesis used this method.

The assay kit measured total nitrate/nitrite concentration in a two-step process. The first step was the conversion of nitrate to nitrite utilising nitrate reductase. The second step was the addition of the Griess Reagents which convert nitrite into a deep purple azo compound (Figure 9). Photometric measurement of the absorbance due to this azo compound then accurately determines NO$_2^-$ concentration.
Figure 9. The chemistry of the Griess reagents used for the detection of nitrate and nitrite

\[
\text{NO}_3^- \xrightarrow{\text{Nitrate Reductase}} \text{NO}_2^-
\]

Nitrate \hspace{1cm} \text{Nitrite}

\[
\text{NO}_2^- + \text{SO}_2\text{NH}_2 \xrightarrow{\text{H}^+} \text{Azo product}
\]

Sulfanilamide \hspace{1cm} [Griess Reagent 1]

N-(1-Naphthyl) ethylenediamine \hspace{1cm} [Griess Reagent 2]

Azo product \hspace{1cm} \lambda_{\text{max}}: 540 \text{ nm}
5.1b Pre-assay preparation

Some of the components used within the kit to measure the nitrate/nitrite concentration come in lyophilized form and therefore need to be reconstituted prior to use. Directions on what the kit contains and which needs to be reconstituted, and what with, are listed below:

i. Assay buffer.

This is used to dilute the samples as necessary prior to assay.

The contents of the assay buffer vial should be diluted to 100ml with HPLC-grade water. This should be stored at 4°C when not in use.

ii. Nitrate reductase.

This is used for the conversion of nitrate to nitrite (figure 9).

The contents of the vial should be reconstituted with 1.2 ml of assay buffer. When in use, this should be kept on ice. When not in use this should be stored at -20°C.

iii. Enzyme co-factors.

This is used with the nitrate reductase to facilitate the conversion of nitrate to nitrite.

The vial is reconstituted with 1.2 ml of assay buffer. When in use, this should be kept on ice. When not in use this should be stored at -20°C.


In order to quantify the nitrate plus nitrite concentrations a nitrate standard curve must be performed.

For the reconstitution of this vial, the stopper should be removed slowly to minimise the disturbance of the lyophilized powder. The vial then should be reconstituted with 1.0 ml of assay buffer. This should then be vortexed and mixed sufficiently to ensure that all the powder in
the vial, including any on the stopper, is in solution. This should then be stored at 4°C when not in use.


A nitrite standard curve is necessary when nitrite is being assayed on its own. This is to allow quantification of the amount in the samples. For the reconstitution of this vial, the stopper should be removed slowly to minimise the disturbance of the lyophilized powder. The vial then should be reconstituted with 1.0 ml of assay buffer. This should then be vortexed and mixed sufficiently to ensure that all the powder in the vial, including any on the stopper, is in solution. This should then be stored at 4°C when not in use.

vi. Griess Reagents R1 and R2.

These are the reagents that convert the nitrite into the purple azo compound for photometric measurement. These reagents are supplied ready for use. They should be stored at 4°C when not in use.

5.1c Measurement of nitrate

i. Preparation of the nitrate standard curve

The nitrate standard curve must be performed in order to quantify the sample nitrate plus nitrite concentrations. In a clean test tube place 0.9 ml of assay buffer. To this add 0.1 ml of reconstituted nitrate standard and vortex. This diluted standard is at a concentration of 200 μM. This can then be used for the preparation of the nitrate standard curve as described below (Table 4):
### Table 4. Nitrate standard curve

<table>
<thead>
<tr>
<th>Well</th>
<th>Nitrate Standard (µl)</th>
<th>Assay Buffer (µl)</th>
<th>Final Nitrate Concentration (µM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>0</td>
<td>80</td>
<td>0</td>
</tr>
<tr>
<td>B1</td>
<td>5</td>
<td>75</td>
<td>5</td>
</tr>
<tr>
<td>C1</td>
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<td>70</td>
<td>10</td>
</tr>
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</tbody>
</table>

*The concentration is calculated for the final 200 µl assay volume after the addition of the Griess Reagents.

---

**ii. Preparation of the samples for total nitrate + nitrite measurement**

The samples of effluent perfusate obtained every hour from the kidneys during pulsatile machine perfusion were used for this assay. Prior to performing the assay for the first time the approximate concentration of nitrate and nitrite was completely unknown, therefore several different dilutions of the samples were made to see which dilution resulted in an absorbency between 0.05 and 1.2 absorbance units. This range was chosen as the detector on the plate reader will give the most accurate values when the absorbance is in this range. From the initial work on different dilutions it became apparent that the undiluted samples gave the best absorbencies. Therefore, for the rest of these experiments the samples were not diluted.
iii.  Performing the assay

- The nitrate standards for the standard curve were deposited into the first column of the microtiter plate.

- To standardise the procedure, 200 µl of assay buffer were added to two blank wells. No other reagents were then added to these wells.

- 80 µl of the sample was added to each remaining well. Each sample was done in duplicate.

- 10 µl of the enzyme co-factor mixture was added to each well, excluding the blanks.

- 10 µl of the nitrate reductase was added to each well, excluding the blanks.

- The plate was then covered and incubated at room temperature for 1 hour.

- After the incubation time, 50 µl of Griess reagent R1 was added to each well, excluding the blanks.

- Immediately after Griess reagent R1 was added, 50 µl of Griess reagent R2 was added to each well, excluding the blanks.

- The colour was allowed to develop for 10 minutes at room temperature.

- Finally, the absorbance was read at 540 nm with a differential wavelength of 630 nm using the plate reader (Labsystems Multiskan EX combined with the Genesis computer programme from Syngene).

5.1d  Measurement of nitrite

i.  Preparation of the nitrite standard curve

The nitrite standard curve must be performed in order to quantify the sample nitrite concentrations. In a clean test tube place 0.9 ml of assay buffer. To this add 0.1 ml of reconstituted nitrite standard and vortex. This diluted standard is at a
concentration of 200 μM. This can then be used for the preparation of the nitrite standard curve as described below (Table 5):

Table 5. Nitrite standard curve

<table>
<thead>
<tr>
<th>Well</th>
<th>Nitrite Standard (μl)</th>
<th>Assay Buffer (μl)</th>
<th>Final Nitrite Concentration (μM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>B1</td>
<td>5</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>C1</td>
<td>10</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>D1</td>
<td>15</td>
<td>85</td>
<td>15</td>
</tr>
<tr>
<td>E1</td>
<td>20</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>F1</td>
<td>25</td>
<td>75</td>
<td>25</td>
</tr>
<tr>
<td>G1</td>
<td>30</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>H1</td>
<td>35</td>
<td>65</td>
<td>35</td>
</tr>
</tbody>
</table>

*The concentration is calculated for the final 200 μl assay volume after the addition of the Griess Reagents.

**ii. Measurement of the sample nitrite**

Measurement of nitrite concentrations in the standards and the unknowns can be measured directly by performing the assay in the absence of substrate or enzymes. As with the measurement of the total nitrate and nitrite concentrations, for the measurement of nitrite alone undiluted samples were used as these were found to be best for keeping the absorbance of the sample between 0.05 and 1.2.
ii. Performing the assay

- The nitrite standards for the standard curve were deposited into the first column of the microtiter plate.
- To standardise the procedure, 200 µl of assay buffer were added to two blank wells. No other reagents were then added to these wells.
- 100 µl of the sample was added to each remaining well. Each sample was done in duplicate.
- 50 µl of Griess reagent R1 was added to each well, excluding the blanks.
- Then 50 µl of Griess reagent R2 was added to each well, excluding the blanks.
- The colour was allowed to develop for 10 minutes at room temperature
- Finally, the absorbance was read at 540 nm with a differential wavelength of 630 nm using the plate reader (Labsystems Multiskan EX combined with the Genesis computer programme from Syngene).

5.1e Calculations

i. Subtracting the blanks

The absorbance value of the blank wells was averaged. This mean value was then subtracted from the absorbance values of all the other wells.

ii. Plotting the standard curves

The standard curves were drawn by plotting the absorbance of the standards at 540 nm as a function of either nitrate or nitrite using the software program GraphPad Prism 3.00 for Windows (GraphPad software, San Diego, California, USA). The nitrate/nitrite concentrations of the unknown samples were then calculated by performing linear regression analyses of the standards and selecting the “standard...
curve. X from Y" function in the program. This then determined X (i.e. nitrate/nitrite concentration) for all unpaired Y (i.e. absorbance at 540 nm) data.

The nitrate standard curve was used to determine the total nitrate + nitrite concentration of the sample, whereas the nitrite standard curve was used to determine the nitrite concentration alone. The nitrate concentration of the samples could then be calculated by subtracting the nitrite concentration from the nitrate + nitrite concentration of a sample.

5.2 Western Blotting

5.2a Introduction

Blotting was first performed by Southern in 1975. His method allowed identification of specific DNA fragments which had been resolved by gel electrophoresis via transfer of the band pattern from agarose gels to nitrocellulose membrane filters [244]. This technique gained immediate recognition and was eponymously named Southern blotting. Since then, blotting has been successfully applied to RNA (Northern blotting) [245] and proteins (Western blotting) [246]

Western blotting can detect very small quantities of a protein in a cell or in bodily fluids. It is able to identify protein antigens in a complex mixture by immunoassay techniques. It is a three-stage process:

- Stage 1: Electrophoresis of the protein mixture on a gel. This is to separate each component into individual bands.
- Stage 2: Blotting or transfer of the protein to an immobilising nitrocellulose membrane.
- Stage 3: Visualisation of the transferred proteins by radiolabelled or enzyme conjugated probes.
Western blotting was used within this study for the semi-quantitative measurement of endothelial NOS (eNOS) in the tissue samples taken by wedge biopsy hourly during pulsatile MP. Initially, the intention was to also measure inducible NOS (iNOS) in the same samples. However, an antibody against porcine iNOS was not commercially available and therefore this part of the study had to be abandoned.

5.2b  **Protein electrophoresis**

    *i. Sample preparation*

    The tissue samples were stored at either -70°C or in a container of liquid nitrogen. To avoid protein degradation by proteolysis, the samples for analysis were transferred to a Dewar containing liquid nitrogen and then thawed individually and homogenised as quickly as possible. Following thawing, the samples were weighed. They were then cut up to aid homogenisation and placed in an Eppendorf tube with phosphate buffered saline (PBS) at a weight: volume ratio of 1:10. A protease inhibitor cocktail (Sigma-Aldrich, Inc, Gillingham, UK) was added at a quantity of 1 μl. The samples were then homogenised using a pellet pestle motor.

    To ensure adequate solubilisation of the proteins and protein subunits in the SDS-PAGE (sodium dodecyl sulphate - polyacrylamide gel electrophoresis) two processes were necessary. Firstly, the homogenised samples had to be denatured by an anionic detergent containing Tris, glycerol, SDS and bromophenol blue (Laemmli Sample Buffer. Bio-Rad Laboratories, Hemel Hempstead, UK). Secondly, the disulphide bonds which maintain protein conformation by covalently crosslinking two different parts of the polypeptide chains were reduced by the addition of β-mercaptoethanol to the sample buffer. This process of denaturation and reduction results in an unfolding of the polypeptide chains to form rod-like complexes of
proteins that have a uniform charge-to-mass ratio proportional to their molecular weight. During electrophoresis, proteins are then separated according to their molecular weight.

