THE EFFECTS OF ERYTHROPOIETIN (EPO) AND CARBON MONOXIDE (CO) ON RENAL ISCHAEMIA/REPERFUSION (I/R) INJURY IN AN ISOLATED PORCINE KIDNEY MODEL

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by

Atul Bagul

Department of Infection, Immunity and Inflammation

Division of Transplant Surgery

University of Leicester

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ABBREVIATIONS

2-MAC 20-methyl aminochroman

AE-ITU Aminoethyl-isourea

ADP Adenosine diphosphate

ADP: ATP ratio Adenosine diphosphate: Adenosine triphosphate ratio

Akt Protein kinase B

AMP Adenosine monophosphate

ANOVA Analysis of variance

AP Arterial pressure

AR Acute rejection

ARF Acute renal failure

AST Aspartate aminotransferase

ATN Acute tubular necrosis

ATP Adenosine triphosphate

AUC Area under curve

BFU-E's Burst-forming-unit-erythoids

CAN Chronic allograft nephropathy

CFU-E's Colony-forming-unit-erythoids

cGMP Cyclic guanine monophosphate

CI Cold ischaemic injury

CIT Cold ischaemic time

CO Carbon monoxide

CORM Carbon monoxide releasing molecule

CORM-1 Carbon monoxide releasing molecule-1

CORM-2 Carbon monoxide releasing molecule-2

CORM-3 Carbon monoxide releasing molecule-3

CORM-A1 Carbon monoxide releasing molecule-A1

CP Cold preservation

CS Cold storage

DFO Deferoxamine

DGF Delayed graft function

DMSO Dimethyl sulfoxide

DNA Deoxyribonucleic acid

ELISA Enzyme-Linked ImmunoSorbent Assay

EPC's Erythrocyte progenitor cells

EPO Erythropoietin

EPO-R Erythropoietin receptor

FBC Full blood count

FOXO3a Forkhead transcription factor

GFR Glomerular filtration rate

Hb Haemoglobin

Hb-CO Carboxyhaemoglobin

HBD Heart-beating donors

HO-1 haem-oxygenase 1

ICAM 1 Intercellular adhesion molecule 1

iCORM Inactive carbon monoxide releasing molecule

IFN γ Interferon γ

IP Ischaemic preconditioning

I/R injury Ischaemia/Reperfusion injury

IRR Intra renal resistance

L-NAME N^G-nitro-L-arginine methyl ester

L-NIL L-N^6-(1-iminoethyl)lysine

MAC Membrane attack complex

MAP Mean arterial pressure

MBL Mannose binding lectin

MHC Major histocompatibility complex

MI Myocardial infarct

NAG N-Acetyl-β-D glucosaminidase

NF-κB Nuclear factor light chain enhancer of activated B cells

NKC Natural killer cell

NHBD Non-heart-beating donor

NO Nitric oxide

NOS Nitric oxide synthase

NP Normothermic resuscitation preservation

O_2 Oxygen

p38-MAPK p38-Mitogen activated protein kinase

PCT Proximal convoluted tubule

PGE1 Prostaglandin E1

ppm Parts per million

RBC Red blood cell

RBF Renal blood flow

PI3K Phosphoinositol 3 kinase

PNF Primary non-function

ROS Reactive oxygen species

sGC Soluble guanylate cyclase

STAT5 Signal transducer and activator of transcription 5

TNF α Tumour necrosis factor α

UNOS United Network of Organ Sharing

UW University of Wisconsin

µm µmol

VCAM1 Vascular cell adhesion molecule 1

vWF Von Willebrand factor

WBC White blood cell

WI Warm ischaemic injury

WIT Warm ischaemic time

XIAP X-chromosome linked inhibitors of apoptotic protein

ABSTRACT

Kidney transplantation remains the best modality for renal replacement therapy, the key problem being lack of suitable organs for transplantation. Thus renewed interest in non-heart-beating-donor (NHBD) organs is on the increase to bridge this gap. NHBD organs are subjected to ischaemia-reperfusion (I/R) injury; the underlying pathophysiology, a complex inter-related sequence leading to short and long term renal allograft dysfunction. The aim of the study was to validate normothermic resuscitation perfusion (NP) as a preservation model followed by investigating the use of erythropoietin (EPO) and carbon-monoxide (CO).

Porcine kidneys were perfused with normothermic-autologus blood on an isolated-organ perfusion system (IOPS), design based on cardio-pulmonary bypass technology. Renal haemodynamics and functions were then measured during 3hr reperfusion.

NP restored renal blood flow and improved renal function, as assessed by % serum creatinine fall, area under curve (AUC) of serum creatinine. EPO when added to NP did not seem to add any major benefit other than marginally improve oxygen consumption. Carbon monoxide delivered as carbon monoxide-releasing molecule-3 (CORM-3) was able to improve urine output, renal blood flow, reduce intrarenal resistance as well as improve renal function reflected by significant improvement in AUC of creatinine clearance.

Normothermic resuscitation preservation not only reversed some of the deleterious effects of I/R injury, it also plays an important role as a versatile delivery system to assess various manipulatory agents that have potential in ameliorating I/R injury. This study provides further evidence that CO may be protective in renal perfusion injury and supports the use of low-dose CO releasing molecules as a method of CO delivery. Thus CORM-3 has the potential application in the field of NHBD kidney transplantation, which continues to be an expanding source of transplant kidneys. While EPO did not add any major benefits when used as a manipulating agent, may have its shortfall when applied to a NHBD kidney programme.

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PUBLICATIONS, PRESENTATIONS AND PRIZES

Publications:

1. **A Bagul** , S A Hosgood , M Kaushik , M D Kay , H L Waller , M L Nicholson
   Renal preservation by normothermic resuscitation perfusion with autologous blood.
   Br J Surg. 2008 Jan; 95(1):111-118

2. **A Bagul**, SA Hosgood, M Kaushik, ML Nicholson.
   Effects of erythropoietin (EPO) on ischemia/reperfusion (I/R) injury in a controlled non-heart beating donor (NHBD) porcine kidney model.
   Transpl Int. 2008 May; 21(5):495-501

3. **A Bagul**, SA Hosgood, M Kaushik, ML Nicholson
   Carbon monoxide protects against ischemia-reperfusion injury in an experimental model of controlled non-heart beating donor kidney.
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4. SA Hosgood, **A Bagul**, M Kaushik, RS Gadepalli, JM Rimoldi, ML Nicholson
   The novel application of nitric oxide and carbon monoxide in a model of renal preservation.
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Abstracts

1. **A Bagul**, S Hosgood, M Kaushik, M Kay, H Waller, M Nicholson.
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2. **A Bagul**, S Hosgood, M Kaushik, RS Gadepalli, J Rimoldi, ML Nicholson.
   Effects of carbon monoxide releasing molecule (CORM-3) on reperfusion in a controlled non-heart beating donor (NHBD) haemoperfused porcine kidney model.
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3. S Hosgood, **A Bagul**, HL Waller, J Rimoldi, ML Nicholson.
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4. S Hosgood, **A Bagul**, M Kaushik, R Pande, J Rimoldi, ML Nicholson.
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   (a) *Br J Surg.* 2007; 94(S2): 185
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5. **A Bagul**, SA Hosgood, M Kaushik, RS Gadepalli, JM Rimoldi, ML Nicholson
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   Effects of erythropoietin (EPO) on ischemia/reperfusion (I/R) injury in a controlled non-heart beating donor (NHBD) porcine kidney model.
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7. B Yang, S Hosgood, **A Bagul**, HL Waller, ML Nicholson.
   Effects of normothermic haemoperfusion with/without erythropoietin on caspase-3, apoptosis and heat shock proteins in ischaemic porcine kidneys.
   (a) *Transpl Int.* 2007, 20(2): 183

Presentations:

Oral:

1. Renal preservation by normothermic resuscitation perfusion with autologous blood: A comparison with static hypothermic storage and hypothermic machine perfusion.
   A Bagul, S Hosgood, M Kaushik, M Kay, H Waller, M Nicholson.
   (a) Novartis Transplant Research Awards, London: September 2006 – **Overall Winner 2006 of National Research in Transplantation.**
   (b) SARS, Cambridge: January 2007
   (c) ASGBI, Manchester: April 2007- **3rd prize Associations prestigious Moynihan Prize Session**
   (c) ESOT, Praque: October 2007

2. Effects of carbon monoxide releasing molecule (CORM-3) on reperfusion in a controlled non-heart beating donor(NHBD) haemoperfused porcine kidney model.
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3. Effects of carbon-monoxide (CO) in a controlled non- heart beating donor (NHBD) normothermic haemoperfused porcine kidney model.
   A Bagul, SA Hosgood, M Kaushik, MD Kay, ML Nicholson
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Posters:

1. The role of normothermic preservation in renal transplantation.
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2. Effects of erythropoietin (EPO) on ischemia/reperfusion (I/R) injury in a controlled non-heart beating donor (NHBD) porcine kidney model.
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3. Effects of carbon monoxide releasing molecule (CORM-3) on reperfusion in a controlled non-heart beating donor (NHBD) haemoperfused porcine kidney model.
   A Bagul, SA Hosgood, R Pande, M Kaushik, HL Waller, JM Rimoldi, ML Nicholson
   (a) SARS, Cambridge: January 2007
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4. The effects of nitric oxide and carbon monoxide in renal preservation.
   S Hosgood**, A Bagul**, M Kaushik, H Waller, John Rimoldi, ML Nicholson
   (a) SARS, Cambridge: January 2007
   (b) BTS, Manchester: March 2007
   (c) ASGBI, Manchester: April 2007
   (d) ATC, San Francisco: May 2007
   (e) ESOT, Praque: October 2007

5. The evaluation of carbon monoxide concentrations in an isolated porcine kidney model
   S Hosgood**, A Bagul**, M Kaushik, H Waller, John Rimoldi, ML Nicholson
   (a) SARS, Cambridge: January 2007
   (b) BTS, Manchester: March 2007
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   (d) ESOT, Praque: October 2007

5. Effects of erythropoietin on apoptosis and inflammation in ischaemic porcine kidneys preserved by normothermic machine haemoperfusion
   B Yang, S Hosgood, **A Bagul**, M Kaushik, H Waller, M Nicholosn
   (a) BTS, Manchester: March 2007
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6. A comparison of normothermic resuscitation perfusion using autologous blood and traditional hypothermic methods for renal preservation
   A Bagul, S A Hosgood , M Kaushik , M D Kay , H L Waller , M L Nicholson
   (a) BTS, Manchester: March 2007
   (b) ATC, San Francisco USA: May 2007: **ATC Abstract of Distiniction**

7. Effects of normothermic haemoperfusion with/without erythropoietin on caspase-3, apoptosis and heat shock proteins in ischaemic porcine kidneys.
   B Yang, S Hosgood, **A Bagul**, HL Waller, ML Nicholson.
   ESOT, Praque: October 2007

8. Effects of carbon-monoxide (CO) in a controlled non- heart beating donor (NHBD) normothermic haemoperfused porcine kidney model.
   A Bagul, SA Hosgood, M Kaushik, RS Gadepalli, JM Rimoldi, ML Nicholson
   (a) ESOT, Praque: October 2007, **Best Poster Prize**
   (b) SARS, Birmingham: January 2008
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9. Erythropoietin in normothermic preservation enhanced reperfusion tolerance of isolated ischaemic porcine kidneys.
   B Yang, S Hosgood, **A Bagul**, M Nicholson.
   ASGBI, Bournemouth: May 2008

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July 2005 : **UHL Renal and Transplant Research Program Grant**
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September 2006 : **Overall Winner 2006, National Research in Transplantation**
Normothermic resuscitation preservation (NP) in an isolated haemoperfused porcine kidney model.
Novartis Transplantation Research Awards
London, UK

April 2007 : **3rd prize Prestigious Moynihan Prize Session**
May 2007 : **ATC Poster of Distinction**
Renal preservation by normothermic resuscitation perfusion with autologus blood: A comparison with static hypothermic storage and static hypothermic machine perfusion
ASGBI, Manchester, UK
ATC, San Francisco, USA

October 2007 : **Best Poster Prize**
Effects of Carbon-monoxide (CO) in a controlled non- heart beating donor (NHBD) normothermic haemoperfused porcine kidney model
ESOT, Praque, Czeck Republic

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Effects of carbon monoxide in a controlled non-heart beating donor normothermic haemoperfused porcine kidney model
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SUMMARY OF FINDINGS

a) Normothermic resuscitation preservation (NP) in an isolated haemoperfused porcine kidney model.

Background:

Normothermic preservation has been shown to improve the metabolic support and maintain the viability of ischaemically-damaged organs retrieved from non-heart beating donors (NHBD) prior to transplantation. This study investigated the effects of NP with blood in a model of controlled NHBD kidneys.

Methods:

Porcine kidneys (n=6) were subjected to 10min warm ischaemia and preserved as follows:

Group 1: 2hr cold storage (CS) (minimal ischaemic damage),

Group 2: 18hr CS (static hypothermic storage),

Group 3: 18hr Cold perfusion (CP) (hypothermic machine perfusion),

Group 4: 16hr CS + 2hr NP (normothermic resuscitation perfusion).

Renal haemodynamics and function were then measured during 3hr reperfusion with autologous blood.

Results:

Increasing CS from 2hr to 18hr reduced renal blood flow [Area under curve (AUC) 444±57 vs. 325±70; P<0.01], but this was restored by NP (563±119; P=0.035 vs. 18hr CS) with no difference seen compared to CP (600±319). Renal function was also better in groups 1, 3 and 4 vs. group 2 (% serum creatinine fall 92± 6, 79±9 and 64±17 vs.

44±13% respectively, P=0.001). AUC serum creatinine was significantly lower in group1 compared to group2 (1102±260 vs. 2156±401; P = 0.002) and to group 4 (1756±280; P = 0.009), while group 4 was similar to group 3 (1354±300). Tubular function was also improved in groups 1, 3 and 4 vs. group 2 (P=0.001). Renal ADP: ATP ratio was significantly lower following NP compared to pre-perfusion levels in all groups (P<0.05).

Conclusion:

Normothermic resuscitation preservation was able to reverse some of the deleterious effects of cold storage in this model of controlled NHBD kidneys, as well as adequately resuscitate kidneys compared to 18 hours of cold preservation.

b) Effects of erythropoietin (EPO) on ischaemia/reperfusion (I/R) injury in a controlled non-heart beating donor (NHBD) porcine kidney model.

Background:

Erythropoietin (EPO) has been shown to have anti-apoptotic action mediated via various mechanisms hence protecting against I/R (Ischaemia/reperfusion) injury. This study investigated the effect of high dose of EPO (5000 units), administered as a bolus in a model of controlled NHBD kidneys.

Methods:

Porcine kidneys (n=6) were subjected to 10min warm ischaemia and preserved after as follows:

Group 1: 16hr Cold storage (CS) + 2hr Normothermic resuscitation preservation (NP)

Group 2: 16hr CS + 2 hr NP (EPO)

Group 3: 18hr CS

Group 4: 18hrs CS (EPO during cold flush)

Various haemodynamic and functional parameters were then assessed during 3hr reperfusion with autologous blood.

Results:

Renal blood flow improved in groups 1& 2 vs. group 3 & 4 though no difference was noted between groups 3 & 4 (563±119 vs. 491±95 vs. 325±70 vs. 418±112, groups 1, 2, 3 & 4 respectively; P=0.012). The total urine output showed no difference between groups (271±172 vs. 359±184 vs. 302±211 vs. 421±88; group 1, 2, 3 & 4 respectively; P=0.576). Percentage of serum creatinine fall at 3 hrs was significantly better in groups 1 & 2 vs. group 3 (64±17 vs. 60±11 vs. 44±13 vs. 52±8; p= 0.04). The fractional excretion of sodium was significantly lower for group 1 & 2 vs. group 3 & 4 (17±14 vs. 18±9 vs. 49±21 vs. 45±16 respectively; p=0.002). There was a significant improvement in oxygen consumption in groups 2 vs. group 3 (P=0.037) and a marginal significance between groups 1 vs. group 3 (39±10 vs. 46±10 vs. 24±12 vs. 24±7 respectively). Kidneys in group 1 & 2 maintained their acid base balance although group 3 kidneys became severely acidotic (P=0.025) following 3 hrs reperfusion, in contrast to group 4 kidneys which showed a trend towards acidosis (P=0.0812) All these parameters showed no difference between group 1 and 2.

Conclusion:

EPO did not seem to add any major benefits when used as a manipulating

agent during two hours of NP or as a flush prior to CS in a controlled NHBD kidney. However, it increased the oxygen consumption when added during NP and maintained the acid base balance in kidneys flushed with EPO prior to CS.

c) Effects of carbon monoxide releasing molecule (CORM-3) on reperfusion in a controlled non heart beating donor (NHBD) haemoperfused porcine kidney model.

Background:

CORM-3 a transitional metal carbonyl possesses the ability to liberate CO under appropriate conditions and function as CO-releasing molecule in biological systems and thereby have a direct ability to influence intracellular pathways that involve apoptosis and inflammation, hence I/R (Ischaemia/reperfusion) injury. This study investigated the effects of CORM-3 administered in blood at the time of reperfusion in a model of controlled NHBD kidneys.

Methods:

Porcine kidneys (n=6) were subjected to 10 min warm ischaemia and reperfused after 18hr Cold storage (CS) as follows:

Group 1: CORM-3 (tricarbonylchloro(glycinato)ruthenium(II)

Group 2: iCORM-3 (Inactive CO-releasing molecule)

Group 3: Control

Renal haemodynamics and function were then measured during 3hr reperfusion with autologous blood.

Results:

The total urine output was significantly better in group 1 (793±212) vs. group 2 (368±72) and group 3 (302±211) [P=0.01]. Renal blood flow improved from group 1 vs. group 2 & 3 (774±19 vs. 448±88 vs. 325±70; P=0.002). AUC of creatinine clearance was significantly better in group 1 vs. group 2 & 3 (14±6 vs. 3.3±0.1 vs. 2.2±2; p= 0.006). While the fractional excretion of sodium was significantly lower for group1 vs. group 2 & 3 (50.7±27 vs. 105±6 vs. 117±38; p=0.04).

Conclusion:

CORM-3 as a manipulating agent significantly ameliorates the effects of reperfusion in a controlled NHBD Kidney.

d) Effects of carbon monoxide (CO) in a controlled non-heart beating donor (NHBD) normothermic-haemoperfused porcine kidney model.

Background:

CO has a direct ability to influence intracellular pathways that involve apoptosis via p38 mitogen-activated protein kinase pathway (p38MAPK) and inflammation with a decrease in cytokines such as IL-6 and inducible nitric oxide synthase, hence downregulate ischaemia/reperfusion (I/R) injury. This study investigated the effects of CO gas and CO-releasing molecules (CORM-3) administered in blood at the time of normothermic resuscitation preservation (NP) in a model of controlled NHBD kidneys.

Methods:

Porcine kidneys (n=6) were subjected to 10min warm ischaemia and 16 hours

cold storage, after which subjected to 2 hours NP as follows:

Group 1: Control

Group 2: CO gas (300ppm)

Group 3: CORM-3 [tricarbonylchloro(glycinato)ruthenium](II)

Group 4: iCORM-3 (Inactive CO-releasing molecule)

NP was *ex-vivo* normothermic perfusion using the isolated-organ-perfusion-system (IOPS) designed based on clinical grade cardiopulmonary bypass technology. Renal haemodynamics and function were then measured during 3hr reperfusion with autologous blood.

Results:

Renal blood flow significantly improved when CORM-3 was added to NP (group 3) compared to control, though no difference was observed in other groups (435±95 vs. 439±194 vs. 751±222 vs. 558±165; P=0.024). AUC of creatinine was numerically better in group 3 vs. group 4 with no significant difference to the control (1892±215 vs. 1895±247 vs. 1662±538 vs. 2255±169; p= 0.053). The oxygen consumption was significantly improved in group 3 (31.7±9 vs. 43.5±15 vs. 72.2±18 vs. 54.3±13; P=0.0036).While the fractional excretion of sodium showed a trend to improve in group 3 (117±56 vs. 111±36 vs. 65±33 vs. 149±25; p=0.02).

Conclusion:

This study not only provides firm evidence of low dose CO-releasing molecules (CORM-3) as a superior method of CO delivery, but additional benefit of carbon monoxide (delivered as CORM-3) in amelioration of ischaemia reperfusion injury targeted at a transplant organ during normothermic resuscitation perfusion.

CHAPTER 1

NON-HEART BEATING DONORS AND ISCHAEMIA REPERFUSION INJURY

1.0 Non-heart beating donors (NHBD)

Kidney transplantation continues to be the best modality of renal replacement therapy for end stage renal failure (ESRF) [1]. Renal transplant programmes continue to be restricted by a lack of suitable organ donors and as a consequence, the transplant waiting list continues to grow. At the end of 2006 in England and Ireland, 5500 patients were awaiting a kidney transplant, but only 1/3 of these received a transplant in that calendar year (UKT data). Due to a lack of suitable organs for transplantation from traditional sources [2] i.e intensive care unit-based brainstem dead heart-beating donors, renewed interest into other alternatives such as live donors and non-heart beating donors (NHBD) [3] are on the increase.

Non heart beating donors include two different groups; a) controlled NHBD (Maastricht Category 3 & 4 [4,5] Table 1) were usually patients here have suffered massive and irreversible anoxic cerebral damage, referred by intensive care units and b) uncontrolled (Maastricht category 1& 2) [4,5] where patients suffered an irreversible cardiorespiratory arrest immediately before or shortly after admission to the A&E department. Following pronouncement of death, cardiopulmonary resuscitate measures are stopped for a period of 10 minutes in line with the guidelines [6]. After which external cardiac massage and ventilation with 100% oxygen is re-established using a mechanical cardiorespiratory resuscitator and NHBD kidneys were perfused with hyperosmolar citrate solution and cooled in situ using a double balloon-triple lumen intra-aortic catheter using the femoral vessels via a groin approach [4,7,8].

Table1: Maastricht classification of non-heart-beating donors (1.0)

I	**Dead on arrival at hospital**
II	**Unsuccessful resuscitation**
III	**Awaiting cardiac arrest- In patient withdrawal of support**
IV	**Cardiac arrest after brain stem death**
V	**Cardiac arrest in a hospital inpatient**

After the next of kin gave written consent, the organs were retrieved in theatre and kidneys were stored in static cold storage until a suitable recipient could be prepared for a transplant. These organs are thus subjected to substantial warm ischaemic injury (WI) where warm ischaemic times (WIT) range from 5 min to 53 min, median for controlled NHBD at 10 min and uncontrolled NHBD at 25 min [4]. In addition to this the organ faces a cold ischaemic time (CIT) of 16-18 hours on average [4].

Thereby kidneys face substantial damage due to these varying periods of WIT and CIT though the long term function and survival of NHBD is comparable to heart beating donors [9,10]. Though results are encouraging, delayed graft function (DGF) and primary non function (PNF) are a significant problem as a consequence of the prolonged warm ischaemic insult during renal organ retrievals [10,11,12,13,14]. Delayed graft function leads to significant service related burdens such as prolonged hospital stay, additional invasive procedures, ongoing dialysis costs and psychological impact on the patient [15,16]. In addition DGF is associated with an increase in acute rejection and acceleration of chronic allograft nephropathy [11,17-19]. PNF is disastrous as recipients undergo a surgical operation offering no benefit, with all associated morbidity and a risk of immunologically sensitising the patient for a further transplant [11.16,19].

Published literature has clearly shown that organs retrieved from NHBD are justified for transplantation considering various clinical outcomes. Several transplant centres are reporting an increase in NHBD activity, thereby bridging the disparity in people on the waiting list and the availability of an organ for transplantation [5,20]. Extensive NHBD programmes in countries like Spain and Japan have significantly reduced transplant waiting lists [3,21].