The steps involved in preparing the samples are outlined below:

- 20 µl of β-mercaptoethanol (βME) was added to 380 µl of Laemmli Sample Buffer in the fume cupboard.
- 15 µl of homogenised sample was added to a new Eppendorf in duplicate.
- 15 µl of the of Laemmli Sample Buffer and βME mixture was then added to each sample.
- The Eppendorfs were then vortexed for 10 seconds before being centrifuged up to 13,000 rpm.
- Each sample was then boiled at 95 °C in a water bath for 5 minutes.
- Lastly, the Eppendorfs were centrifuged up to 8,000 rpm to gravitate the condensation that formed on the lid of the Eppendorf during boiling.
- The samples were now ready for electrophoresis.

**ii. Making the running buffer**

The Tris/glycine/SDS running buffer (Bio-Rad Laboratories, Hemel Hempstead, UK) was supplied as a 10x concentrate. To make 1 litre of working buffer, 100 ml of buffer concentrate was added to 900 ml of purified water and mixed thoroughly. The final concentration of this solution was: 25 mM tris, 192 mM glycine and 0.1% (w/v) SDS, pH 8.3. This solution was then used as the buffer for the protein electrophoresis.
iii. Preparing the Ready Gels

The gels used for the electrophoresis were Ready Gels (Bio-Rad Laboratories, Hemel Hempstead, UK). These were 7.5% Tris-HCl gels. Each gel contained 10 wells with capacity for 30 μl of sample. The gels were immersed in the running buffer and the comb was removed by positioning the thumbs on the ridges on each end of the comb and pushing upwards with a smooth, continuous motion. The tape was then cut along the black line across the entire length of the bottom of the gel. The tab was then pulled upwardly to remove the tape. The tape must be completely removed to ensure that the gel makes contact with the running buffer in the mini tank. Using a disposable pipette, the wells were rinsed with running buffer to eradicate any air bubbles and any deformed wells were straightened.
iv. Assembling the inner buffer chamber (Figure 10)

Figure 10. Assembling the inner buffer chamber (Adapted from the Bio-Rad Mini-PROTEAN 3 instruction manual)
The Ready Gel cassette was placed into the slots at the bottom of each side of the electrode assembly, ensuring that the short side of the glass plate of the Ready Gel cassette faced inwards toward the notch on the U-shaped gasket. This procedure was then repeated for the second Ready gel cassette on the other side. The Ready Gel cassettes were then pressed up against the gaskets. The electrode assembly, together with the Ready Gels, were then placed inside the clamping frame. The two cam levers on the clamping frame were then closed whilst pushing down on the electrode assembly. This is to ensure good contact between the Ready gel plate and the rubber gasket to prevent any leakage. The electrode assembly, ready gels and the clamping frame together form the inner chamber. The inner chamber was filled with approximately 125 ml of running buffer, so that the buffer reached a level between the tops of the short and long plates of the Ready Gel.
Sample loading (Figure 11)

The samples were loaded into the wells with a pipette using a long, thin gel loading tip. The sample loading guide was placed across the top of the electrode assembly to help with the locating of the wells. 15 µl of Benchmark prestained protein ladder (Gibco Life Sciences, Pisley, UK) was loaded into well 1. This protein ladder shows 10 major bands when separated by electrophoresis on SDS-PAGE, ranging in apparent molecular weight from about 10 to 200 kDa. The proteins were rendered blue by a method covalently coupling dyes to the proteins. The fourth band from the top was coupled with a pink dye for easy orientation and to ensure proper identification of the eNOS. Samples were then loaded into wells 2 to 10, with 25 µl of
sample loaded into each well. The samples were loaded slowly and allowed to settle evenly on the bottom of the well. Each sample was analysed in duplicate.

**vi. Gel electrophoresis**

The inner chamber was inserted into the mini-tank and approximately 200 ml of running buffer was poured into the mini-tank, ensuring that about 1 cm of the gel was covered by buffer. The electrode plugs and jacks were aligned, ensuring that the colours of the plugs on the lid matched the colours of the jacks on the electrode assembly. The electrical leads were attached to the power supply, the Power Pac 300 (Bio-Rad Laboratories, Hemel Hempstead, UK), set at 100 volts constant. The power was switched on and the Gel was allowed to run for 1 hour.

Once electrophoresis was completed, the power supply was switched off and the electrical leads were disconnected. The lid was removed and the inner chamber carefully removed. The buffer was poured out of the inner chamber prior to opening the cams of the clamping frame and the electrode assembly was removed. The gel was then removed from the Ready gel cassette by cutting the white tape along the sides of the Ready Gel cassette where the inner glass plate met the outer plastic plate. This allowed separation of the two plates and the gel was now ready to be floated off the glass plate by submersion and gentle agitation in the transfer buffer.

**5.2c Protein transfer**

**Making the transfer buffer**

The Tris/glycine transfer buffer (Bio-Rad Laboratories, Hemel Hempstead, UK) was supplied as a 10x concentrate. To make 1 litre of working buffer, 100 ml of buffer concentrate was added to 900 ml of purified water and mixed thoroughly. The final concentration of this solution was: 25 mM tris, 192 mM glycine, pH 8.3. This solution was then used as the buffer for the protein transfer. Initially, methanol was
also added to the transfer buffer. However, it was found that optimisation of the transfer of eNOS, which has a relatively high molecular weight, was achieved by omitting the methanol. This did, however, result in a longer transfer time. Therefore to improve the heat dissipation over this prolonged period, transfer buffer that had been stored at 4°C was used.

\textit{ii. Assembling the transfer gel sandwich}

As mentioned previously, following electrophoresis the gel was removed from the Ready Gel cassette and immersed in running buffer. Two fibre pads, two pieces of filter paper (Mini Trans-Blot filter paper, Bio-Rad Laboratories, Hemel Hempstead, UK) and one nitrocellulose membrane (Trans-Blot transfer medium, 0.2 μl pure nitrocellulose membrane, Bio-Rad Laboratories, Hemel Hempstead, UK) were also soaked in running buffer. The gel sandwich was then assembled as outlined below (Figure 12):
Figure 12. Assembling the gel sandwich for electrophoretic transfer (Adapted from the Bio-Rad Mini-PROTEAN 3 instruction manual).

- The gel holder cassette was placed, with the black side down, on to clean paper towels.
- One soaked fibre pad was placed on the black side of the cassette.
- A sheet of filter paper was placed on top of the fibre pad.
- The gel was then placed on the filter paper.
- The nitrocellulose membrane was carefully placed over the gel.
- The second piece of filter paper was placed on top of the membrane.
- Any air bubbles which may have formed were removed by gently rolling a pen over the uppermost filter paper.
- To complete the sandwich, the second fibre pad was placed on top and the gel holder cassette was firmly closed and locked with the white latch.

This process was repeated for each gel which had undergone SDS-PAGE.
iii. Electrophoretic transfer (Figure 13)

Figure 13. Assembling the equipment for electrophoretic transfer from the Ready Gel to the membrane (Adapted from the Bio-Rad Mini-PROTEAN 3 instruction manual).
Two gel holder cassettes were fitted into the electrode module, with the black side of the cassette facing the black side of the module for both cassettes. This ensured that the gel was orientated toward the cathode and the membrane was orientated toward the anode. The proteins were solubilised in an alkaline pH (transfer buffer pH was 8.3), therefore they had a net negative charge and moved toward the anode i.e. from the gel to the membrane.

The electrode assembly, with gel holder cassettes in situ, was placed in the buffer tank, together with the Bio-Ice cooling unit and a magnetic stir bar. Inadequate cooling during transfer leads to swelling of the gel, therefore the cooling unit was used to help with heat dissipation. This was stored in a -20 °C freezer. The stir bar was used to help maintain even buffer temperature and ion distribution within the tank. The tank was then filled with transfer buffer and the lid was put on. The tank was placed on to a magnetic stirrer and the stir rate was set as fast as possible. The electrical leads were attached to the power supply, which was set at 60 volts constant. The power was switched on and the blot was given a transfer time of 2 hours. The ice in the cooling unit was replaced after 1 hour to maximise heat dissipation. At the end of the transfer time the blotting sandwich was disassembled and the membrane was removed for development.

5.2d Protein visualisation

i. Washes

A successful Western blot needs to have an adequate signal/background ratio. Removal of non-specifically bound ligand is therefore crucial. This is achieved by washing or rinsing the blot between each step of the development process. A balance must be achieved as insufficient rinses will result in a strong background, whereas excessive rinses will result in loss of the signal. A non-ionic detergent was used as the
rinsing solution for the blots within this study. Tween-20 (350 μl) was added to 700 ml of Tris buffered saline (TBS). This TBS-Tween solution was then used for washing the blots. Firstly, the blots were rinsed with a small quantity of TBS-Tween solution, and then the blot was soaked in the solution and shaken on a Gyro-Rocker (Jencons-PLS, UK) for 10 minutes prior to a final rinsing. This system of rinsing and soaking of the blots resulted in blots with an acceptable signal/background ratio.

**ii. Quenching**

Nitrocellulose membranes have a high binding capacity, of approximately 80-100 μg/cm², for proteins. This is a benefit when the protein is being transferred from the gel to the nitrocellulose membrane, but is inconvenient when the primary and secondary antibodies are added. This problem is surmounted by quenching the membrane before addition of the antibodies. Quenching is the process by which all the unoccupied binding sites on the membrane are blocked by an “inert” protein. The protein used within this study was bovine serum albumin (BSA). The blots were soaked in a solution of 0.9 g of BSA in 30 ml of TBS and shaken on the Gyro-Rocker for 1 hour. Following quenching, the blots were rinsed as described above.

**iii. Primary antibody solution**

The first antibody used in the process of detection of the protein band (the antigen) on the blot was a mouse monoclonal antibody which recognised eNOS in various mammals, including porcine (called anti-eNOS, Calbiochem-Novabiochem, Nottingham, UK). The antibody was incubated with the blot in the presence of a probing buffer: for this study, 50 μg of anti-eNOS was added to a solution of 0.25 g of BSA in 25 ml of TBS. The blot was soaked in this solution for 1 hour whilst being shaken on the Gyro-Rocker. Following this, the blots were rinsed as described above.
iv. **Secondary antibody solution**

The secondary antibody used was a goat anti-mouse IgG, IgM and IgA that was conjugated with alkaline phosphatase (Calbiochem-Novabiochem, Nottingham, UK). The blot was soaked in a solution of 0.3 g of BSA in 30 ml of TBS with the addition of 10 μl of the secondary antibody. The blot was soaked in this solution for 1 hour whilst being shaken on the Gyro-Rocker. Following this, the blots were rinsed as described above.

v. **Detection system**

The two commonest detection systems used are radiolabelled probes and enzyme conjugated probes. For this study, an enzyme conjugated probe was used. Binding of the enzyme conjugated ligand to the target on the blot is detected by a chromogenic reaction, in which the substrate after the reaction changes colour and precipitates in the vicinity of the enzyme conjugated ligand, producing a colour band. In this study, the substrate used was 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT, Calbiochem-Novabiochem, Nottingham, UK). The alkaline phosphatase conjugated to the secondary antibody hydrolyses the phosphate ester of the BCIP to produce powerful reducing compounds that react rapidly with NBT to convert it to the insoluble NBT-formazan. This is then visible as a dark purple deposit that does not fade upon drying. The blots were incubated with the BCIP/NBT solution at 37°C in a darkened environment for 10 minutes. Following this, the blots were kept in a darkened environment until dry. The process of visualising the proteins by the primary and secondary antibodies and the BCIP/NBT is shown diagrammatically below (Figure 14).
Figure 14. Immunostaining to detect the presence of eNOS

5.2e Densitometry

For the semi-quantitative analysis of the completed blots, each blot was scanned and the individual eNOS bands were measured for size and density using the Bio-Rad GS-700 Imaging Densitometer (Bio-Rad Laboratories, Hemel Hempstead, UK). An arbitrary “percentage adjusted volume” (PAV) was calculated for each band. To standardise these values for each blot, the PAV for the background was also calculated and subtracted from the sample PAV.

5.2f Total protein quantification

It was necessary to determine the amount of total protein in the samples used so that the PAV calculated could be expressed as PAV per mg tissue. This allowed for comparison between blots.

The assay used was the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hemel Hempstead, UK). This assay is based on the observation that the absorbance
maximum for an acidic solution of Coomassie brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs [247, 248].

Reagent preparation

The two reagents required for this assay were the dye reagent and the protein standard. The dye reagent was supplied as a 5x concentrate. This was diluted by adding 200 ml of dye concentrate to 800 ml of high quality purified water and filtering through Whatman No. 1 paper. Once diluted, this was stored at room temperature for up to 2 weeks.

The protein standard used was bovine gamma globulin (BGG). This was supplied in a lyophilized form and needed to be reconstituted with 20 ml of purified water. This then yielded a concentration of 1.4 mg/ml. This was then stored at -20 °C for up to 60 days.

i. Sample preparation

The homogenised samples that were used for the Western blots were frozen. These same samples were then defrosted and used for the protein assay. For the analysis with the spectrophotometer (Philips Scientific & Analytical equipment) a response of between 0.1 – 1.0 optical density (OD) units was needed at a wavelength of 595 nm (OD95). To achieve this response, the samples were diluted by a factor of 8.

ii. Assay procedure

Several dilutions of the protein standard were prepared containing between 0.2 – 1.4 mg/ml as shown below (Table 6):
Table 6. Protein standard dilutions

<table>
<thead>
<tr>
<th>Reconstituted BGG (µl)</th>
<th>Purified water (µl)</th>
<th>Final protein content (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>140</td>
<td>0</td>
<td>140</td>
</tr>
<tr>
<td>120</td>
<td>20</td>
<td>120</td>
</tr>
<tr>
<td>100</td>
<td>40</td>
<td>100</td>
</tr>
<tr>
<td>80</td>
<td>60</td>
<td>80</td>
</tr>
<tr>
<td>60</td>
<td>80</td>
<td>60</td>
</tr>
<tr>
<td>40</td>
<td>100</td>
<td>40</td>
</tr>
<tr>
<td>20</td>
<td>120</td>
<td>20</td>
</tr>
</tbody>
</table>

The assay was then performed as shown below:

- 60 µl of each standard was placed in a test tube.
- 60 µl of each 8x diluted sample was placed in a test tube.
- 60 µl of sample buffer was placed in a test tube. This is the “blank”.
- 3 ml of diluted dye reagent was then added to each test tube.
- Each tube was vortexed and then left for 10 minutes.
- Using the spectrophotometer, the OD₅₉₅ was found for each standard and sample.
- The standard curve was drawn by plotting the OD₅₉₅ against the concentration of the standards using the software program GraphPad Prism 3.00 for Windows (GraphPad software, San Diego, California, USA).
- The unknown samples were then calculated by performing linear regression analysis of the standards and selecting the “standard
curve. X from Y” function in the program. This then determined X
(i.e. protein content) for all unpaired Y (i.e. OD<sub>995</sub>) data
SECTION C

RESULTS
CHAPTER SIX

THE INFLUENCE OF WARM ISCHAEMIC INJURY

6.1 Introduction

6.2 The effect of warm ischaemia on IRR during pulsatile MP

6.3 The effect of warm ischaemia on nitrate/nitrite during pulsatile MP

6.4 The effect of warm ischaemia on eNOS during pulsatile MP

6.5 Conclusion
6.1 Introduction

As described in detail in chapters 4 and 5, each kidney underwent pulsatile MP for 6 hours after sustaining a period of warm ischaemia of between 10 and 90 minutes. During pulsatile MP, perfusate systolic pressure, diastolic pressure and flow rate were measured every minute and IRR was calculated using the formula: IRR (mmHg/ml/min) = mean perfusate pressure/flow rate. Samples of perfusate effluent were taken every hour and tissue biopsies were taken every two hours. The perfusate effluent was then analysed for total nitrate/nitrite content using the Griess reagent. Western blotting was performed on the tissue biopsies, looking at the enzyme eNOS.

Statistical analysis was performed using the following statistical packages: GraphPad Prism version 3.00 for Windows and GraphPad Instat version 4.10 for Windows, GraphPad Software, San Diego, California, USA, www.graphpad.com. SPSS version 10.0 for Windows, SPSS Inc, Chicago, Illinois, USA, www.spss.com.

6.2 The effect of warm ischaemia on IRR during pulsatile MP

All the data within this section was non-parametric in nature. Therefore, for statistical analysis the appropriate non-parametric tests were used.

This study showed that IRR at the start of pulsatile MP was higher for those kidneys which were subjected to the longer periods of warm ischaemia than for those with the shorter warm ischaemic injury times (Figure 15). During the 6 hours of pulsatile MP, IRR decreased in all groups. However, the greatest falls in IRR occurred in those kidneys which had been subjected to the longest periods of warm ischaemia (Figure 16).
Figure 15. Relationship between the duration of warm ischaemia and the IRR at the start of pulsatile MP. Values expressed as median, quartiles and range.

Figure 16. Relationship between the duration of warm ischaemia and the IRR over six hours of pulsatile MP. Values are expressed as means.

Using multiple regression analyses, with IRR as the dependent variable and WIT, MP time, total nitrate/nitrite concentration and eNOS level as the independent...
Figure 15. Relationship between the duration of warm ischaemia and the IRR at the start of pulsatile MP. Values expressed as median, quartiles and range.

Figure 16. Relationship between the duration of warm ischaemia and the IRR over six hours of pulsatile MP. Values are expressed as means.

Using multiple regression analyses, with IRR as the dependent variable and WIT, MP time, total nitrate/nitrite concentration and eNOS level as the independent
variables a linear correlation between these variables was found, with $r^2=0.2322$ and $p<0.0001$ (Figure 17). The variables which made the significant contributions were WIT and MP time as shown below (Table 7):

**Table 7. Results of multiple regression analyses with IRR as the dependent variable**

<table>
<thead>
<tr>
<th>Variable</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP time</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>WIT</td>
<td>0.0066</td>
</tr>
<tr>
<td>Total nitrate/nitrite</td>
<td>0.4722</td>
</tr>
<tr>
<td>eNOS level</td>
<td>0.9088</td>
</tr>
</tbody>
</table>
Figure 17. Results of multiple regression analyses with IRR as the dependent variable. A: Correlation with length of MP. B: Correlation with length of warm ischaemia. C: Correlation with total nitrate/nitrite concentration. D: Correlation with eNOS level. The r-squared value was 0.2322 and p was <0.0001. P values for individual contributions of the independent variables are shown on the graphs.

Non-parametric analysis of variance (Kruskal-Wallis test) demonstrated that the variation between the median IRR for each warm ischaemic time (WIT) at the start of machine perfusion was significantly greater than would have been expected due to chance alone, with a p value of <0.0055 and a Kruskal-Wallis statistic (KW) of 16.529. Dunn’s multiple comparisons post test showed that the significant differences
were between the shortest WIT i.e. <10 minutes and the longer WIT’s i.e. 45, 60 and 90 minutes (Table 8):

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Mean rank difference</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>10m WIT vs. 15m WIT</td>
<td>-11.167</td>
<td>NS</td>
</tr>
<tr>
<td>10m WIT vs. 30m WIT</td>
<td>-8.667</td>
<td>NS</td>
</tr>
<tr>
<td>10m WIT vs. 45m WIT</td>
<td>-18.000</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>10m WIT vs. 60m WIT</td>
<td>-20.833</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>10m WIT vs. 90m WIT</td>
<td>-18.333</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>15m WIT vs. 30m WIT</td>
<td>2.500</td>
<td>NS</td>
</tr>
<tr>
<td>15m WIT vs. 45m WIT</td>
<td>-6.833</td>
<td>NS</td>
</tr>
<tr>
<td>15m WIT vs. 60m WIT</td>
<td>-9.667</td>
<td>NS</td>
</tr>
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<td>15m WIT vs. 90m WIT</td>
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<td>NS</td>
</tr>
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<td>30m WIT vs. 45m WIT</td>
<td>-9.333</td>
<td>NS</td>
</tr>
<tr>
<td>30m WIT vs. 60m WIT</td>
<td>-12.167</td>
<td>NS</td>
</tr>
<tr>
<td>30m WIT vs. 90m WIT</td>
<td>-9.667</td>
<td>NS</td>
</tr>
<tr>
<td>45m WIT vs. 60m WIT</td>
<td>-2.833</td>
<td>NS</td>
</tr>
<tr>
<td>45m WIT vs. 90m WIT</td>
<td>-0.333</td>
<td>NS</td>
</tr>
<tr>
<td>60m WIT vs. 90m WIT</td>
<td>2.5</td>
<td>NS</td>
</tr>
</tbody>
</table>

Comparisons were also made between the IRR at the start and end of pulsatile MP for each WIT. This confirmed that IRR was significantly lower in all but the <10
minute warm ischaemia group at the end of 6 hours of perfusion. (p=0.0313 in all but 10 minute group, Wilcoxon signed rank test; Figure 18).

The data was then divided into two equal groups, one with a short warm time of ≤ 30 minutes and the other with a long warm time of ≥ 45 minutes. A repeated measures GLM analysis showed that over the 6 hours of pulsatile MP there was a significant change in IRR within the two warm ischaemic groups (Wilks’ Lambda F value=10.405, p=0.000) and also between the groups (Wilks’ Lambda F=3.380, p=0.012). Over the 6 hours, the line of best fit had a linear (p=0.000) and quadratic (p=0.009) component (Figure 19). Comparison between the IRR at the start and end of pulsatile MP in the long and short warm ischaemia groups showed that, again, IRR was significantly lower in both groups at the end of 6 hours of perfusion (p=0.0005 in <30 minutes group and p=0.0002 in >45 minutes group, Wilcoxon signed rank test; Figure 20).
Figure 19. Relationship between the duration of warm ischaemia and the IRR over six hours of pulsatile MP for the short (<30 minutes) and long (>45 minutes) sub-groups. Values are expressed as medians.