PNF rates reported by Koffman *et. al.* showed variation from 4-14% based on their review of European experience [4]. Nicholson *et. al.* published a PNF rate in NHBD of 5.8 % versus 1.3% from heart beating donors (HBD); as per literature looking at various studies from 1990-2002 [20]. The large variation in these figures suggests that the high incidence of PNF is probably potentially avoidable by careful selection of NHBD kidneys and avoiding those with irreversible ischaemic damage (Maastricht category III/IV at 0% to Maastricht category I/II at 19.5%) [22]. Thus PNF in NHBD kidneys, which has been considered as a barrier to wide spread acceptance of a NHBD programme, is on the decline; keeping in mind organ viability assessments based on criteria such as flow dynamics and perfusate enzyme concentrations [23-24].

DGF remains the other key concern in NHBD kidneys, where its incidence ranges from 50-94% which is significantly higher compared to HBD kidneys [25]. In NHBD grafts, DGF is associated with impaired graft function, reduced graft survival and higher rates of acute and chronic rejection [11,19,26]. Yet DGF does not seem to affect the long term function and survival of these NHBD kidneys; Nicholson *et. al.* showed that despite significantly differing early function there was no significant difference in serum creatinine levels from year 1 to 5 years in comparison of living donor, HBD and NHBD transplant recipients [2]. Other centres have reported a similar 5 year survival of 54-78% for NHBD compared to 55-85% for HBD kidney transplants [7,8,27].

To date there has been no difference reported in the 10 year graft survival for HBD versus NHBD kidneys [28]. Evidence as to why NHBD grafts are associated with higher acute rejection remains unclear. DGF association with high rates of acute rejection can be explained by the relation to cytokine and reactive oxygen species (ROS) and

up-regulation of MHC class II molecules during IRI [29-30]. Data from UNOS (United Network of Organ Sharing) showed a higher but acceptable incidence of rejection in NHBD (19% versus 14%) [31]. A review from European experience showed that only PancholcyK *et. al.* had a significant difference in incidence of AR; NHBD at 66% versus HBD at 46% [4,32]. Barrientos A *et. al.* showed donor brainstem death was the variable that was most commonly associated with an increased risk of vascular rejection [3]. Chronic allograft nephropathy (CAN) the leading cause of renal allograft loss is associated with prolonged warm ischaemia and reperfusion injury [33-34]. NHBD grafts that sustain such injury, however show similar long term survival data compared to HBD kidneys and would thus be expected to show little differences in the incidence of CAN [28,35-36]. Bains *et. al.* showed no difference in graft fibrosis in 1 year post transplant biopsies of NHBD and HBD kidneys, a key mode of injury leading to chronic allograft dysfunction [37].

Warm ischaemic time (WIT) is a constant but very variable parameter sustained to the NHBD organ, ranging from 5-53 minutes [38-39]. This parameter has been found to show a direct relationship with graft outcome. Harper *et. al.* in a isolated perfused porcine model showed that an increase in WIT significantly reduced function during a 6 hour post perfusion assessment [40]. Castelao *et. al.* showed that grafts from controlled NHBD had a similar rate of DGF to HBD (34%), while uncontrolled NHBD had a much higher rate (88%), demonstrating that increased WIT hampered early graft function [6]. The effects of increasing WIT are reflected by higher rates of PNF but the explanations remain unclear. Shiroki *et. al.* showed that WIT greater than 30 min was associated with proportionally higher rates of PNF in a series of NHBD transplants [41]. Takai *et. al.* has also reported that longer WIT was associated with significantly higher PNF, although

the primary risk factor for DGF was in fact a Prolonged CIT [42]. In contrast, studies by Yoshida *et. al.* and Tanabe k *et. al.* showed no relationship between the variables of WIT and PNF [21,43].

NHBD organs are thus becoming an important source of organs for transplantation, hence making ongoing research and development very important. Many studies have been directed to organ retrieval techniques such as total body donor cooling with cardiopulmonary bypass and extracorporal membrane oxygenation [44-45]. Precise management and titration of nephrotoxic immunosuppressant medication, as immunosupression is directly proportional to both early grafts function and CAN [46]. Key interest lies in organ perfusion, where studies based on hypothermic and normothermic machine perfusion compared to static hypothermic storage are being used to evaluate organ viability and resuscitate these grafts prior to transplantation [47-53]. Finally a range of interventions designed to ameliorate ischaemia/reperfusion injury (I/R) in allograft is under investigation.

1.1 Ischaemia reperfusion (I/R) injury

1.1.1 Introduction

Renal injury can be caused by various factors which include ischaemia, autoimmune disease and toxins. In NHBD kidneys the underlying pathophysiology of early graft injury is thought to be a complex interrelated sequence of events called ischaemia-reperfusion (I/R) injury. Ischaemia entails abolition of blood flow to the organ, starving the tissue of oxygen and nutrients and leading to the accumulation of metabolic waste products. There are two types of ischaemia: warm ischaemia (WI) and cold ischaemia (CI).

The detrimental combination of warm and cold ischaemic damage represents a key obstacle for expanding the non heart-beating organ donor pool. Kidneys generally tolerate a period of warm ischaemia but they show markedly reduced viability with a combination of hypothermia and warm ischaemia [54].

Warm Ischaemia (WI)

This parameter is very variable in NHBD kidneys and various studies using different animal models have been performed to assess its effects on organ outcome. WI is tolerated without serious alteration of rabbit function for no more than 30 min, thereafter gross changes are seen in tubules and at 60 min complete necrosis of all segments of the majority of the tubules is seen. The irreversibility of the damage is reflected in survival where 90% survive 30 min while only 75% survive 60 min of WI

[55]. Jablonski *et. al.* showed that after 60 min of WIT there was permanent damage of the rat kidney [56]. Similarly Harper *et. al.* confirmed these findings in an isolated haemoperfused porcine kidney model where damage was directly proportional to the length of WIT [40]. However in contrast other studies demonstrated that 18 hours of *ex-vivo* normothermic perfusion in rat kidneys resulted in significant improvement in renal function after transplantation [57-59].

Cold Ischaemia (CI)

It is well known that cold preservation reduces ischaemic damage in kidneys. Pegg *et. al.* has shown that renal oxygen demand can be reduced by around 97% using hypothermia [57,58] and oxygen consumption drops in relation to renal perfusion [59]. The principle behind static hypothermic storage is to slow the metabolic reactions that need ATP and thereby rapidly reduce the energy dependent metabolic activity of the kidney. It has been shown that the metabolism is slowed by a factor of 1.5-2 for each 10°C in temperature [60]. Despite these beneficial effects of cold storage, cold I/R injury of transplanted kidneys is associated with acute tubular necrosis leading to delayed graft function and short term graft survival [61]. DGF may also be due to ischaemic and immunological causes that occur synergistically [62]. It thus appears crucial to attenuate cold I/R injury in order to prevent delayed graft function and consequently improve the outcome of kidney transplantation. Static hypothermic storage after flushing of kidneys at 4°C using preservation solutions is an easy and effective way to reduce ischaemic injury during cold storage, certainly 24 hours [62]. A prominent feature of CI is cell swelling secondary to the arrest of the Na^+/K^+ pump due to lack of ATP. These features are more severe after a period of WI and worse out come is seen in NHBD Kidneys [54].

1.1.2 Pathophysiology

During the ischaemic period the main biochemical changes at cellular level are inhibition of oxidative metabolism, depletion of ATP, a decrease in antioxidant activity, an increase in anaerobic metabolism and inhibition of the Na^+/K^+ ATPase pump [63]. ATP is broken down rapidly to AMP and subsequently adenosine and inosine; which accumulate. In addition anaerobic glycolysis leads to accumulation of lactic acid, lowering intracellular pH and causing lysosomal instability with activation of lytic enzymes [25]. I/R injury involves an accumulation of ATP breakdown products in an ischaemic environment and following reperfusion they are converted to xanthine and superoxide anion by xanthine oxidase. The conversion of xanthine dehydrogenase to xanthine oxidase is under the influence of a calcium dependent protease which is activated by ischaemia [64-65]. This commences a cascade of free radical formation, causing direct injury to lipids, proteins, DNA and initiating pro-inflammatory and apoptotic pathways [64,66]. Depletion of ATP in this process leads to cell membrane instability by incapacitating the sodium/potassium (Na^+/K^+) pump and secondary intracellular calcium accumulation which in turn inhibits mitochondrial function and acts as a second messenger in apoptotic pathways [67].

Apoptosis, programmed cell death has a dual role in renal injury. On one hand it serves as a healing mechanism related to the resolution of inflammation [68-70], while on the other hand inappropriate and accelerated apoptosis causes cell depletion and graft injury [68,71-74]. Although different signals initiate apoptosis, the phenotype of apoptosis is surprisingly similar even in different cell types, suggesting that the final stage of apoptotic death is highly conserved [68,75]. Two phases of the apoptosis process have been

described [24]. The initiation phase involves death factors/death receptors or mitochondrial dysfunction. Death receptors are members of the tumour necrosis factor super-family of which the TNF receptor 1 (TNF-R1) and Fas (CD95/APO-1) are best characterised. Upon activation by their cognate ligands, both Fas and TNF-R1 recruit an intracellular death complex consisting of adapter proteins and procaspases. The death complex then activates apical caspases, mainly caspase-8, which subsequently activates downstream effector caspases; caspase-3. In the alternative initiation pathway, cellular stress triggers release of cytochrome c to bind Apaf-1, which in turn activates caspase-9. Here onwards both the pathways converge because caspase-9 also activates effector caspases. Caspases (14 different members) are a class of proteases contributing to cell injury and execution of the death programme [54,76-77]. All pro-forms of caspases contain both recognition and cleavage sites implying their activation occurs either autocatalytically or by other capases.

Thus Caspase-3 activation is by two major pathways, either mediated by death receptors (caspase-8) or by mitochondria (caspase-9) [60,75]. Caspase-3 is major execution enzyme acting upstream of DNA fragmentation [60,78-79] and can also be activated via endoplasmic reticulum pathways (caspase-12) [80]. Previous studies demonstrate an increase of caspases in I/R injury in various organs [81-82].

Leucocytes play a vital role and neutrophils have been identified as the initial mediators of the inflammatory component of I/R injury. The generation of ROS and subsequent lipid peroxidation in endothelial and parenchymal tissues leads to rapid production of cytokines and chemokines which attract neutrophils and monocytes to tissues that have sustained I/R injury [83]. This early up-regulation of chemo-attractants like interleukin-8,

lead to a neutrophil infiltration within an hour of reperfusion [84-85]; followed by leucocyte immobilisation and diapedesis [86]. A second phase of chemokine expression involving gamma-interferon induces protein 10 and chemo-attractant protein 1 which facilitate recruitment of leucocytes such as neurtophils, monocytes, natural killer cells (NKC) and T cells [83]. The neutrophils themselves perpetuate the process through further production of superoxide, nitric oxide, hypochlorous acid and cytokines [78]. This inflammatory response subsequently leads to direct tissue damage through release of proteases and also up-regulates apoptosis. Neutrophils cause microvascular occlusion by the magnitude of leucocyte adhesion, the so called 'no reflow' phenomenon [87]. Release of vasoactive factors like leukotrines contribute to microvascular spasm and impaired perfusion [88].

Complement has been shown to be an important mediator of renal I/R injury [89-91]. The complement cascade is activated via three pathways;

The classical (activated by antigen-antibody interaction)

The alternative (activated by microbial products)

The mannose binding lectin (MBL) (activated by mannose on microbial surfaces)

These pathways converge at the cleavage of C3 and lead to activation of C5, generating anaphylatoxins C3a and C5a as well as the membrane attack complex (MAC) or C5b-C9. Factors C3a and C5a; mediate, recruit and activate neutrophils and macrophages to produce cytoxines and adhesion molecules [92] and induce apoptosis [93]. MAC in addition causes cell death via both necrosis and apoptosis, attracts and activates neutrophils and generates cytoxines and adhesion molecules [94-96]. The alternative and MBL pathways predominate in renal I/R injury, while the classical pathway is implicated in I/R injury

involving other tissues [97]. De Vries *et. al.* demonstrated that complement activation mediates renal I/R injury through neutrophil dependent and independent pathways [93].