Figure 20. Comparison between the IRR at the start and end of six hours of pulsatile MP for the short (<30 minutes) and long (>45 minutes) sub-groups. Values are expressed as median, quartiles and range. An asterisk indicates a significant difference from the initial IRR (p=0.0005 for short WIT group, p=0.0002 for long WIT group).
6.3 The effect of warm ischaemia on nitrate/nitrite during pulsatile MP

Initially, both nitrite and total nitrate/nitrite concentrations were measured in the perfusate effluent samples by the Griess reagent. However, the concentration of nitrite was very low and below the range for accurate analysis by the Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical Company, Alexis Corporation (UK) Ltd, Nottingham, UK). Therefore, only total nitrate/nitrite concentrations were measured in subsequent samples and these are presented here. All the data within this section was normally distributed. Therefore, for statistical analysis the appropriate parametric tests were used.

Samples were taken and analysed every hour for nitrate/nitrite concentration. After one hour of pulsatile MP when the first samples were taken, the total nitrate/nitrite concentration tended to be higher for those kidneys which were subjected to the longer periods of warm ischaemia than for those with the shorter warm ischaemic times (Figure 21). During the 6 hours of pulsatile MP, the total nitrate/nitrite concentration tended to increase over the 6 hours in most groups (Figure 22).
Figure 21. Relationship between the duration of warm ischaemia and the total nitrate/nitrite concentration after one hour of pulsatile MP. Values are expressed as means ± SEM.

Figure 22. Relationship between the duration of warm ischaemia and the total nitrate/nitrite concentration over six hours of pulsatile MP. Values are expressed as means.
Using multiple regression analyses, with nitrate/nitrite concentration as the dependent variable and WIT, MP time, IRR and eNOS level as the independent variables a linear correlation between these variables was found, with $r^2=0.1271$ and $p=0.014$ (Figure 23). The only variable which made a significant contribution was the WIT as shown below (Table 9)

Table 9. Results of multiple regression analyses with nitrate/nitrite concentration as the dependent variable

<table>
<thead>
<tr>
<th>Variable</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP time</td>
<td>0.7181</td>
</tr>
<tr>
<td>WIT</td>
<td>0.0029</td>
</tr>
<tr>
<td>IRR</td>
<td>0.4722</td>
</tr>
<tr>
<td>eNOS level</td>
<td>0.1389</td>
</tr>
</tbody>
</table>
Figure 23. Results of multiple regression analyses with total nitrate/nitrite concentration as the dependent variable. A: Correlation with length of MP. B: Correlation with length of warm ischaemia. C: Correlation with IRR. D: Correlation with eNOS level. The r-squared value was 0.1271 and the p value was 0.014. P values for the individual contributions of the independent variables are shown on the graphs.
One way analysis of variance (ANOVA) demonstrated that the variation between the mean nitrate/nitrite concentrations for each WIT after 1 hour of pulsatile MP was not significantly greater than would have been expected due to chance alone, with a p value of 0.8537. Therefore, it was not appropriate to perform any post tests.

Comparisons were also made between the total nitrate/nitrite concentration after 1 hour and at end of pulsatile MP for each WIT. The nitrate/nitrite concentration was higher at the end of the pulsatile MP in all but the 10 minutes WIT group. However, using a paired t test, this did not reach statistical significance in any of the groups (Figure 24).

![Figure 24. Comparison between the total nitrate/nitrite concentration at the start and end of six hours of pulsatile MP. Values are expressed as means ± SEM.](image)

When the data was divided into a short (<30 minutes) and a long (>45 minutes) WIT group, the total nitrate/nitrite concentration was higher in the group with the longer WIT at all time points of pulsatile MP (Figure 25). A repeated measures GLM analysis showed that over the 6 hours of pulsatile MP for the short
and long WIT groups there was no significant change in total nitrate/nitrite concentrations within the warm ischaemic groups (Wilks' Lambda F value=2.046, p=0.111) and also between the groups (Wilks' Lambda F=1.023, p=0.428). Over the 6 hours, the line of best fit was a linear line (p=0.023). There was no difference in the shape of the lines between the different warm ischaemic groups. Comparison between the total nitrate/nitrite concentration after 1 hour and after 6 hours of pulsatile MP in the long and short warm ischaemic groups showed an increase in total nitrate/nitrite in both groups after 6 hours; however, using a paired t test this was not statistically significant in either group (Figure 26).

Figure 25. Relationship between the duration of warm ischaemia and the total nitrate/nitrite concentration over six hours of pulsatile MP for the short (<30 minutes) and the long (>45 minutes) sub-groups. Values are expressed as means ± SEM.
6.4 The effect of warm ischaemia on eNOS during pulsatile MP

Wedge biopsies were performed every hour and stored in liquid nitrogen prior to being analysed for eNOS using Western blotting techniques, as described in detail in chapter 5.

In this study eNOS was expressed in arbitrary units of percentage adjusted volume per milligram of tissue (PAV/mg). All the data within this section was normally distributed. Therefore, for statistical analysis the appropriate parametric tests were used.

At the start of pulsatile MP the level of eNOS tended to be higher for those kidneys which were subjected to the longer periods of warm ischaemia than for those with the shorter warm ischaemic times (Figure 27). During the 6 hours of pulsatile
MP, the level of eNOS increased slightly in the 10, 15, 30 and 60 minute WIT group but decreased in the 45 and 90 minute WIT group (Figure 28).

**Figure 27.** Relationship between the duration of warm ischaemia and the eNOS level at the start of pulsatile MP. Values are expressed as means ± SEM.

**Figure 28.** Relationship between the duration of warm ischaemia and the eNOS level over six hours of pulsatile MP. Values are expressed as means.
Using multiple regression analyses, with eNOS level as the dependent variable and WIT, MP time, IRR and total nitrate/nitrite concentration as the independent variables a linear correlation was not found as the $r^2$ value was 0.0529 and p value was 0.2877 (Figure 29). Therefore, no variables made a significant contribution, as shown below (Table 10):

Table 10. Results of multiple regression analyses with eNOS level as the dependent variable

<table>
<thead>
<tr>
<th>Variable</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP time</td>
<td>0.4750</td>
</tr>
<tr>
<td>WIT</td>
<td>0.3886</td>
</tr>
<tr>
<td>IRR</td>
<td>0.9088</td>
</tr>
<tr>
<td>Total nitrate/nitrite</td>
<td>0.1389</td>
</tr>
</tbody>
</table>
Figure 29. Results of multiple regression analyses with NOS level as the dependent variable. A: Correlation with length of MP. B: Correlation with length of warm ischaemia. C: Correlation with IRR. D: Correlation with total nitrate/nitrite concentration. The r-squared value was 0.0509 and p was 0.0302. P values for the individual independent variables are shown on the graphs.

One way ANOVA demonstrated no significant difference (p=0.3276) in the variation of the eNOS levels for each WIT at the start of pulsatile MP. Therefore, it was not appropriate to perform any post tests.

Comparisons were made between the eNOS level at the start and end of pulsatile MP for each WIT. The eNOS level was higher at the end of pulsatile MP in
all but the 90 minutes WIT group. However, using a paired \( t \) test, this was not statistically significant in any of the groups (Figure 30).

![Chart showing eNOS levels over time](chart)

**Figure 30.** Comparison between the eNOS level at the start and end of six hours of pulsatile MP. Values are expressed as means ± SEM.

When the data was divided into the short (<30 minutes) and long (>45 minutes) WIT groups, the eNOS level was higher in the group with the longer WIT at all time points of pulsatile MP (Figure 31). A repeated measures GLM analysis showed that over the 6 hours of pulsatile MP for the short and long WIT groups there was no significant change in the eNOS level within the warm ischaemic groups (Wilks’ Lambda F value=2.001, \( p=0.135 \)) and also between the groups (Wilks’ Lambda F=0.922, \( p=0.478 \)). Comparison between the eNOS level at the start and end of 6 hours of pulsatile MP in the long and short warm ischaemic groups showed an increase in the level of eNOS in both groups after 6 hours; however, using a paired \( t \) test this was not statistically significant in either group (Figure 32).
Figure 31. Relationship between the duration of warm ischaemia and the eNOS level over six hours of pulsatile MP for the short (<30 minutes) and long (>45 minutes) sub-groups. Values are expressed as means ± SEM.

Figure 32. Comparison between the eNOS level at the start and end of six hours of pulsatile MP for the short (<30 minutes) and long (>45 minutes) sub-groups. Values are expressed as means ± SEM.
6.5 Conclusion

There is a clear and significant relationship between the length of WIT and IRR at the start of pulsatile MP in this study. As the WIT lengthens, the IRR increases proportionally. During the six hours of pulsatile MP the IRR reduces significantly, with the largest reduction in IRR being in those kidneys which initially had the highest IRR.

The nitrate concentration tended to be higher the longer the period of WIT. Multiple regression analysis showed that the WIT was the only independent factor to have a linear relationship with the nitrate concentration. However, one way ANOVA did not show any significant differences between the nitrate concentrations after one hour of pulsatile MP within the different WIT groups. Over six hours of pulsatile MP the nitrate concentration did tend to increase, but this did not reach statistical significance.

The expression of eNOS also increased as the WIT lengthened and the eNOS level became higher in all but the 90 minute WIT group over the six hours of pulsatile MP. Nevertheless, none of these trends reached statistical significance.
CHAPTER SEVEN

THE INFLUENCE OF COLD ISCHAEMIC INJURY

7.1 Introduction

7.2 The effect of cold ischaemia on IRR during pulsatile MP

7.3 The effect of cold ischaemia on nitrate/nitrite during pulsatile MP

7.4 The effect of cold ischaemia on eNOS during pulsatile MP

7.5 Conclusion
7.1 Introduction

As described in detail in chapter 4 and 5, each kidney underwent pulsatile MP for 6 hours after sustaining a period of warm ischaemia of either 15 or 45 minutes. Following this, they underwent a second ischaemic insult of between 2 and 48 hours of static cold storage. During pulsatile MP, perfusate systolic pressure, diastolic pressure and flow rate were measured every minute and IRR was calculated using the formula: $\text{IRR (mmHg/ml/min)} = \frac{\text{mean perfusate pressure}}{\text{flow rate}}$. Samples of perfusate effluent and tissue biopsies were taken every two hours. The perfusate effluent was then analysed for total nitrate/nitrite content using the Griess reagent. Western blotting was performed on the tissue biopsies, looking at the enzyme eNOS.

Statistical analysis was performed using the following statistical packages: GraphPad Prism version 3.00 for Windows and GraphPad Instat version 4.10 for Windows, GraphPad Software, San Diego, California, USA, www.graphpad.com. SPSS version 10.0 for Windows, SPSS Inc, Chicago, Illinois, USA, www.spss.com.

7.2 The effect of cold ischaemia on IRR during pulsatile MP

All the data within this section was non-parametric in nature. Therefore, for statistical analysis the appropriate non-parametric tests were used.

This study showed that in the group which had been subjected to 15 minutes of warm ischaemia that IRR at the start of MP was higher for those kidneys which were subjected to the longer periods of cold ischaemia (Figure 33). Linear regression analysis found that the slope was significantly non-zero, confirming the linear relationship between CT and IRR in the 15 minute WT group (Figure 34). In the group subjected to 45 minutes of warm ischaemia, IRR was similar for all cold ischaemic times (Figure 35) and no linear relationship existed between the CT and
IRR (Figure 34). However, non-parametric analysis of variance (Kruskal-Wallis test) did not show any significant difference between the IRRs at the start of machine perfusion for any of the kidneys.

Figure 33. Relationship between the duration of cold ischaemia, in kidneys subjected to 15 minutes of warm ischaemia, and the IRR at the start of pulsatile MP. Values are expressed as median, quartiles and range.
Figure 34. Results of linear regression analyses looking at the potential relationship between cold ischaemia and IRR at the start of pulsatile MP. The r-squared and p values for each warm ischaemia group are shown on the graph.

Figure 35. Relationship between the duration of cold ischaemia, in kidneys subjected to 45 minutes of warm ischaemia, and the IRR at the start of pulsatile MP. Values are expressed as median, quartiles and range.
During the 6 hours of pulsatile MP, IRR reduced in all groups (Figure 36 & Figure 37).

**Figure 36.** Relationship between the duration of cold ischaemia, in kidneys subjected to 15 minutes of warm ischaemia, and the IRR over six hours of pulsatile MP. Values are expressed as means.
Figure 37. Relationship between the duration of cold ischaemia, in kidneys subjected to 45 minutes of warm ischaemia, and the IRR over six hours of pulsatile MP. Values are expressed as means.

Comparisons were made between the IRR at the start and end of pulsatile MP for each CT at both 15 minutes and 45 minutes of WIT. This confirmed that IRR was significantly lower in all groups at the end of 6 hours of perfusion. (p=0.0313, Wilcoxon signed rank test; (Figure 38 & Figure 39).
Figure 38. Comparison between the IRR at the start and end of six hours of pulsatile MP in the kidneys subjected to 15 minutes of warm ischaemia. Values are express as median, quartiles and range. An asterisk indicates a significant difference from the initial IRR (p=0.0313).

Figure 39. Comparison between the IRR at the start and end of six hours of pulsatile MP in the kidneys subjected to 45 minutes of warm ischaemia. Values are express as median, quartiles and range. An asterisk indicates a significant difference from the initial IRR (p=0.0313).
Using multiple regression analyses, with IRR as the dependent variable and CT, WIT, MP time, total nitrate/nitrite concentration and eNOS level as the independent variables a linear correlation between these variables was found, with $r^2=0.2799$ and $p<0.0001$ (Figure 40). The variables which made the significant contributions were WIT and MP time as shown below (Table 11):

**Table 11. Results of multiple regression analyses with IRR as the dependent variable**

<table>
<thead>
<tr>
<th>Variable</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP time</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>WIT</td>
<td>0.0397</td>
</tr>
<tr>
<td>CT</td>
<td>0.2752</td>
</tr>
<tr>
<td>Total nitrate/nitrite</td>
<td>0.9586</td>
</tr>
<tr>
<td>eNOS level</td>
<td>0.4799</td>
</tr>
</tbody>
</table>
Figure 40. Results of multiple regression analyses with IRR as the dependent variable. A: Correlation with length of MP. B: Correlation with length of warm ischaemia. C: Correlation with length of cold ischaemia. D: Correlation with total nitrate/nitrite concentration. E: Correlation with eNOS level. The r-squared value was 0.2598 and p was <0.0001. P values for the individual contributions of the independent variables are shown on the graphs.
7.3 The effect of cold ischaemia on nitrate/nitrite during pulsatile MP

All the data within this section was normally distributed. Therefore, for statistical analysis the appropriate parametric tests were used.

Samples were taken and analysed every two hours for nitrate/nitrite concentration. After two hours of pulsatile MP when the first samples were taken, the total nitrate/nitrite concentration tended to be higher for those kidneys subjected to the longer periods of cold ischaemia. This was true whether the kidney had sustained 15 or 45 minutes of warm ischaemia (Figure 41). During the six hours of pulsatile MP, the total nitrate/nitrite concentration decreased in all but the 2 hour cold ischaemic groups. This was again true regardless of whether the kidneys had sustained 15 or 45 minutes of WT (Figure 42 & Figure 43).

Figure 41. Relationship between the duration of cold ischaemia and the total nitrate/nitrite concentration after two hours of pulsatile MP. Values are expressed as means ± SEM.
7.3 The effect of cold ischaemia on nitrate/nitrite during pulsatile MP

All the data within this section was normally distributed. Therefore, for statistical analysis the appropriate parametric tests were used.

Samples were taken and analysed every two hours for nitrate/nitrite concentration. After two hours of pulsatile MP when the first samples were taken, the total nitrate/nitrite concentration tended to be higher for those kidneys subjected to the longer periods of cold ischaemia. This was true whether the kidney had sustained 15 or 45 minutes of warm ischaemia (Figure 41). During the six hours of pulsatile MP, the total nitrate/nitrite concentration decreased in all but the 2 hour cold ischaemic groups. This was again true regardless of whether the kidneys had sustained 15 or 45 minutes of WT (Figure 42 & Figure 43).

![Graph](image)

**Figure 41. Relationship between the duration of cold ischaemia and the total nitrate/nitrite concentration after two hours of pulsatile MP. Values are expressed as means ± SEM.**
Figure 42. Relationship between the duration of cold ischaemia and the total nitrate/nitrite concentration over six hours of pulsatile MP for kidneys subjected to 15 minutes of warm ischaemia. Values are expressed as means ± SEM.

Figure 43. Relationship between the duration of cold ischaemia and the total nitrate/nitrite concentration over six hours of pulsatile MP for kidneys subjected to 45 minutes of warm ischaemia. Values are expressed as means ± SEM.
Using multiple regression analyses with nitrate/nitrite concentrations as the dependent variable and WIT, CT, MP time, IRR and eNOS as the independent variables a linear correlation between these variables was found, with $r^2=0.0946$ and $p=0.0086$ (Figure 44). The only variable to make a significant contribution was cold time as shown below (Table 12): 

Table 12. Results of multiple regression analyses with nitrate/nitrite concentration as the dependent variable

<table>
<thead>
<tr>
<th>Variable</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP time</td>
<td>0.1636</td>
</tr>
<tr>
<td>WIT</td>
<td>0.1097</td>
</tr>
<tr>
<td>CT</td>
<td>0.0041</td>
</tr>
<tr>
<td>IRR</td>
<td>0.6473</td>
</tr>
<tr>
<td>eNOS level</td>
<td>0.9586</td>
</tr>
</tbody>
</table>
Figure 44. Results of multiple regression analyses with total nitrate/nitrite concentration as the dependent variable. A: Correlation with length of MP. B: Correlation with length of warm ischaemia. C: Correlation with length of cold ischaemia. D: Correlation with IRR. E: Correlation with eNOS level. The r-squared value was 0.0946 and the p value was 0.0086. P values for the individual contributions of the independent variables are shown on the graphs.
In the group of kidneys subjected to 15 minutes of warm ischaemia, one way analysis of variance (ANOVA) demonstrated that the variation between the mean nitrate/nitrite concentration after 2 hours of pulsatile MP was significantly greater than would have been expected due to chance alone, with a p value of 0.0006 and r-squared equal to 0.5282. Tukey's multiple comparisons test showed that the significant differences were between the shortest CT i.e. 2 hours and all the other CT's (Table 13):

Table 13. Result of Tukey's multiple comparisons post test following one way ANOVA of CT and nitrate/nitrite concentration after 2 hours of pulsatile MP.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Mean difference</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 vs 12 hours CT</td>
<td>-3.333</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>2 vs 24 hours CT</td>
<td>-3.075</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>2 vs 36 hours CT</td>
<td>-3.082</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>2 vs 48 hours CT</td>
<td>-5.302</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>12 vs 24 hours CT</td>
<td>0.2583</td>
<td>NS</td>
</tr>
<tr>
<td>12 vs 36 hours CT</td>
<td>0.2517</td>
<td>NS</td>
</tr>
<tr>
<td>12 vs 48 hours CT</td>
<td>-1.968</td>
<td>NS</td>
</tr>
<tr>
<td>24 vs 36 hours CT</td>
<td>-0.0067</td>
<td>NS</td>
</tr>
<tr>
<td>24 vs 48 hours CT</td>
<td>-2.227</td>
<td>NS</td>
</tr>
<tr>
<td>36 vs 48 hours CT</td>
<td>-2.220</td>
<td>NS</td>
</tr>
</tbody>
</table>
In the group of kidneys subjected to 45 minutes of warm ischaemia, one way analysis of variance (ANOVA) demonstrated that the variation between the mean nitrate/nitrite concentrations after 2 hours of pulsatile MP was not significantly greater than would be expected due to chance alone, with a p value of 0.2149. Therefore, it was not appropriate to perform any post tests.

Comparisons were made between the total nitrate/nitrite concentration after 2 hours and at the end of pulsatile MP for each cold time in both the 15 and 45 minute warm ischaemic groups. The concentration was lower after six hours of pulsatile MP in all but the 2 hour cold ischaemic group, regardless of whether they sustained 15 or 45 minutes WT. However, using a paired t test, this did not reach statistical significance in any of the group (Figure 45 & Figure 46).

![Graph showing nitrate/nitrite concentration over cold ischaemic time](image)

**Figure 45.** Comparison between the total nitrate/nitrite concentration after two and six hours of pulsatile MP for kidney subjected to 15 minutes of WIT. Values are expressed as means ± SEM.
Figure 46. Comparison between the total nitrate/nitrite concentration after two and six hours of pulsatile MP for kidney subjected to 45 minutes of WIT. Values are expressed as means ± SEM.

### 7.3 The effect of cold ischaemia on eNOS during pulsatile MP

Biopsies were performed every hour and stored in liquid nitrogen prior to being analysed for eNOS using Western blotting techniques, as described in detail in chapter 5.

Again, in this study eNOS was expressed in arbitrary units of percentage adjusted volume per milligram of tissue (PAV/mg). All the data within this section was normally distributed. Therefore, for statistical analysis the appropriate parametric tests were used.

At the start of pulsatile MP the level of eNOS tended to be lower for those kidneys which were subjected to longer periods of cold ischaemia than for those with the shorter CT's, regardless of whether the kidneys had been subjected to 15 or 45 minutes of warm ischaemia (Figure 47). However, as shown in chapter 6, the level of eNOS was higher in the group subjected to 45 minutes of WIT than in the 15 minute
WIT group. During the six hours of pulsatile MP, the level of eNOS increased slightly in all but the group of kidneys subjected to 2 hours of cold ischaemia and 45 minutes of warm ischaemia (Figure 48 & Figure 49).

Figure 47. Relationship between the duration of cold ischaemia and the eNOS level at the start of pulsatile MP. Values are expressed as means ± SEM.
Figure 48. Relationship between the duration of cold ischaemia and the eNOS level over six hours of pulsatile MP for kidneys subjected to 15 minutes of warm ischaemia. Values are expressed as means ± SEM.