1.1.3 Histopathology

The primary focus of injury is the proximal tubule and renal microvasculature as a result of oxidative injury, inflammation and cell loss secondary to necrosis and apoptosis. A variable degree of irreversible injury to the distal segment of the proximal tubule is seen during ischaemia-reperfusion. This results in shedding of necrotic tubular cells into the lumen causing impaired filtrate flow and back pressure which in turn reduce glomerular filtration rate (GFR) [98]. Impedance to flow is increased further as a result of the gelatinisation of Tamm-Horsfall protein in the loop of Henle. This protein is secreted by the ascending limb and a significant change in the solubility of this protein occurs as a result of elevated sodium concentration in the post-ischaemic nephron [99]. Endothelial injury, micro-vascular spasm, and sludging of erythrocytes and leucocytes leads to capillary occlusion, particularly in the outer medullary inner stripe [100]. The resultant reduction in glomerular trans-capillary pressure reduces GFR further and slows clearance of tubular debris and recovery of renal function.

1.1.4 Methods to attenuate I/R injury (Table 2)

The pathophysiology of I/R injury has been investigated by a large number of *in vivo* and *in vitro* studies. Methods described to attenuate this process include removal and inhibition of leucocytes, inhibition of classical and alternative complement pathways, inhibition of platelets, down regulation of endothelial cell adhesion molecules,

inhibition of free radical forming enzymes, free radical chelation and anti-apoptotic agents and ischaemic pre-conditioning [82,101-107].

The use of various pharmaceutical interventions with agents like statins [108-109], iloprost [110], prostaglandins E1 (PGE1) [111-113], erythropoietin (EPO) [81-82,101], allopurinol [114-115], carbon-monoxide (CO) [116-123] and anti-inflammatory agents [93,124-129] are being evaluated in various experimental studies.

The use of some anti-oxidants play a promising role to reduce I/R injury in the experimental setting, but their effects have not been proven in clinical trials, such as glutathione added to University of Wisconsin (UW) solution [130-131]. Others such as 20-methyl aminochroman (2-MAC), desferoxamine (DFO) [132], lazaroid U74006F [132-134], N-acetyl cysteine [135], superoxide dismutase [136-137], propionyl-L-carnitine [138], nitric oxide (NO) [139], inhibitors of inducible nitric oxide synthase (iNOS), such as l-N6-(1-iminoethyl)lysine (L-NIL), aminoethyl-isothiourea (AE-ITU) [140] and GW274150 [79].

Post ischaemia protection is possible because genes are up regulated after ischaemia, allowing a window of opportunity for intervention [104]. Transfer of several protective genes including Il-10, ICAM antisense, HSP20 and CTLA4-IG and Fas-ligand gene using adenoviral vectors has been shown to ameliorate I/R injury [141-145].

Table 2: Strategies to prevent ischaemia-reperfusion injury (1.1.4)

Strategy	Example	Reference
Endogenous defence mechanism	• **Glutathione** • **Catalase** • **Superoxide dismutase** • **Haemoxygenase enzymes**	**146** **147** **147** **116**
Preservation solutions	• **UW solution** • **Celsior solution**	**148** **149**
Vasodilators agents	• **NO donors** • **CO** • **Ca^{++} channel blockers** • **Antagonists of vasoconstriction**	**150** **151,152** **153** **154**
Anti-inflammatory agents	• **Cytokine inhibitors** • **Complement inhibitors** • **Immunosuppressants**	**155** **156** **157**
Anti-apoptotic agents	• **Caspase inhibitors** • **P53 inhibitors**	**78** **60**
Mitochondrial protection	• **Adenosine** • **Anti-ischaemic agents**	**58** **158**
Ischaemic preconditioning	• **Short ischaemic pre-treatment**	**159**
Antioxidants	• **Glutathione** • **α-tocopherol** • **Haemoxygenase-1 inducers**	**160** **161** **162**
Gene transfer therapy	• **Il-10, ICAM antisense, HSP20** • **CTLA4-IG** • **Fas Ligand gene**	**141-143** **144** **163**

1.1.5 Conclusion

Ischaemia-reperfusion injury is a complex central sequence of events influencing the short and long term outcome of renal transplantation, particularly in the context of non-heart-beating donors due to the cumulative effects of warm and cold ischaemia.
The underlying mechanism which is multi-factorial, inter-related, and complex includes multiple cell types involving an array of intra and extra-cellular signalling and effector pathways. The understanding of I/R injury is of utmost importance, requiring extensive investigative experimentation, thereby generating various hypotheses and strategies to create various therapeutic interventions.

CHAPTER 2

ERYTHROPOIETIN (EPO)

2.0 Introduction

Erythropoietin is a 30.4 kDa acidic glycoprotein hormone that was first detected as a haemopoietic factor in the blood of rabbits by Deflandre *et. al.*, from Paris, France in 1906 [164-165]. The protein was isolated in 1977 [166] and its gene was cloned in 1985 [167-168]. Recombinant human erythropoietin thereafter became available from 1985 for clinical trials and was introduced into clinical practice for the correction of anaemia in 1989 [169].

Human erythropoietin (hEPO) consists of 165 amino acids and three N-linked glycans and one O-linked glycan [170-171]. The relative molecular mass of the protein backbone deduced from the primary sequence is 18,398 [168-167] but due to glycosylation, recombinant EPO (hEPO) and urinary EPO migrate in SOS gel with a molecular size of 34,000-37,000 [170,172].

2.1 EPO production

EPO is mostly made of the kidney type I, cortical interstitial cells and participates in a classical negative feedback control system [173-176]. Hypoxia is the fundamental physiological stimulus that causes a rapid increase in renal production of EPO through an exponential increase in the number of EPO-producing cells [177]. In addition it is also secreted in the brain, liver, uterus, testis and mammary glands (Table 3).

Table 3: Possible physiological roles of EPO (2.1)

Production site	Function	Stimuli for production	Reference
Foetal Liver	Erythropoiesis	Retinoic acid	178,179
Kidney	Erythropoiesis	Hypoxia	180,181
CNS	Neuroprotection	Hypoxia	182-184
Retina	Neuroprotection	Hypoxia	185,186
Uterus	Angiogenesis	Hypoxia & oestrogen	187,188
Testis	Steroidogenesis	Hypoxia	189-191
Mammary gland	Gut maturation		192,193

2.2 Physiological regulatory mechanisms:

EPO in erythropoiesis:

EPO is the primary regulator of erythropoiesis and exerts its effects by binding to a surface receptor (EPO-R) on erythroid progenitors and precursors [194-195]. Red blood cell production is then ultimately stimulated by inhibition of apoptotic cell death of immature erythroblasts through the bcl-2 family of anti-apoptotic genes, thereby permitting their progression to mature erythrocytes [194]. The net effect, a compensatory adaptation to renal tissue hypoxia is an augmentation of the oxygen-carrying capacity of blood. Evidence suggests that EPO may act both as a survival factor [196] and a mitogen [197]. Recent studies in knock out mice have shown that EPO is crucial for *in vivo* proliferation and survival of colony-forming-unit-erythroids (CFU-E's) and their irreversible terminal differentiation, and thus is not required for generation of burst-forming-unit-erythoids (BFU-E's) and their differentiation to CFU-E's [198]. EPO can promote erythoid progenitor survival by repressing apoptosis through bcl-XL and bcl-2 [199]. The uses of EPO in clinical conditions associated with defective endogenous EPO production are outlined in table 4.

Pleiotropic cyto-protective actions of EPO:

Abundant experimental evidence demonstrates that EPO interacts with its receptors in many tissues to induce a range of cytoprotective responses including mitogenesis, angiogenesis, and inhibition of apoptosis and promotion of vascular repair by mobilisation of endothelial progenitor cells [164,200].

Table 4: Use of EPO in clinical conditions associated with defective endogenous EPO production (2.2)

Anaemia due to uraemia
Anaemia due to prematurity
Anaemia due to inflammation • Rheumatoid arthritis • Inflammatory bowel disease • Chronic infection • AIDS
Anaemia due to malignancy • Solid tumours • Multiple myeloma • Malignant lymphoma

EPO administration has also been documented to ameliorate I/R injury and mechanical injuries of the spinal cord, retinal neurons, peripheral nerves, gut, liver and heart [164,201-203]. Some of these benefits may be related to mobilisation of erythrocyte progenitor cells (EPCs) from bone marrow and subsequent promotion of neovascularisation [200]. Increased neovascularisation by EPCs has been shown to improve both cardiac function after experimental myocardial infarction (MI) and clinical outcomes after MI [200].

2.3 Mechanisms of reno-protection by EPO

The molecular signals by which EPO provides protection against renal injury are not very clear but a number of explanations have been put forward. It would appear that at least some benefit is mediated by activation of EPO-R, because experimental renal injury is not ameliorated by an EPO analogue that does not bind to EPO-R [204-205]. EPO can act directly on proximal tubule epithelial cells and protect against I/R injury [82]. Some investigations have shown that EPO has some receptor independent tissue protective effects and seems to protect myocardium and brain against I/R injury [205]. Studies from some authors have observed that the reno-protective benefit of EPO occurs prior to, or in the absence of a significant change to the haemocrit [82,204,206-208]. Activation of EPO-R by EPO leads to dimerization of the receptor and its subsequent phosphorylation and activation of Janus kinase 2 (JAK-2) (a tyrosine kinase bound to β-subunit of the EPO receptor). This in turn activates a number of signalling pathways, including signal transducer and activator of transcription 5 (STAT5), mitogen-activated protein kinase (MAPK) and the phosphoinositol 3 kinase (PI3K)/protein kinase B (Akt) signalling pathways [164,204-205]. Ates *et . al.* recently demonstrated that the protective effect of EPO in a rat model of renal I/R injury was abrogated by the tyrosine kinase

inhibitor, genisten [208]. Sharples *et. al.* similarly observed that the anti-apoptotic effects of EPO on human HK-2 proximal tubule cells were blocked by an inhibitor of Janus kinase 2 phosphorylation (tyrphostin or AG490) and by specific inhibitors of PI3K (LY294002 and wortmannin) [82]. PI3K activation results in phosphorylation of Akt which in turn promotes cell survival and anti-apoptotic effects via inhibition of forkhead transcription factor (FOXO3a), inactivation of glycogen synthetase kinase-3β, reduced activity of apoptotic proteins (Bad, Bax), increased activity of anti-apoptotic proteins (Bcl-2, Bcl-X_L), induced expression of X-chromosome linked inhibitors of apoptotic protein (XIAP), inactivation of caspases, maintenance of mitochondrial membrane potential ($\Delta\Psi m$), prevention of cytochrome C release and prevention of glycolysis and ATP synthesis [164,201-202]. Bahlmann *et. al.* demonstrated that the protection against ischaemic acute renal failure (ARF) afforded by EPO was associated with decreased expression of pro-apoptotic protein Bax[200], although no changes were seen in the expression of the anti-apoptotic proteins Bcl-2, Bcl-X_L [207]. Johnson *et. al.* similarly observed diminished expression of Bax in experimental ischaemic ARF after EPO therapy [207], where as Sharples *el. al* reported up regulation of Bcl-X_L and XIAP [82]. Further studies also showed that EPO and darbepoetin expressed reno-protection by prevention of activation of caspases-3, 8 and 9 *in vivo* studies [204,209].

NF-κB, a key mediator of inflammatory and cytokine responses, is additionally activated by the Akt pathway and adds to EPO cytoprotection [209]. This may be due to the induction by NF-κB of a number of anti-apoptotic proteins such as XIAP, growth arrest and DNA damage protein 45 (Gadd45β), heat shock protein70 and Bcl-X_L [209]. NF-κB may also ameliorate tissue damage by inhibiting NO [210] and by enhancement of hypoxia-inducible factor-1-regulator EPO expression [211]. They also demonstrated an up regulation of Bcl-X_L, XIAP and reduction of caspase-3 activation, which enhance the

protective effects of EPO because it acts on both death receptor (Fas-FasL) mediated and mitochondrial pathways [212]. XIAP has also shown its protective effects in renal I/R via indirect anti-apoptotic effects by induction of p21cip1 leading to cell cycle arrest at the G1/S transition [210,213].

Other potential pathways described included activation of STAT5 (which translocates to the nucleus and stimulates transcription of pro-mitogenic and anti-apoptotic genes) [201], MAPK [202] and protein kinase C (causing opening of mitochondrial potassium channels) [202]. It is also possible that EPO exerts a number of EPO-R-independent cytoprotective actions via antioxidation, maintenance of mitochondrial membrane permeability and inhibition of cytochrome C release [164,201].