Figure 49. Relationship between the duration of cold ischaemia and the eNOS level over six hours of pulsatile MP for kidneys subjected to 45 minutes of warm ischaemia. Values are expressed as means ± SEM.
Using multiple regression analyses, with eNOS as the dependent variable and WIT, CT, MP time, IRR and total nitrate/nitrite concentration as the independent variables a linear correlation between these variables was found, with $r^2=0.1721$ and $p<0.001$ (Figure 50). The variables which made the significant contributions were cold ischaemic time and warm ischaemic time as shown below (Table 14):

Table 14. Results of multiple regression analyses with eNOS level as the dependent variable

<table>
<thead>
<tr>
<th>Variable</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP time</td>
<td>0.7429</td>
</tr>
<tr>
<td>WT</td>
<td>0.0122</td>
</tr>
<tr>
<td>CT</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Total nitrate/nitrite</td>
<td>0.6473</td>
</tr>
<tr>
<td>IRR</td>
<td>0.4799</td>
</tr>
</tbody>
</table>
Figure 50. Results of multiple regression analyses with eNOS level as the dependent variable. A: Correlation with length of MP. B: Correlation with length of warm ischaemia. C: Correlation with length of cold ischaemia. D: Correlation with total nitrate/nitrite concentration. E: Correlation with IRR. The r-squared value was 0.1721 and the p value was <0.001. P values for the individual contributions of the independent variables are shown on the graphs.
One way ANOVA demonstrated no significant difference ($p=0.1212$) in the variation of the eNOS levels at the start of pulsatile MP for each CT within the 15 minute WT group. However, in the kidneys subjected to 45 minutes of WIT, the variation between means at the start of pulsatile MP was significantly greater than would have been expected due to chance alone, with a $p$ value of $<0.0001$. Tukey’s multiple comparisons test showed that the significant difference was between the shortest CT of 2 hours and one of the longest CT’s i.e. 36 hours of cold ischaemia (Table 15).

Table 15. Result of Tukey’s multiple comparisons post test following one way ANOVA of CT and eNOS level at the start of pulsatile MP.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Mean difference</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 vs 12 hours CT</td>
<td>7.016</td>
<td>NS</td>
</tr>
<tr>
<td>2 vs 24 hours CT</td>
<td>10.13</td>
<td>NS</td>
</tr>
<tr>
<td>2 vs 36 hours CT</td>
<td>12.09</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>2 vs 48 hours CT</td>
<td>10.47</td>
<td>NS</td>
</tr>
<tr>
<td>12 vs 24 hours CT</td>
<td>3.110</td>
<td>NS</td>
</tr>
<tr>
<td>12 vs 36 hours CT</td>
<td>5.073</td>
<td>NS</td>
</tr>
<tr>
<td>12 vs 48 hours CT</td>
<td>3.456</td>
<td>NS</td>
</tr>
<tr>
<td>24 vs 36 hours CT</td>
<td>1.963</td>
<td>NS</td>
</tr>
<tr>
<td>24 vs 48 hours CT</td>
<td>0.3458</td>
<td>NS</td>
</tr>
<tr>
<td>36 vs 48 hours CT</td>
<td>-1.617</td>
<td>NS</td>
</tr>
</tbody>
</table>
Comparisons were also made between the eNOS level at the start and end of pulsatile MP for each cold and warm ischaemic time. The eNOS level was higher at the end of pulsatile MP in all but the group of kidneys subjected to 2 hours cold and 45 minutes warm ischaemia. However, using a paired t test, this did not reach statistical significance in any of the groups (Figure 51 & Figure 52).

Figure 51. Comparison between the eNOS level at the start and end of pulsatile MP for kidney subjected to 15 minutes of WIT. Values are expressed as means ± SEM.
Figure 52. Comparison between the eNOS level at the start and end of pulsatile MP for kidney subjected to 45 minutes of WIT. Values are expressed as means ± SEM.

7.5 Conclusion

When studying kidneys subjected to a short WIT of 15 minutes, a significant linear relationship was found between the length of CIT and the IRR at the start of pulsatile MP. When kidneys subjected to the same CIT’s were also subjected to a longer WIT of 45 minutes the initial IRR was consistently higher but no such linear relationship existed. Nonetheless, in all kidneys the IRR reduced significantly over six hours of pulsatile MP.

The nitrate concentration increased as the CIT lengthened for both WIT groups. Multiple regression analysis showed that the CIT was the only independent factor to have a linear relationship with the nitrate concentration. Substantiating this, one way ANOVA showed a significant difference between the nitrate concentrations after two hours of pulsatile MP within the different CIT groups which had been subjected to 15 minutes of WIT, but not those subjected to 45 minutes of WIT. Over
six hours of pulsatile MP the nitrate concentration decreased in all but the 2 hour CIT group (regardless of WIT), but this did not reach statistical significance.

The expression of eNOS decreased as the CIT increased, with multiple regression analyses showing a significant contribution to the linear relationship by both the CIT and the WIT. Although the eNOS level was slightly higher at the end of six hours of pulsatile MP in all but those kidneys subjected to 2 hours CIT and 45 minutes WIT, these were not statistically significant.
CHAPTER EIGHT

THE INFLUENCE OF NO DONORS AND NOS INHIBITORS

8.1 Introduction

8.2 The effect of NO donors and NOS inhibitors on IRR during pulsatile MP

8.3 The effect of NO donors and NOS inhibitors on eNOS during pulsatile MP

8.4 Conclusion
8.1 Introduction

As described in detail in chapters 4 and 5, each kidney underwent pulsatile MP for 6 hours after sustaining a period of warm ischaemia of 45 minutes and cold ischaemia of 24 hours.

The perfusate used this time had one of the following added:

Group A: sodium nitroprusside (SNP), a NO donor.
Group B: L-NAME (LN), an inhibitor of both iNOS and cNOS.
Group C: ADMA, a cNOS inhibitor.
Group D: Aminoguanide (AG), an iNOS inhibitor.
Group E: Control, with no additives to perfusate.

During pulsatile MP, perfusate systolic pressure, diastolic pressure and flow rate were measured every minute and IRR was calculated using the formula: IRR (mmHg/ml/min) = mean perfusate pressure/flow rate. Tissue biopsies were taken every two hours. Western blotting was performed on the tissue biopsies, looking at the enzyme eNOS.

In this experiment, effluent perfusate was not taken and analysed by the Griess reagent for total nitrate/nitrite concentration because of the additives already within the perfusate which would interfere with these results and make them non-valid.

8.2 The effect of NO donors and NOS inhibitors on IRR during pulsatile MP

All the data within this section was normally distributed, but with differing standard deviations (SD). Therefore, for statistical analysis the appropriate parametric tests (with correction for the differing SD's where necessary) were used.

This study showed that compared to the control group, IRR at the start of pulsatile MP was higher for those kidneys where SNP or ADMA was added to the perfusate and slightly lower for those kidneys where L-NAME or aminoguanide was added to the perfusate (Figure 53). During the six hours of pulsatile MP, IRR decreased in all groups. However, the greatest falls occurred in those which had the highest initial IRR i.e. the SNP and ADMA groups (Figure 54).

![Figure 53. Relationship between different additions to the perfusate and the IRR at the start of pulsatile MP. Values are expressed as median, quartiles and range.](image)
Figure 54. Relationship between the different additions to the perfusate and the IRR over 6 hours of pulsatile MP. Values are expressed as means.

One way analysis of variance (ANOVA) demonstrated that the variation between the mean IRR for each intervention group at the start of machine perfusion was significantly greater than would have been expected due to chance alone, with a p value of 0.0398. However, the Tukey-Kramer multiple comparisons post tests did not elucidate any significant differences between the groups (Table 16). This may be due to the small sizes of the groups.
Table 16. Result of Tukey-Kramer multiple comparisons test following one way ANOVA of IRR for different interventional groups at the start of pulsatile MP. A q value of >4.155 would give a p value of <0.05.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Q value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNP vs L-NAME</td>
<td>3.139</td>
<td>NS</td>
</tr>
<tr>
<td>SNP vs ADMA</td>
<td>0.4898</td>
<td>NS</td>
</tr>
<tr>
<td>SNP vs Aminoguanide</td>
<td>2.927</td>
<td>NS</td>
</tr>
<tr>
<td>SNP vs Control</td>
<td>2.469</td>
<td>NS</td>
</tr>
<tr>
<td>L-NAME vs ADMA</td>
<td>3.629</td>
<td>NS</td>
</tr>
<tr>
<td>L-NAME VS Aminoguanide</td>
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<td>NS</td>
</tr>
<tr>
<td>L-NAME vs Control</td>
<td>0.6694</td>
<td>NS</td>
</tr>
<tr>
<td>ADMA vs Aminoguanide</td>
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<td>NS</td>
</tr>
<tr>
<td>ADMA vs Control</td>
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<td>NS</td>
</tr>
<tr>
<td>Aminoguanide vs Control</td>
<td>0.4575</td>
<td>NS</td>
</tr>
</tbody>
</table>

Comparison of IRR between each interventional group and the control at the start of machine perfusion using an unpaired t test with Welch correction also did not show any significant differences, with p values as shown below (Table 17):
Table 17. Comparison between interventional groups and control at start of pulsatile MP. P values for unpaired t test with Welch correction

<table>
<thead>
<tr>
<th>Comparison</th>
<th>P value</th>
</tr>
</thead>
<tbody>
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<td>SNP vs Control</td>
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</tr>
<tr>
<td>L-NAME vs Control</td>
<td>0.3613</td>
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<tr>
<td>ADMA vs Control</td>
<td>0.1465</td>
</tr>
<tr>
<td>Aminoguanide vs Control</td>
<td>0.5636</td>
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</tbody>
</table>

Comparisons were also made between the IRR at the start and end of pulsatile MP for each interventional group (Figure 55). This confirmed that IRR was significantly lower at the end of six hours of perfusion in all groups (paired t test; see Table 18 for p values).

Table 18. Comparison between IRR at start and end of MP. P values for paired t test.

<table>
<thead>
<tr>
<th>Group</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNP</td>
<td>0.0218</td>
</tr>
<tr>
<td>L-NAME</td>
<td>0.0080</td>
</tr>
<tr>
<td>ADMA</td>
<td>0.0309</td>
</tr>
<tr>
<td>Aminoguanide</td>
<td>0.0271</td>
</tr>
<tr>
<td>Control</td>
<td>0.0151</td>
</tr>
</tbody>
</table>
8.3 The effect of NO donors and NOS inhibitors on eNOS during pulsatile MP

All the data within this section was normally distributed, but with differing standard deviations (SD). Therefore, for statistical analysis the appropriate parametric tests (with correction for the differing SD's where necessary) were used.

This study showed that compared to the control group, the level of eNOS at the start of pulsatile MP was slightly less in all groups (Figure 56). However, there were not any significant differences between the groups. During the six hours of pulsatile MP, the level of eNOS did not change very much within any group (Figure 57).
Figure 56. Relationship between different additions to the perfusate and the eNOS level at the start of pulsatile MP. Values are expressed as median, quartiles and range.

Figure 57. Relationship between the different additions to the perfusate and the eNOS level over 6 hours of pulsatile MP. Values are expressed as means.
One way analysis of variance (ANOVA) demonstrated no significant difference ($p=0.0924$) in the variation of the eNOS levels for each intervention group at the start of pulsatile MP. Therefore, it was not appropriate to perform any post tests.