Summarising that EPO directly protects proximal tubule epithelial cells:

1) Activating EPO receptor / JAK-2 kinase
2) Activating PI3K leading to activation of Akt
3) Up-regulation of Bcl-X_L and XIAP
4) Preventing activation of caspases (3,8,9) and ultimately apoptosis

2.4 Potential clinical applications of EPO reno-protection:

The consistent findings of the beneficial effects of EPO when administered prior to the onset of experimental ischaemic and toxic renal injury, suggest that it could be a suitable manipulating agent to pharmacologically precondition the kidney in patients at high clinical risk of ischaemic ARF. There are many and quite common clinical situations where pre-emptive EPO treatment could potentially prove to be of substantial

clinical benefit. These clinical scenarios include renal transplantation, aortic or coronary artery surgery and radiographic contrast administered to patients who either have a transplant or are diabetic or have chronic kidney disease.

2.5 Adverse effects:

The beneficial effects of EPO in acute and chronic renal disease must be balanced against potential adverse effects, particularly with relatively high doses of EPO (up to 5000 u/kg). Serious adverse clinical sequel could range from hypertension, seizures, thrombosis to stroke; all are related to a haematocrit increase [164]. Recent concerns have been raised about EPO stimulating growth of various tumours [214], including renal cell carcinomas [215]. A recent pilot safety study for a multicenter, randomized controlled trial in acute stroke used a single dose of 100,000 Units (1500 u/kg) demonstrated no adverse effects (including no significant rise in haematocrit over 30 days) , despite a transient 500- fold increase in serum EPO concentrations [216].

2.6 Conclusion:

Abundant evidence shows that the administration of EPO at the time of or up to 6 hours prior to experimental acute renal injury significantly augmented various histological and functional parameters leading to organ recovery and animal survival. The reno-protective effects are primarily mediated by targets of EPO-R signalling pathways including AKT, STAT5 and MAPK leading to anti-apoptotic, mitogenesis and angiogenesis and mobilisation and differentiation of EPCs. EPO may thus be useful in a NHBD kidney transplant programme. A dual effect may thus be obtained in patients

with kidney disease where EPOs cytoprotective benefits can be used in protecting the kidney from an intervention induced ischaemia and also provide additional benefit from promoting erythropoiesis. Strategies to avoid systemic exposure to high doses of EPO can easily be tested in a NHBD programme by direct perfusion of the organ on an isolated perfusion as proposed in this thesis.

CHAPTER 3

CARBON MONOXIDE (CO)

3.0 Introduction:

The first clinical description of coal gas poisoning was in 1755 by Harmant [217] in France although Aristotle did mention the lethal effects of coal fumes in the 3rd century BC. It was not until 1842 that the toxic substance in coal gas was identified as CO by Le Blanc in France. In 1857 Claude Bernard a French physiologist showed that CO produces hypoxia by combining with haemoglobin; this research thus became the background for assigning CO to the category of poisons and toxins [217]. It was the British scientist John Haldane (1895) who explained that the toxic effects were hypoxic related due to the binding of CO to haemoglobin. Nicloux in 1927 discovered carboxyhaemoglobin (HbCO) in experiments using dogs [218]. During 1944 Roughton and Darling reported that HbCO shifted the oxyhaemoglobin dissociation curve to the left [219]. Sjostrand *et. al.* in 1951 reported that CO is endogenously produced in normal man and CO originates from the carbon atom of haem [220]. In 1968 and 1969 Tenhunen discovered that haem oxygenase (HO) was the enzyme responsible for haem catabolism and in the mid 80s two isoforms of HO were cloned [221]. Young and Caughey in 1986 documented that CO is oxidised to CO_2 by mitochondria [222]. In the last decade evidence has mounted to show that HO enzymes and their by-products are important players in biological life, despite the fact that CO is a dangerous exogenous poison. In addition there is evidence suggesting it an essential endogenous physiological regulator [157].

3.1 Carbon monoxide sources

CO is an odourless, colourless, tasteless, non-irritant diatom and is chemically an inert gas; sources are classified as exogenous and endogenous (Table 5)

Exogenous: CO comes naturally from volcanic gases, photo-dissociation in the upper atmosphere, coalmines and forest fires [217]. Man made sources include incomplete fuel combustion, burning of solid waste and smoking cigarettes [157].

Endogenous: Cells and tissue can produce CO as a product of haem metabolism [223]. Haem degradation is responsible for more than 85% of endogenous CO while the remaining 15% is produced by non haem sources such as cytochrome P-450 dependent oxidation of certain xenobiotics and lipid substrate or as a by-product of the iron dependent peroxidation process [224].Under normal conditions more than 80% is stored as HbCO via binding to Hb and the rest is bound to other haem-proteins like myoglobin or cytochrome-c oxidase [219].

3.2 The Haem oxygenase system

CO is a product of haem oxygenase enzymes, the first and rate limiting step being haem degradation. Each haem is degraded into 1 mole of CO, 1 mole of biliverdin-IXα and 1 mole of ferrous iron. Three isoforms of HO have been characterised to date; the constitutive forms (HO-2) and (HO-3) and the inducible form (HO-1) [225]. HO-1 is also known as heat shock protein 32 and is sensitive to up regulation in all tissues by a variety of stimuli that cause oxidative stress and pathological conditions such as heat shock, hypoxia, ischaemia radiation [225]. HO-2 is expressed at high levels in neuronal,

Table 5: Properties of carbon monoxide (3.1)

Synonyms	Carbonic oxide, Carbon oxide, Exhaust gas
Molecular formula	CO
Colour	Colourless gas
Odour	Odourless gas
Taste	Tasteless gas
Molecular weight	28.01
Melting point	-205 $^{\circ}$C
Boiling point	-191 $^{\circ}$C
Flammability	Flammable
Solubility	H_2O= 3.3 ml^{-1} at 0°C, soluble in chloroform & acetic acid
Specific gravity	Gas= 1.25gL^{-1} at 0°C, Liquid =0.793gL^{-1} at 0°C
Conversion factor	1ppm= 1.25mg m^{-3} at 25°C
Vapour pressure	Greater than 1 atm at 20°C
Vapour density	0.968
Antidote	100% oxygen
Biological half life	2-5 hours

vascular and hepatic tissues [226] and is involved in regulation of neural system function; the only known inducers are adrenal glucocorticoids [218]. HO-3 isoform has only been found in rats [227]. All isoforms of HO are inhibited by certain synthetic and naturally occurring metalloporphyrins such as zinc protoporphrin-IX [227].

The two other products of haem degradation (iron & biliverdin) are also biologically active, iron sequestrated by ferritin protects against oxidative damage [228]. The water soluble biliverdin is converted by NADPH: biliverdin reductase to lipophilic bilirubin, a powerful endogenous antioxidant that protects mammalian tissue against oxidative stress by scavenging ROS and reducing cell death [229-231]. Clarke *et. al.* has reported that HO-1 derived bilirubin provides cardiac protection against reperfusion injury in an isolated rat model [232]. Furthermore Adin *et. al.* has shown pre-treatment with low doses bilirubin can improve renal function after I/R injury in an isolated perfused rat model[233].

3.3 Carbon monoxide as a poison

CO has been historically identified as a poison, where *in vivo* concentration beyond 4000 p.p.m are immediately lethal [218]; thus labelled as a silent and invisible killer. In the UK each year about 50 people die from CO poisoning [234]. The well known mechanism of CO toxicity is that after inhalation, the gas diffuses across the alveolar membrane of the lung and binds reversibly to haemoglobin in the blood. The affinity being 200-250 time that compared to O_2, CO completely competes with O_2 and binds to the four iron haems forming HbCO. This causes a shift of oxyhaemoglobin dissociation curve to the left thus decreasing the oxygen carrying capacity of the blood producing hypoxia. Compensatory response to the hypoxic stress involve increasing the cardiac output and blood flow rate to the vital organs and eventually life threatening hypoxia results in

brain damage and cardiovascular collapse [235]. The toxicity elicited by CO is dependent on both duration of exposure and the concentration of HbCO. The clinical manifestations range from no symptoms at HbCO below 10% to nausea, vomiting, head ache, shortness of breath, convulsions and coma at levels between 16-60% to lethal effects if not treated at levels 67-70% [235].

3.4 Carbon monoxide as a novel therapeutic

It is only in the last decade that the traditional view of CO as deadly gas started to change, as experiments revealed the various pleiotropic physiological mechanisms. Therefore the careful use of CO at low concentrations (10-500p.p.m) could be developed into a novel therapy [221]. The mechanisms responsible include activation of soluble guanylate cyclase (sGC), potassium channels, mitogen-activated protein kinase (MAPK), various inflammatory mediators and mitochondria. (Table 6)

Table 6: Target sites for carbon monoxide (3.4)

CO Targets	Physiological Function	Reference
Soluble guanylate cyclase (sGC)	• Vasorelaxation • Anti-inflammatory • Anti-apoptotic • Anti-proliferative • Neurotransmitter • Inhibiting platelet aggregation • Protection against I/R injury	236-237 238 239 240 241 242 238
K^+ channels	• Vasorelaxation	237,243-245
MAPK	• Anti-inflammatory • Anti-apoptotic • Anti-proliferative • Protection against I/R injury	219 245-246 248-249 250
Inflammatory mediators	• Anti-inflammatory • Protection against I/R injury	251-252 152,253,250,254,255
Mitochondria	• Anti-proliferative	256

3.5 Physiological regulatory mechanisms

CO has been shown to play a major vasoregulatory function by inducing vasorelaxation through a mechanism involving cyclic guanine monophosphate (cGMP) and calcium-activated potassium channels [146, 257-259].

CO an important signalling mediator [236,237,260-264] is generated in mammals during degradation of haem by a family of constitutive (HO-2) and inducible (HO-1) haem oxygenase enzymes [225,265-266]. A number of experiments have demonstrated the ability of HO expression to be cytoprotective against oxidative stress [267-268] and have indicated a role in ischaemic reperfusion injury in various organs such as heart [232,269], liver [270], kidney [118,271], nerve tissue [272] and lungs [273-275]. These protective actions are associated with a decrease in inflammation and apoptosis [276]. CO acts through the p38 mitogen-activated protein kinase pathway (p38MAPK) thereby decreasing apoptosis by inhibition of Fas/Fas ligand, as well as stimulation of anti-apoptotic proteins such as Bcl-2 [124,277]. While anti-inflammatory mechanism is associated with a decrease in cytokines such as IL-6 and inducible nitric oxide synthase [238]. In addition it is accompanied by its antiproliferative action [240] and inhibition of platelet aggregation by activation of guanylate cyclase [242].

3.5.1 Role of carbon monoxide in organ preservation, transplantation and I/R injury

With increasing evidence supporting the various pleiotropic actions of CO, the hypothesis that CO may provide protection in inflammatory diseases, I/R injury, organ transplant models and organ preservation various investigations are on the increase.

Various models showing protective role of low concentration of CO have demonstrated a beneficial role as listed in Table 7.

Table 7: Role of CO in I/R injuries and organ transplantation (3.5.1)

Organ	Effect of CO	Reference
Heart	• Protects heart from transplant associated I/R injury • Suppresses mice/rat cardiac transplant rejection • Prevents atherosclerosis post aorta transplant • Suppresses stenosis after carotid balloon injury • Protection against cardiac I/R injury	278 279 248 248 250
Lung	• Cyto-protection in a rat lung transplant model • Protection against lung I/R injury • Protection against hyperoxic lung injury	280 247 274
Liver	• Cyto-protection against cold I/R in rat liver model • Protects liver from transplant associated cold I/R • Limits haemorrhagic shock-induced liver dysfunction • Protects against liver failure	281 282 283 284
Kidney	• Protects kidney from transplant associated cold I/R • Prevents development of CAN	94,255 285
Intestine	• Protects intestine from transplant associated cold I/R	238,254

3.6 Various approaches

Currently different approaches that have been used to study these pleiotropic effects of this diatomic gas are

1) Direct administration of the gas [251]
2) Use of pro-drugs (methylene chloride) that are catabolised by hepatic enzymes to generate CO [286]
3) Transport and delivery of CO by means of specific CO carriers (transition metal carbonyls) – carbon monoxide releasing molecules (CORM) [287]

3.7 Carbon monoxide-releasing molecule (CORM)

3.7.1 Introduction

Increasing experimental evidence supporting the biological role of CO highlighting its cyto-protective effects needed a more precise method for CO administration. CO used as the diatom gas itself is limited due to rapid conversion to HbCO and associated toxicity, in addition to difficulty storing and safe delivery of CO gas in a controlled manner [219]. Similarly the use of pro-drugs is limited as functional hepatic component is required for the generation of CO [286].

Thus progress in the field of CO research has led to a new way to deliver it at the target organ by safe compounds which carry and release it under physiological conditions. This class of compounds thus termed carbon monoxide releasing molecules (CORMs), which are able to transport and release CO both *in vivo* and *in vitro* in a safe and

controlled fashion [288-289]. These carriers can potentially enhance the specificity of CO delivery and facilitate its use as an upcoming therapeutic agent [290].

3.7.2 Types of CORM

Metal carbonyls were discovered 100 years ago and used in purification of metals, currently in clinical practice they are used in treatment of cancer and inflammation [291]. Motterlini *et .al.* showed that metals surrounded by carbonyl groups have an interesting property of releasing CO which was identified spectrophotometrically by measuring the conversion of deoxymyoglobin to carbonmonoxy myoglobin using nuclear magnetic resonance analysis; hence termed carbon monoxide-releasing molecules (CORM) [267,288,292].

CORM-1 (dimanganese decacarbonyl) [$Mn_2(CO)_{10}$] and CORM-2 (tricarbonyldichlororuthenium(II) dimmer) [$Ru(CO)_3Cl_2$] are soluble in organic solvents such as dimethyl sulfoxide (DMSO) and appear to be safe to cells in concentration ranges from 40-210 μmol L [288].

CORM-3 [Tricarbonylchloro(glycinato)ruthenium(II)] [Ru(CO)3Cl(glcinate)] is the first water soluble molecule [289] and thus has the advantage of avoiding toxic organic solvents. Studying the kinetics, the same group showed that one mole of CO is released per mole of CORM-3. Motterlini *et. al.* further showed that the rate of release of CO from CORM-3 varies according to the type of solution used for preparation, where it is relatively stable in distilled water but rapidly releases CO in human plasma ($t_{½} \approx 1$ min) [289]. CORM-A1 (sodiumboranocarbonate) [$Na_2(h_3CO_2)$] is another water soluble CO releaser but unlike the others lacks a transitional metal and liberates CO at a much slower rate under physiological conditions ($t_{½} \approx 21$ min) [290,292]. The same group have

published that CORM-3 and CORM-A1 are relatively safe and do not significantly affect cell viability *in vitro* at concentrations up to 500μm [290].

Inactive CORMs (iCORMs) have been used as negative controls in order to prove effects are elicited by CO released by CORMs, where the inactive forms are chemically identical and left in a physiological solution for 24 hours prior experimentation, thus liberating all the CO [267].

3.7.3 Biological activities of CORMs (Table 8)

These transition metal carbonyls possess the ability to liberate CO under appropriate conditions and function as CO-releasing molecules (CO-RM's) in biological systems. They work through mechanisms that can be simulated by activation of the HO-1/CO pathway [266,276,288] (involvement of mitoK_{ATP} channel activation), in addition they induce the levels of HO-1 protein [276].

Role in vasorelaxation:

Motterlini *et. al.* showed that CORM-1 added to an isolated perfused heart caused marked attenuation in the increase in coronary pressure when challenged with an inhibitor of NO synthase (L-NAME) [288]. Motterlini *et. al.* reported a concentration dependent vasodilatation in isolated cerebral arterioles [292], mechanism explained by activation of guanylate cyclase [393]. The vasodilatory action of CO is endothelium dependent and includes a permissive role of NO, suggesting that NO facilitates the vasodilatory action of CO [294]. Xi *et. al.* demonstrated CO activates Ca^{++}-activated K^{+} channels in porcine cerebral arteriole smooth muscle cells [295]. Arregui *et. al.* demonstrated that administration of CORM-1 to a rat renal artery lead to significant

renal vasodilatation increased GFR, urinary excretion of Na^+ and water as well as urinary cGMP excretion [96]. Motterlini *et. al.* demonstrated profound vasodilatation, dependent on cGMP and activation of the HO-1/CO pathway following the stimulation of haem in a rat model [288-289]. Further studies by the same group demonstrated concentration dependent relaxation in pre-contracted aortic vessels, mediated by K^+ channels and guanylate cyclase activation [292,297].

Role in proliferation and apoptosis:

CORM-2 has been proven to inhibit proliferation and attenuates the release of vasoconstrictor endothelin-1 in human pulmonary artery smooth muscle [298-299]. Taille *et. al.* studies revealed both mitochondrial respiratory chain and free radical generation are targets for anti-proliferative effects of CORM-2 [256]. Li *et. al.* showed that CORM-3 increases angiogenesis in HO-1 deficient endothelial cells starting at a concentration of 25 μm [300]. Vadori *et. al.* experiments revealed anti-proliferative and anti-apoptotic effects by CORM-3 in porcine aortic endothelial cells [301].

Role in Inflammation:

Lee *et. al.* showed that CORM-2 completely suppresses the inflammatory response by inhibiting the translocation of NF-κB into the nucleus, indicating a direct contributory effect of CO [302]. Sawle *et. al.* provided evidence for anti-inflammatory action of both CORM-2 and CORM-3 by inhibiting the increase of TNF-α and NO levels in endotoxin-stimulated macrophages [303]. Seveso *et. al.* similarly showed that pigs treated with CORM-3 prevented the release of TNF-α in mononuclear cells stimulated by LPS [303].

Role in ischaemic reperfusion injury:

Clark *et. al.* demonstrated that CORM-3 protects against hypoxia-reoxygenation and oxidative stress in cardiac cells [266]. In addition they showed that the attenuation of I/R injury in rat hearts by CORM-3 involves the activation of ATP-dependent K^+ channels present in mitochondria [266]. CORM-3 used in *ex vivo* models protects against I/R injury and prevents cardiac allograft rejection in vivo [267]. Guo *et. al.* demonstrated a decrease in myocardial infarct size when given just before reperfusion in vivo [304]. Stein *et. al.* further showed a delayed and sustained 72 hour protection against myocardial infarct, which is similar to ischaemic preconditioning using CORM-3 [305]. In a mouse model of ischaemia induces acute renal failure Vera *et. al.* showed that administration of CORM-3 significantly decreased levels of plasma creatinine and restricted renal damage [276].

3.8 Conclusion

The need to continually improve renal transplantation in context to NHBD organs drives expanding interest with use of such manipulating agents like CO & CORM-3. Taking renal transplantation into consideration these relate to methods of graft preservation aimed at reducing I/R injury and improving clinical outcomes via manipulation of the underlying pathophysiological procedure. Thus using CO direct ability to influence intracellular signalling pathways that involve apoptosis and inflammation, key in I/R injury a major concern in NHBD organs, leads to various experimental strategies so as to assess its use as a possible novel therapeutic.

Table 8: Biological properties of CORMs (3.7.3)

Active Molecule	Biological Properties	Reference
CORM-1	• Cerebral vasorelaxation	294
	• Attenuation of coronary vasoconstriction	288
	• Improves renal haemodynamics	296
	• Increased glomerular filtration	296
	• Internal anal sphincter relaxation	306
	• Activates Ca^{++}-activated K^{+} channels	295
CORM-2	• Aortic vasorelaxation	288
	• Modulates immune system	307
	• Increased angiogenesis	300
	• Anti-proliferative effects	256,298
	• Anti-inflammatory action	303
	• Limits renal ischaemic damage	276
	• Anti-hypertrophic effects	308
	• Inhibits the release of endothelin-1	308
	• Tracheal relaxation	309
CORM-3	• Aortic vasorelaxation	297
	• Protects against hypoxic-reoxygenation	267
	• Protects against oxidative stress	267
	• Attenuates cardiac I/R injury	267,304,305
	• Prevents CAN	267
	• Anti-inflammatory action	303
	• Increased angiogenesis	300
	• Decreases myocardial infarct size	304
	• Limits renal ischaemic damage	276
	• Anti-proliferative action	301
	• Anti-apoptotic action	301
CORM-A1	• Aortic Vasorelaxation	292

CHAPTER 4

AIMS AND METHODS

4.0 Aims

The aims of this thesis are:

1) To design a system for perfusing isolated porcine kidneys with normothermic perfusion using autologus blood, simulating the logistics of NHBD transplantation.

2) To investigate the effects of a period of normothermic resuscitation perfusion (NP) in comparison to hypothermic techniques.

3) To investigate whether a high dose of erythropoietin (EPO) administered as a bolus will protect against I/R injury using an isolated perfused porcine kidney model.

4) To investigate whether low concentration of carbon monoxide (CO) released from carbon monoxide releasing molecule-3 (CORM-3) protects against I/R injury.

5) To investigate whether carbon monoxide administered either as CO gas or CORM-3 in addition to NP can be used as a possible novel therapeutic strategy for kidney preservation, particularly in the context to NHBD transplantation.

4.1 Methods

4.1.1 Isolated porcine kidney model

The model chosen for this investigation used autologus whole blood perfused *ex-vivo* isolated porcine kidneys, the isolated organ perfusion system (IOPS). The kidneys were subjected to 10 minutes of warm ischaemia (WIT) and 16hours of cold storage ischaemic time (CSIT), thus representing median WIT and CSIT as seen in controlled NHBD transplantation [2].

4.1.2 Retrieval

Large white pigs (60-70 Kg) were sacrificed by electrocution followed by exsanguination via an incision in the external jugular vein. Then approximately 1 litre of blood was collected into a sterile receptacle containing 25,000 units of heparin (Multiparin; CP Pharmaceuticals, Wrexham, UK) and then transferred to CPDA-1 blood bags (Baxter Healthcare) for storage. This was carried out by a professional slaughter man in an abattoir as per schedule 1 of the UK Home Office guidelines. A rapid retrieval technique was then employed, involving *en bloc* resection of intraperitoneal organs (by a professional slaughter man) and then simultaneous removal of both kidneys by two surgeons ensuring preservation of vascular and ureteric pedicles. After a warm ischaemic period of 10 min, the kidneys were then flushed with 300 ml of hyperosmolar citrate solution (HOC; Soltran, Baxter Healthcare Ltd, Thetford, UK) at 4°C, delivered at a hydrostatic pressure of 100 cm H_20. The organs were then stored on ice in HOC and transported to the perfusion laboratory.

4.1.3 Isolated organ perfusion

After planned cold storage time, the renal artery and vein were dissected and cannulated with appropriated sized renal cannula and the ureter cannulated with a 10 Fr urinary catheter. The kidneys were then flushed with a plasma substitute (Gelofusin; B. Braun, Sheffield, UK) and then be placed on the isolated organ perfusion system (IOPS) [40] for a period of 2 hours of normothermic perfusion (the preservation arm) or thereafter for 3 hours of reperfusion (the viability testing arm) and various parameters assessed.

The manipulating agents to be tested would be given in the preservation arm during the 1st hour of normothermic resuscitation perfusion with autologous blood.

4.1.4 Normothermic resuscitation perfusion (NP) circuit

The IOPS was designed using commercially available clinical-grade cardiopulmonary bypass technology (Medtronic bio console system; Medtronic, Watford, UK). This consisted of a centrifugal blood pump (550 Bio-pump), a heat exchanger (Grant, GD120, Cambridge, UK), a 5 litre venous reservoir (Medtronic) and a minimax plus membrane oxygenator (Medtronic). The circuit hardware included a speed controller, a TX50P flow transducer, a pressure transducer (Medtronic) and a temperature probe (Cole-Parmer, London, UK). Two PC-2 Gemini infusion pumps (Alaris, Basingstoke, UK), a urinometer (Bard, Crawley, UK) and two modified leukoguard RS white cell filters (Pall, UK & Phoenix cardiovascular systems, Preston, UK) were incorporated into the system (Figure 1, picture 1 & 2).