Comparison of eNOS level between each interventional group and the control at the start of machine perfusion using an unpaired t test with Welch correction also did not show any significant differences, with $p$ values as shown below (Table 19):

<table>
<thead>
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<th>Comparison</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
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<td>SNP vs Control</td>
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<tr>
<td>L-NAME vs Control</td>
<td>0.4927</td>
</tr>
<tr>
<td>ADMA vs Control</td>
<td>0.5881</td>
</tr>
<tr>
<td>Aminoguanide vs Control</td>
<td>0.0576</td>
</tr>
</tbody>
</table>

Comparisons were also made between the level of eNOS at the start and end of pulsatile MP for each interventional group (Figure 58). This showed that the level of eNOS was slightly higher at the end of six hours of perfusion in all but the control group, the level here was slightly lower. These differences only reached significance in the group where aminoguanide was added to the perfusate (paired t test, $p=0.0118$).
8.4 Conclusion

Compared to the control group, IRR at the start of pulsatile MP was higher for those kidneys that had SNP or ADMA added to the perfusate and lower for those that had L-NAME or aminoguanide added. Although one way analysis of variance did demonstrate that the variation between the mean IRR for each intervention group at the start of machine perfusion was significantly greater than would have been expected due to chance alone, the Tukey-Kramer multiple comparisons post tests did not elucidate any significant differences between the groups. During the six hours of pulsatile MP, IRR decreased significantly in all groups, but there were no significant differences between the groups.
Compared to the control group, the level of eNOS at the start of pulsatile MP was slightly less in all groups. However, there were not any significant differences between the groups. During the six hours of pulsatile MP, the level of eNOS increased slightly in all groups apart from the control, however this only reached significance in the aminoguanide group.
CHAPTER NINE

DISCUSSION OF RESULTS AND PROSPECTS FOR
FUTURE RESEARCH

9.1 Discussion about the influence of warm ischaemic injury

9.2 Discussion about the influence of cold ischaemic injury

9.3 Discussion about the influence of NO donors and NOS inhibitors

9.4 Strengths and weaknesses of the studies performed

9.5 Recent developments and prospects for future research
9.1 Discussion about the influence of warm ischaemic injury

There is a clear and significant relationship between the length of WIT and IRR at the start of pulsatile MP in this study. As the WIT lengthens, the IRR increases proportionally. During the six hours of pulsatile MP the IRR reduces significantly, with the largest reduction in IRR being in those kidneys which initially had the highest IRR.

The nitrate concentration tended to be higher the longer the period of WIT. Multiple regression analysis showed that the WIT was the only independent factor to have a linear relationship with the nitrate concentration. However, one way ANOVA did not show any significant differences between the nitrate concentrations after one hour of pulsatile MP within the different WIT groups. Over six hours of pulsatile MP the nitrate concentration did tend to increase, but this did not reach statistical significance.

The expression of eNOS also increased as the WIT lengthened and the eNOS level became higher in all but the 90 minute WIT group over the six hours of pulsatile MP. Nevertheless, none of these trends reached statistical significance.

It is possible to postulate that the increase in eNOS expression over the six hours could lead to an increase in NO production (as measured by nitrate concentration) which in turn could cause vasodilatation and a reduction in IRR. However, this study does not at any stage find any significant correlation between IRR, NO production or eNOS expression. This would also not explain why NO production and IRR both increased with WIT, because if NO was influencing IRR by causing vasodilatation it would be expected that NO production would decrease with warm ischaemic time, rather than the opposite. This could possibly be explained if the concentration of nitrate that we are measuring in the perfusate effluent is actually leaching out of the vasculature of the kidneys and is therefore not available to fulfil its
vasodilatory role. Also, as explained previously, eNOS is a constitutive NOS more usually responsible for a continuous, basal release of NO rather than an inducible enzyme [194]. In addition, as explained in the previous chapter on organ preservation, hypothermia significantly inhibits enzymatic processes such as NOS [129].

Of the indices measured, IRR had the closest and most convincing relationship with WIT. It would seem possible that this could be used as a viability assessment measure, especially when previous studies have shown a relationship between IRR and delayed graft function [120, 178, 181]. The fact that in this study (and also in the experiments on CIT and NO metabolism manipulation) IRR decreased over the six hours of pulsatile MP provides positive evidence of the potential benefit of MP in minimising the deleterious effects of warm and cold ischaemia. Unless the kidneys were subsequently transplanted this could not be proven conclusively.

9.2 Discussion about the influence of cold ischaemic injury

When studying kidneys subjected to a short WIT of 15 minutes, a significant linear relationship was found between the length of CIT and the IRR at the start of pulsatile MP. When kidneys subjected to the same CIT's were also subjected to a longer WIT of 45 minutes the initial IRR was consistently higher but no such linear relationship existed. Nonetheless, in all kidneys the IRR reduced significantly over six hours of pulsatile MP.

The nitrate concentration increased as the CIT lengthened for both WIT groups. Multiple regression analysis showed that the CIT was the only independent factor to have a linear relationship with the nitrate concentration. Substantiating this, one way ANOVA showed a significant difference between the nitrate concentrations after two hours of pulsatile MP within the different CIT groups which had been subjected to 15 minutes of WIT, but not those subjected to 45 minutes of WIT. Over
six hours of pulsatile MP the nitrate concentration decreased in all but the 2 hour CIT group (regardless of WIT), but this did not reach statistical significance.

The expression of eNOS decreased as the CIT increased, with multiple regression analyses showing a significant contribution to the linear relationship by both the CIT and the WIT. Although the eNOS level was slightly higher at the end of six hours of pulsatile MP in all but those kidneys subjected to 2 hours CIT and 45 minutes WIT, these were not statistically significant.

These results raise some interesting questions. Firstly, why should IRR increase with longer periods of cold ischaemia in only the short WIT group? Maybe because these kidneys had a relatively low IRR to start with and therefore more scope for the IRR to increase with a further insult, whereas the group with the longer WIT had a higher IRR to start with anyway.

Secondly, whilst IRR decreased and nitrate concentration increased, as occurred with increasing WIT, why in this study did eNOS expression significantly decrease with lengthening CIT? Endothelial NOS is usually bound to the cytoskeleton of cells [201], so it is possible that further tissue injury due to the cold ischaemic damage may reduce eNOS levels. This leaves the question of why the increase in NO production? Again it is possible to postulate that the concentration of nitrate that we are measuring in the perfusate effluent is actually leaching out of the vasculature of the kidneys. Conversely, we must not forget the role of iNOS and it would be possible to hypothesise that this increase in NO is due to the expression of iNOS in response to the injurious stimulus. As we were unable to measure iNOS expression it is not possible to prove this.

Again at no stage did this study demonstrate any significant correlation between IRR, NO production or eNOS expression.
9.3 Discussion about the influence of NO donors and NOS inhibitors

If the IRR is produced via a NO mediated pathway, then it would be expected that adding a NO donor to the perfusate would result in a reduction in the IRR. In this study, conversely, the addition of the NO donor SNP led to an initial increase in IRR (although this was not significantly different from the control). Although the IRR subsequently reduced significantly over the six hours of pulsatile MP, the IRR was at no stage less than that of the control group.

In a similar way, it would be expected that the addition of NOS inhibitors to the perfusate would potentially change the IRR, depending on which NOS was active. However, again this study did not show any significant differences when iNOS and/or cNOS inhibitors were added to the perfusate.

If the eNOS measured in this study was active during the hypothermic conditions, then it would be expected that the inhibition of cNOS would lead to a lower expressed level of eNOS within the tissue biopsies. Therefore, the addition of L-NAME, an iNOS and cNOS inhibitor and ADMA, an exclusively cNOS inhibitor should do this. Yet, this was not found to be the case as there was in actual fact little difference between the groups, either at the start of pulsatile MP or at any stage over the six hours of perfusion.

The only significant difference found was with the addition of aminoguanide, an iNOS inhibitor, where the level of eNOS was found to be higher at the end of six hours of pulsatile MP.

9.4 Strengths and weaknesses of the studies performed

Careful consideration was made about the design of the studies within this thesis. A pig model was used as this was felt to be close to humans in terms of
physiology and the pigs were readily available. As stated previously, landrace pigs were used. The pigs were all between 35 – 45 kg in weight and were aged between 3 – 4 months. Pigs of this type and age were used as landrace pigs are noted for their early, rapid growth: their weight at weaning being higher than that of other breeds [240]. They are also reaching sexual maturity [241], consequently the kidneys retrieved were suitable for the experiments contained within this study and allowed comparisons to be made to human adult kidneys. The sex of the pigs was not noted, although for completeness this could have been done.

The warm ischaemic times used were <10 minutes, 15, 30, 45, 60 and 90 minutes. In the <10 minute group the kidneys were retrieved and then flushed with hypothermic HOC solution as quickly as possible. This group therefore is the nearest to a zero WIT that could be achieved with this animal model. The other time periods were chosen to include – and with the 90 minutes WIT group to extend – the range of WITs that are commonly encountered in clinical practice. These times were chosen to make the studies as clinically relevant as possible. Many centres would use kidneys subjected to 30 minutes or less of warm ischaemia as long as the initial perfusion was deemed to be adequate. However, there would be concerns on using those kidneys subjected to the longer WITs. I wanted to find out if the use of pulsatile MP either improved the quality of these organs with the longer WITs and/or whether any of the parameters used could be incorporated into some sort of criteria for viability testing. I have not answered these question fully, although I think the results on the correlation between IRR and WIT were encouraging. Also, the fact that IRR decreased in all groups over the 6 hours suggests there may be a benefit here. However, this has not been confirmed with this animal model.

Within each experiment, groups of six animals were used. This number has been sufficient to reveal statistically significant results within some of the experiments, especially when looking at the effect of warm and cold ischaemia on
IRR. However, it may be that some of the non-significant trends found within the experiments are type II statistical errors due to the sample sizes being too small. For example in experiment 1, this may be why the nitrate concentration at the end of MP tended to be higher, but was not statistically significant. Also, why eNOS expression between the warm ischaemic times tended to be higher in those kidneys with the longer WIT, but again this was not significant.

This number of pigs was chosen not only from a statistical point of view but also what was practical in terms of time and financial restraints. Ideally, it would be useful to extend the number of animals in the groups to see if any of the smaller differences became statistically significant.

Nitric oxide has a half life of only 4 seconds, therefore as mentioned previously can not be directly measured in biological systems. Consequently, a two step process was performed utilizing the Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical Company, Alexis Corporation (UK) Ltd, Nottingham, UK). First, nitrate was converted to nitrite using nitrite reductase. Secondly, the Griess reagents were added to convert the nitrite into a deep purple azo compound. Photometric measurement of the absorbance due to this azo compound then accurately determines the nitrite concentration. This is a well recognised and validated method [249] of measuring the products of NO. All samples were done in duplicate as recommended by the kit manufacturer. Appendix C shows a sample of the raw data and the duplicated samples which confirms that the divergence between the duplicates was small and a mean of this adequate for statistical analysis.