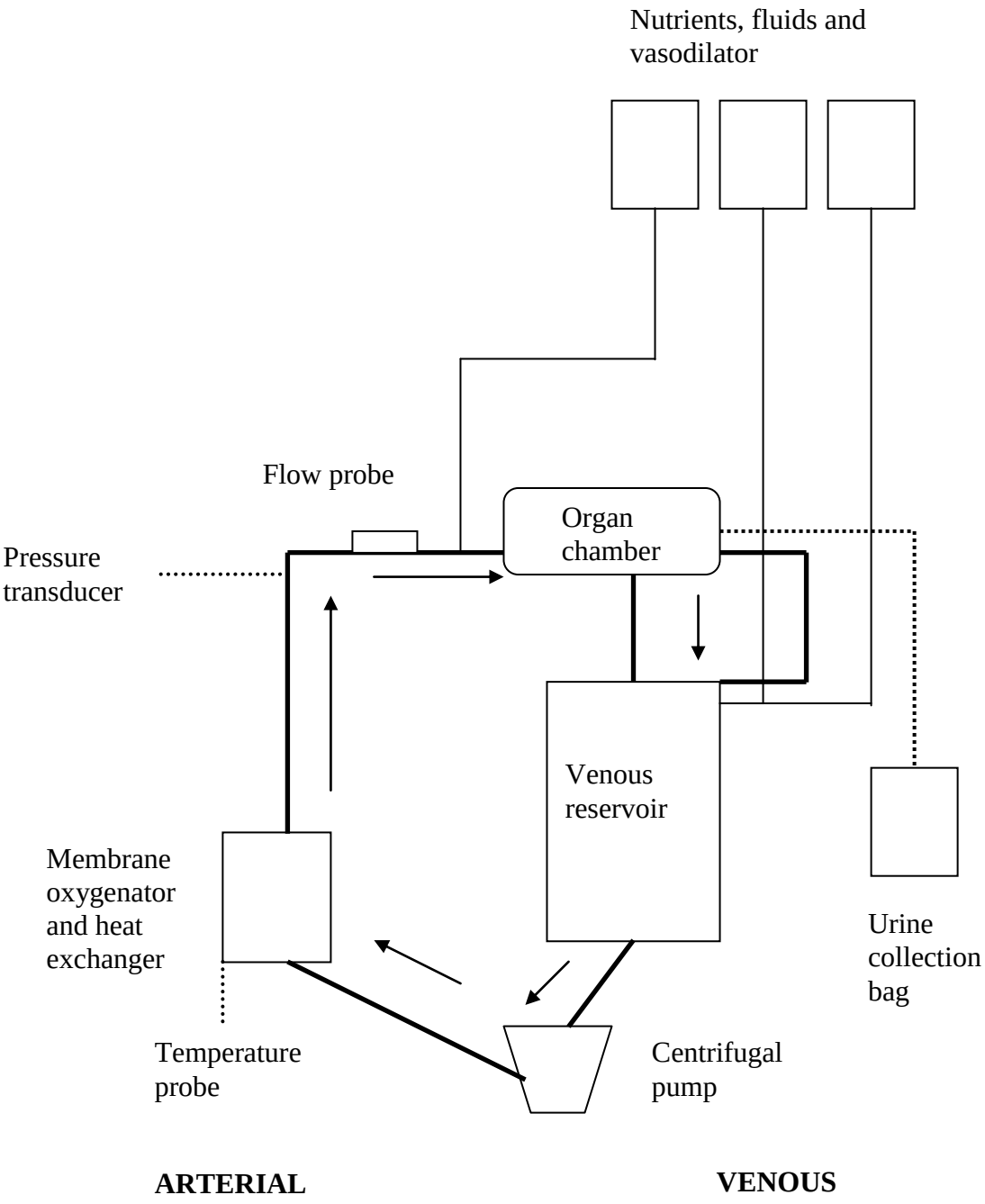
The circuit was primed with 500 ml Ringer's lactate (Baxter) containing mannitol 10mg (Baxter Healthcare Ltd, Norfolk, UK), dexamethasone 10 mg (Organon Labs Ltd, Cambridge, UK), cefuroxime 750 mg (Britannia pharmaceuticals Ltd, Surrey, UK) and 12 ml sodium bicarbonate 8.4% (Fresenius Kabi, Warrington, UK) added to meet physiological conditions. 500 ml heparinised whole blood was added to the circuit after priming and allowed to circulate at a temperature of 39°C.

This system was supplemented with the vasodilator sodium nitroprusside (Mayne Pharma PLC, Leamington Spa, UK) during the first hour (25ml/hr). This was then replaced with a continuous infusion of 5% glucose (Baxter) at a rate of 7ml/hr. Insulin (100 units; Actrapid, Noro Nordisk Bagsvaerd, Denmark) and 25 ml of 8.4% sodium bicarbonate (Fresenius Kabi, Warrington, UK) were added to a nutrient and multivitamin preparation (Nutriflex B; Braun, Sheffield, UK) and this mixture was infused at a rate of 20 ml per hour throughout the perfusion period. A crystalloid solution (Ringer's lactate) was used to replace the urine output ml for ml. The perfusate was oxygenated with a mixture of 95% oxygen/ 5% carbon dioxide. The mean arterial pressure was set at 75 mmHg and kidneys were flushed with gelofusine at 4°C (B Braun, Sheffield, UK) to remove any residual HOC and then perfused for a period of 2 hours. Renal blood flow (RBF) and intra renal resistance (IRR) were measured throughout perfusion. Blood gases were measured for calculation of oxygen consumption in ml/min/g [(PaO_2 art - PaO_2 ven) × flow rate/weight] and acid-base homeostasis. The circuit thus used to resuscitate the kidney included leukocyte depleted blood, lower arterial pressure (75 mmHg), a vasodilator (sodium nitroprusside), dexamethasone and nutrient and multivitamin preparation (Nutriflex B) hence termed the preservation arm.

4.1.5 Reperfusion circuit

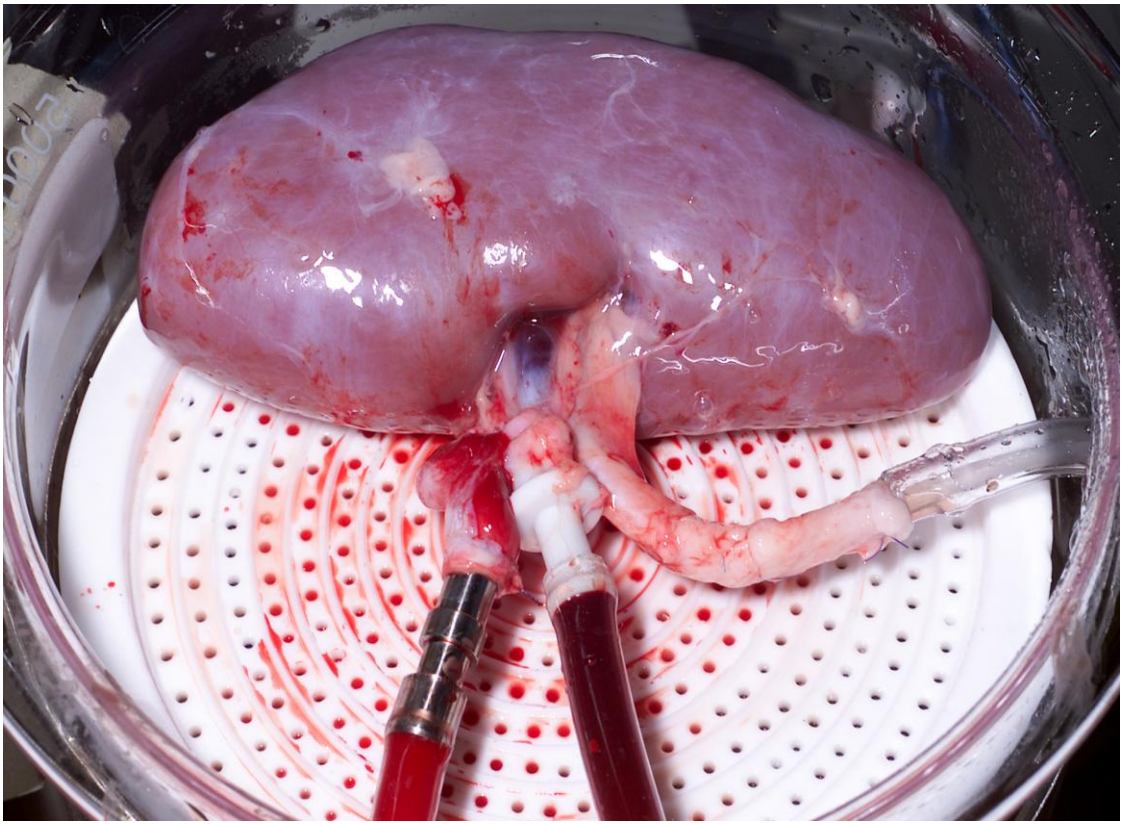
After the preservation period, kidneys were flushed with 200 ml of hyperosmolar citrate solution (4°C) followed by 90 ml of gelofusine to flush out this preservation solution. Kidneys were left at this temperature for a period of 30-45 min (to simulate the surgical anastomosis time) and were then reperfused on the IOPS machine for a period of 3 hrs at a mean arterial pressure of 85 mmHg. The protective agents, sodium nitroprusside (Mayne Pharma PLC), dexamethasone (Organon Labs Ltd) and multivitamins were omitted from the perfusate. Whole autologous blood was used instead of the leukocyte-depleted blood and the circuit was spiked with 1000µmol/L creatinine (Sigma-Aldrich, Steinheim, Germany). These alterations were made to replicate a more physiological environment from which the extent of reperfusion injury could be assessed, thus the viability testing arm.

Figure 1: Diagram of the isolated organ perfusion system (IOPS) outlying the direction of blood flow. (4.1.1)



Picture 1: The isolated organ perfusion system (IOPS) (4.1.1)

Picture 2: The perfused kidney on IOPS circuit (4.1.1)



4.2 Experimental Design

The kidneys were reperfused on the isolated circuit for 3 hours at a set mean arterial pressure of 85 mmHg. Six kidneys in each group are required to have an 80% chance of detecting a difference in mean glomerular filtration rate of 1 ml/min/100g (SD 0.6 ml/min/100g) at the level of 5% level of significance using un-paired t-test hence used.

4.2.1 Phase I (Chapter 5): To assess normothermic resuscitation perfusion (NP)

Kidneys after retrieval were assigned as follows:
Group1 (n=6): 2 hr CS (cold storage) (minimal ischaemic damage)
Group2 (n=6): 18 hr CS (static hypothermic storage)
Group3 (n=6): 18 hr CP (cold perfusion) (hypothermic machine perfusion)
Group4 (n=6): 16 hr CS followed by 2 hr NP (normothermic resuscitation preservation)
They were then reperfused for 3 hr with autologous blood for assessment of renal viability and function.

Minimal ischaemic damage (group 1) & static hypothermic storage (group 2)

Kidneys were stored on ice in HOC in the perfusion laboratory for the cold storage time of 2 and 18 hours respectively.

Hypothermic machine perfusion (CP group)

Kidneys were placed in the Lifeport hypothermic preservation machine (Organ Recovery System) immediately after flushing and perfused with KPS-1 solution below 6°C for the entire storage period. A mean perfusion pressure of 30 mmHg was used throughout the 18 hours.

4.2.2 Phase II (Chapter 6): To investigate the effects of EPO on I/R injury

Porcine kidneys (n=6) were subjected to 10 min warm ischaemia and preserved after as follows:

Group1: 16hr Cold storage (CS) + 2hr Normothermic resuscitation preservation (NP)

Group 2: 16hr CS+ 2 hr NP (EPO)

Group 3: 18hr CS

Group 4: 18hrs CS (EPO during cold flush)

The manipulating agent human recombinant erythropoietin (EPO) (NeoRecorom, Roche) was used in a high dose of 5000 units [207]. This was given as a bolus in group 2 into the blood on reperfusion during NP and as bolus flush prior to CS as in group 4.

The kidneys were then reperfused for 3 hr, at pressure of 85 mmHg with autologous blood on the isolated organ preservation system (IOPS) so as to assess renal viability and function.