Western blotting was used for the measurement of eNOS. This is a process that has changed little since it was first published in 1979 [246]. Although it has limitations, the most significant of which is probably that it has a limited quantitative capability, the technique has remained popular because it is hard to match in specificity and versatility. Common problems that occur usually revolve around the
signal. This may be not present, weak or diffuse. Blots may be uneven and background too high to read the blot accurately. Although I had initial teething problems with setting this process up in the laboratory, I did not have any substantial problems with the above. The main problem I came across was in the protein transfer. This was however, easily resolved by omitting the methanol from the transfer buffer. This consequentially led to a longer transfer time. So, to improve heat dissipation over this prolonged period, transfer buffer that had been stored at 4°C was used.

In the final experiment, an NO donor and NOS inhibitors were added to the perfusate. This was then used to MP ischaemically damaged kidneys that had been subjected to 45 minutes of warm ischaemia and 24 hours of cold ischaemia. The doses of the NO donor and NOS inhibitors was taken from a paper by Weight et al [6]. Little effect was achieved with these in the doses used. Ideally, it would have been beneficial to repeat the experiment with different doses of the NO donor and NOS inhibitors to see if there was any different result. Also, it would have been interesting to see the effect of these substances on kidneys with less ischaemic damage to see if any benefit could have been elicited.

9.5 Recent developments and prospects for future research

The work presented within this thesis raises as many questions as it answers. Whilst IRR seems to be a good indicator of ischaemic damage, to fulfil its potential role as an index of viability further work needs to be undertaken. In the first incidence, further animal studies could be undertaken to re-implant the kidneys following the ischaemic insults and storage with pulsatile MP. Nicholson [250] et al looked at the effect of MP versus CS in porcine kidneys. They performed left nephrectomy of kidneys that were subjected to either 0 or 30 minutes of warm ischaemia followed by either 24 hours of MP or static CS. The left kidney was then
autotransplanted into the right iliac fossa and an immediate right nephrectomy performed. Renal function was then assessed daily for 14 days. Improved renal function was found following MP in the 0 minute WIT group, but not in the 30 minutes WIT group as demonstrated by the area under the creatinine curve during the first 14 days [250]. A correlation between renal function and IRR was not looked at, but a similar model could be used to look at other WITs and IRR.

It may be that a cut off could be established using IRR to find a level above which viability was unlikely. This however, would not be straightforward as it is known that NHBD kidneys have higher rates of DGF, so for this animal model it may be necessary to provide the pigs with an alternate form of renal replacement therapy i.e. dialysis, for a substantial period prior to the kidneys either recovering or becoming definitely non-viable or to delay the second nephrectomy to allow for some recovery of renal function in the autotransplanted kidney.

A comparable study in humans would raise serious ethical questions, because if it were to be done accurately it would involve transplanting kidneys at the very edge of what was deemed viable to ascertain if kidneys above a certain IRR really are non-viable. A similar study was performed by Metcalfe et al [251] where seven pairs of uncontrolled NHBD kidneys were determined at the time of harvesting to be fit (group 1) or unfit (group 2) for transplantation according to their macroscopic appearances. The kidneys from both groups were then MP, but only those kidneys from group 1 were transplanted, regardless of the MP parameters. Eight kidneys were allocated to group 1 and deemed fit for transplant. Of these, 5 functioned (group 1a) and 3 had PNF (group 1b). There was no difference in the median initial IRR between group 1a and 1b, therefore in this study IRR would not have helped to determine which kidneys were viable as opposed to those which were non-viable. However, in group 2, the non-transplanted group the IRR was significantly higher than in either group 1a or 1b [251]. As these were not transplanted, because of the obvious ethical
reasons, we cannot say for certain that all these kidneys would have had PNF but this would certainly be the supposition.

The gold standard to confirm fully that pulsatile MP is actually beneficial in helping to ameliorate the damage caused by ischaemia would be a randomised, controlled trial of MP versus static cold storage. This has been done on a small scale with encouraging, but not definitive, results [108, 109, 111, 119, 153, 156]. A multicentre approach may be the best way in which to get adequate numbers involved to answer this question once and for all. A multicentre study of this kind is actually due to start in 2006 (person communication with Mr CJE Watson).

This study suggests that the increase in IRR is unlikely to be due to the NO pathway, although this is a complex area and it is possible that NO is involved as one of several processes. It would be interesting to repeat the study with an iNOS inhibitor, if one were to become commercially available to see if any different results were found.

Measurement of NO and eNOS following transplantation of the kidneys subjected to the conditions within this thesis would also be informative, as this study has only really looked at the ischaemia part of the ischaemia-reperfusion injury phenomenon.

Further developments in machine perfusion have included looking at the possibility of perfusing kidneys at near physiological temperatures. A lot of this work has been performed by Brasile et al [252, 253]. One study undertaken by this group used canine kidneys subjected to 120 minutes of WI. The kidneys were then either reimplanted immediately, MP for 18 hours at 4°C or underwent 18 hours of MP at 32°C with an acellular perfusate. It was found with this model that the warm perfused kidneys provided life-sustaining function whereas the other two groups did not. They concluded that this demonstrates recovery of renal function which could provide the basis for recovering function in severely ischaemically damaged kidneys and therefore potentially increase the number of transplantable kidneys [252].
The same group also successfully warm perfused canine and human kidneys ex vivo in near physiological temperatures for 48 hours. They identified NOS as the underlying mechanism preserving vascular integrity [253]. This would fit with the reperfusion part of the ischaemia-reperfusion injury phenomenon described earlier.

Metcalfe also looked at warm perfusion in pigs. He undertook a study where groups of pigs (n=5) underwent left nephrectomy following WITs of 0 or 30 minutes. The kidneys were then preserved either by static CS, cold MP or warm MP using a tissue culture fluid and perfluorocarbon emulsion to increase oxygen capacity. After 24 hours the kidney was autotransplanted and a right nephrectomy was performed. This small study did not show any difference in either animal survival or area under the creatinine curve levels between any of the three groups [254].

Research has also continued into which is the best solution to use when performing cold MP. Several papers have compared solutions with Belzer MPS and found improvements in DGF with Vasosol [255]or enhanced preservation of kidneys exposed to longer WIT with UW-solution (Viaspan) [256].

Other investigators have continued to look at the effect of adding substances to the perfusate to elicit if this improves various outcome measures. One such study used the addition of endothelin receptor antagonists [257]. The same investigators had previously shown that urinary excretion of the vasoactive peptide endothelin was significantly increased during MP of kidneys subjected to pre-retrieval warm ischaemia [258]. This study now looked at the effect of adding endothelin receptor antagonists to the perfusate in a rat model following 30 minutes of ischaemia. The kidneys were isografted into genetically identical rats following 2 hours of MP. The GFR was measured two weeks post transplant. It was found that in the treatment groups that the GFR was not significantly different to a control group which did not have any pre-retrieval ischaemia, but that the GFR was significantly higher than in the group which underwent a 30 minute period of ischaemia but did not have the addition of the...
endothelin antagonists [257]. It would be interesting to see a similar study performed in humans to see if the same short term improvement could be replicated and whether this then correlated with any long term improvement in renal function.

Finally, studies continue to observe perfusion parameters such as flow and IRR whilst performing MP to ascertain whether a viability test can be reliably used. Talbot et al have defined criteria which they use when deciding whether to use a kidney from a NHBD or not. These initially included decreasing IRR, but on further experimentation this was changed to a perfusion flow index of greater than 50 ml/min/100g of kidney[259]. The criteria also evolved to include low temperature, low weight increase and total glutathione S-transferase of less than 200 IU/litre/100g of kidney [260]. By adhering to these criteria they have achieved graft success rates of over 90% [259].
APPENDICES
APPENDIX A

Comparison of transonic flow probe with direct timer method to measure flow. Linear regression analysis demonstrated a strong correlation with $r^2 = 0.9838$ and $p < 0.0001$ (Figure 59).

![Linear regression analysis of the transonic and direct timer methods of measuring flow with the RM3 Renal Preservation System.](attachment:image.png)

**Figure 59.** Linear regression analysis of the transonic and direct timer methods of measuring flow with the RM3 Renal Preservation System.

**Experiment 1**

<table>
<thead>
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<th>Direct timer flow ml/min</th>
</tr>
</thead>
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### Experiment 2

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### Experiment 3

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APPENDIX B

Summary of steps involved in the set-up and operation of the Renal Perfusion System:

RM3 Preparation
1. Check circulation pump for connection and installation.
2. Add approximately 1.5 litres of cold water and fill the ice chest with crushed ice.
   Be sure that the drain tube is clamped.
3. Plug in power cord to mains and switch on.

Attaching the Cassette
1. Place the cassette on the RM3 control unit. Place the pump head of the cassette in
   the pump assembly arms and secure the cassette at both mounting screws.
   **DO NOT connect the pressure transducer tubing to the transducer at this
time.**
2. Connect the temperature probe to the cassette and RM3.
3. Connect the heat exchange tubing to the cassette ensuring that the inlet connection
   is located on the top.
4. Attach the flow probes to the RM3 and arterial tubing.

RM3 Initial Set-up
1. Prime cassette with approximately 600 mls of perfusate.
2. Turn POWER switch ON. Check date is correct.
3. RM3 will initialise and the MAIN MENU will be displayed.
4. Press SYSTEM from MAIN MENU.
5. From the SYSTEM MENU, turn ON the pulse pump, zero pressure and enter the
   appropriate identification number.
6. Connect the pressure tubing to the transducer.
7. De-bubble pump head.
8. Increase STROKE VOLUME knob to begin circulating perfusate.
9. Adjust fluid level in bubble trap to centre line.

10. Press MAIN on the SYSTEM MENU to go to the MAIN MENU.

**Renal Perfusion**

1. Clamp one arterial tubing in the cassette.

2. Decrease stroke volume to slow the circulating perfusate.

3. Slowly fill and de-bubble the renal artery at the unclamped tubing and attach the kidney.

4. Adjust stroke volume to systolic pressure of 60 mmHg

5. Readjust fluid level in bubble trap to centre line

6. Verify that all parameters are within acceptable parameters.

**RM3 Final Set-Up**

1. Select SYSTEM from the MAIN MENU.

2. From the SYSTEM MENU select Trend to ON.

3. Select MAIN from the SYSTEM MENU for normal operation.

4. Select ALARM MENU and select Continue to activate the alarm system.

The Renal Perfusion System is now in operation. It can be safely left alone, with periodic checks to ensure that the systolic pressure remains at 60 mmHg. If the systolic pressure decreases/increases then the stroke volume can be increased/decreased as appropriate. Hourly biopsies and sampling of perfusate effluent can be done. The coolant reservoir needs to be drained and refilled with crushed ice every 2 hours.
APPENDIX C

A sample of raw data used in the measurement of the nitrite/nitrate concentration. Figure 60 shows the position of each standard, the blanks and the samples in each well. Figure 61 shows the corresponding absorbance at 540 nm. The top chart shows the results in duplicate and the bottom chart shows the calculated means.

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Figure 60. Position of nitrite standards and samples.
Figure 61. Absorbance of nitrite at 540 nm. Top chart shows raw data in duplicate. Bottom chart shows calculated mean.
Bibliography