4.2.3 Phase III (Chapter 7): To assess a low dose of CORM-3 on I/R injury

Porcine kidneys (n=6) were subjected to 10min warm ischaemia and reperfused after 18hr Cold storage (CS) as follows:

Group 1: CORM-3 [tricarbonylchloro(glycinato)ruthenium(II)]

Group 2: iCORM-3 (inactive CO-releasing molecule)

Group 3: Control (no treatment)

The manipulating agent was used at a concentration of 50µmol(µm), based on a preliminary dose response study [50µm, 100µm, 200µm & 400µm of CORM-3 (n=4), Figure 17, 18, 19] and published literature [304]. This was diluted in 60 ml of normal saline and infused 5 minutes before into the circuit prior to organ being attached for reperfusion for a period of 1 hour at a rate of 50 ml/hour. The inactive parent molecule (iCORM-3) was prepared beforehand in 60ml of saline and left at room temperature for 18 hr to allow for complete liberation of its contained CO[305]; 50µm of iCORM was then administered in the same way.

The kidneys were then reperfused for 3 hr, at pressure of 85 mmHg with autologous blood on the isolated organ preservation system (IOPS) so as to assess renal viability and function.

The CORM-3 molecule was provided by Dr JM Rimoldi, Department of Medicinal Chemistry, University of Mississippi, Mississippi, USA.

4.2.4 Phase IV (Chapter 8): To investigate the beneficial effects of carbon monoxide (CO and CORM-3) and normothermic resuscitation perfusion (NP) on I/R injury

Porcine kidneys (n=6) were subjected to 10 minutes warm ischaemia and 16 hours of cold storage to simulate the clinical situation of the controlled NHBD. After this they were subjected to 2 hours NP as follows:

Group 1: Control, 16hr Cold storage (CS) + 2hr Normothermic perfusion (NP)

Group 2: CO gas (300ppm)

Group 3: CORM-3 [tricarbonylchloro(glycinato)ruthenium](II)

Group 4: iCORM-3 (Inactive CO-releasing molecule)

Solutions of the carbon monoxide releasing molecule were prepared immediately before perfusion, at a concentration of 50µmol (µm) CO-releasing molecule-3 [tricarbonylchloro(glycinato)ruthenium(II)] [288,304,352,389] in 60ml of normal saline at 4°C. Infusion of CORM-3 solution began 5 minutes before kidneys were connected to the IOPS and continued for the first hour of the NP. The inactive parent molecule (iCORM-3) was prepared beforehand in 60ml of saline and left at room temperature for 18 hr to allow for complete liberation of its contained CO[305]; 50µm of iCORM was then administered in the same way. CO gas (Airproducts, Walton on Thames, Surrey, UK) (300ppm)[251] was bubbled through the IOPS via the arterial line during 1st hour of NP.

The kidneys were then reperfused for 3 hr, at pressure of 85 mmHg with autologous blood on the isolated organ preservation system (IOPS) so as to assess renal viability and function.

4.3 Parameters

Renal blood flow (RBF) and arterial pressure (AP) were continuously monitored and the data downloaded on a laptop computer. Hourly serum and urine samples were used for biochemical assays and whole blood for haematology.

- Renovascular haemodynamics: Renal blood flow (RBF), arterial Pressure (AP) and intrarenal vascular resistance (IRR=AP/RBF)
- Renal function: Serum creatinine, urea, fractional excretion of sodium and creatinine clearance at 0,3 hours of reperfusion
- Haematology: Haematocrit, FBC, Hb, Platelets, WBC at 0,3 hours
- Blood gases, oxygen consumption (ml/min/g), bicarbonate and pH were measured during NP and at 0, 3 hours of reperfusion.
- Renal histopathology: haematoxylin/eosin stained sections of paraffin-embedded tissue.

  Wedge biopsies were taken at pre CS, post preservation and post reperfusion and then fixed in 4% formalin, dehydrated and embedded in paraffin wax. Sections were cut at 4 µM and then stained with haematoxylin and eosin for evaluation using the light microscopy. Sections were scored over 5 fields assessing changes in seven morphological parameters (tubular dilation, epithelial shedding, tubular debris, condensed tubular nuclei, RBC presence, intracytoplasmic vacuolation and glomerular shrinkage). Each parameter was thereafter scored using a following semi-quantitative scale: 0 none; 1 mild; 2 moderate; 3 severe.
- Serum aspartate aminotransferase (AST) (marker for tubular reperfusion injury)

- Adenosine diphosphate: adenosine triphosphate (ADP/ATP) ratio

  Renal ADP:ATP ratio was measured in pre and post-perfusion biopsies using an adenylate nucleotide ratio assay kit (Cambrex Bio Science, Berkshire, UK), with detection based upon the bioluminescent measurement of ATP using the enzyme luciferase. This enzyme catalyses the formation of light from ATP and was measured using a luminometer.

  Frozen sections of 10 µM thickness were cut in triplicate and placed in an eppendrof pre-cooled to -20ºC. Nucleotide releasing agent (80 µl) was added to the sections and the sample was vortexed and brought to room temperature. Then nucleotide monitoring reagent (20 µl) was added to the aliquot and the sample was placed in a Bio-Orbit luminnometer. An immediate voltage reading (A) on a 2210LKB Bromma chart recorder was noted. A second reading (B) was taken after 10 min. The sample was removed and ADP converting reagent (20 µl) was added and the sample placed back in the luminometer. A final reading (C) was taken after 5 min. A mean ratio was calculated by subtracting reading B from C and dividing by reading A for duplicate samples (C-B/A).
- Carboxyhaemoglobin levels: Measured on blood gases.
- Endothelial damage: Von Willebrand factor (vWF), a marker of endothelial injury was detected by Ag ELISA (Technoclone Ltd Dorking, Surrey, UK). Plasma samples were obtained by centrifuging venous blood (5ml) for 15 min at 2500g. This was frozen immediately in liquid nitrogen and stored at -20ºC. Prior to starting the test all components were brought to room temperature. The conjugate working solution was prepared by diluting 1 part of volume conjugate with 50 parts by volume of incubating buffer. The control plasma and sample plasma solutions were prepared by diluting 10 µl of either with 250 µl of

incubation buffer. The two were then added to the test wells and incubated at 37 ºC for 45 min. After which washing buffer solution (200 µl) was added, the wells covered with the test strips and further incubated at room temperature for 15 min. Then stopping solution (20 µl) was pippetted into the test wells and readings were measured using an ELISA reader (450 nm filter). The values of vWF: Ag was reported in U/ml.

4.4 Statistical analysis

Values are presented as mean ± SD. Continuous variables such as serum creatinine were plotted as level versus time curves for each kidney and the mean area under the curve was calculated using Microsoft® Excel Software (Reading, UK). These were then compared using the Kruskal-Wallis test with Dunn's post test (Graphpad Instat software package). Other groups were compared with the Mann-Whitney test and ANOVA using the same software package. A P value of ≤ 0.05 was considered significant.

CHAPTER 5

EXPERIMENTAL STUDY: NORMOTHERMIC RESUSCITATION PRESERVATION (NP) IN AN ISOLATED HAEMOPERFUSED PORCINE KIDNEY MODEL

5.0 Introduction

Organ transplantation has progressed largely due to advances in immunology but post operatively graft viability is also greatly influenced by procurement, handling and organ preservation [311]. The lack of organs for transplantation from traditional sources [1] has led to renewed interest in other alternatives such as non-heart beating donors (NHBD) [2]. Whilst the results of NHBD kidney transplantation are encouraging [3,4], there are continuing concerns about the relatively high rates of primary non- function (PNF) and delayed graft function (DGF). These complications arise as a consequence of the combination of an initial warm ischaemic (WI) injury [4-8] followed by cold ischaemia (CI) during organ storage and transport [312-315].

There continues to be a major controversy in the literature and clinical practice regarding the place of various preservation methods in NHBD transplantation. Hypothermic machine preservation which was considered more expensive and offered no significant advantage to CS [316] in the early 1990s now seems to be the method of choice in NHBD organ preservation as a number of studies show improved viability and functional integrity in the organ [317-321]. Other studies confirmed these findings but unfortunately in addition showed increased endothelial damage in cold machine liver perfusion [317,322]. Normothermic perfusion has the potential to provide metabolic support to warm ischaemically-damaged organs and to reverse some of the effects of CI. In addition normothermic preservation has the potential to be developed into an invaluable method for assessing the viability of organs prior to transplantation [311, 323-325]. The cumulative benefits of normothermic preservation techniques include lower levels

of PNF/DGF, extension of the safe preservation period and hence expansion of the donor pool.

A number of experimental studies using rabbit, canine and porcine kidneys have suggested that normothermic kidney resuscitation during organ preservation is more beneficial than the traditional hypothermic techniques [50,59,326-327]. Whilst WI and CI can be controlled in the experimental setting [59, 328-330], in the clinical situation, the logistics of organ retrieval and transfer necessitate a preliminary period of cold preservation averaging 16-18 hours [2].

5.1 Aims

The aim of this study was to determine the effects of a period of normothermic preservation in a model of controlled NHBD kidneys in comparison to hypothermic techniques. The model chosen involved isolated blood perfusion of porcine kidneys subjected to controlled periods of warm and cold ischaemia.

5.2 Methods

5.2.1 Retrieval and perfusion

Kidneys were retrieved from large white pigs and perfused as previously described in chapter 4.

5.2.2 Experimental design

The kidneys after retrieval were assigned to the following groups:

Group1 (n=6): 2 hr CS (cold storage) (minimal ischaemic damage)

Group2 (n=6): 18 hr CS (static hypothermic storage)

Group3 (n=6): 18 hr CP (cold perfusion) (hypothermic machine perfusion))

Group4 (n=6): 16 hr CS followed by 2 hr NP (normothermic resuscitation preservation)

They were then reperfused for 3 hr with autologous blood for assessment of renal viability and function.

5.3 Assessment

5.3.1 Functional and biochemical parameters

Renal blood flow (RBF) and intra renal resistance (IRR) were continuously measured throughout perfusion. Hourly serum and urine samples were used for biochemical assays and whole blood for haematology. Creatinine clearance [urinary creatinine (U_{cr}) ×urinary volume (V)/ plasma creatinine (P_{cr})] and fractional excretion of sodium [(U_t × U flow) / (GFR × P_t) ×100] were calculated and serum creatinine levels recorded hourly. Blood gases were measured for calculation of oxygen consumption in ml/min/g [(PO_2 in arterial blood - PO_2 in venous blood) x (flow rate / weight)] and acid-base homeostasis was measured.

Serum AST levels were also measured at pre-reperfusion and 3 hours post reperfusion in all groups.

5.3.2 Histology

Wedge biopsies were taken pre CS, post preservation and post reperfusion and histology was analysed.

5.3.3 Renal ADP:ATP ratio

Renal ADP:ATP ratio was measured in pre and post-perfusion biopsies using an adenylate nucleotide ratio assay kit (Cambrex Bio Science).

5.3.4 Endothelial damage

Von Willebrand factor (vWF), a marker of endothelial injury was detected by Ag ELISA (Technoclone Ltd Dorking, Surrey).

5.4 Statistical analysis

Values are presented as mean ± SD and analysis was carried out as outlined in chapter 4.

5.5 Results:

5.5.1 CP

The perfusion flow rates and resistance for each individual kidney are summarised in Table 9.

Table 9: Flow rates and resistances during CP (5.5.1)

	Flow rates (ml/min/100g)	**Resistances (mmHg/ml/min)**
1	60.6±27.1	46.4±31.5
2	61.1±20.4	38±20
3	47.1±23.9	65.1±52.3
4	60.6±27.1	46.43±1.5
5	65.8±23.6	42.7±36.6
6	78.8±21.7	32.5±18.6

5.5.2 Normothermic perfusion (NP)

Renal blood flow peaked during the first hour at a rate of 90 ± 29 ml/min/100g then declined to 76 ± 23 ml/min/100g during the second hour, this decrease probably representing the withdrawal of the vasodilator after the first hour. Intra renal resistance remained at a low level throughout perfusion (0.4 ± 0.2 mmHg/ml/min). Oxygen consumption reached a level of 40.6 ± 12.5 ml/min/g at 2 hours and the total mean urine output was 406 ± 200 ml.

5.5.3 3 Hour reperfusion period

Pre-perfusion haematocrit and haemoglobin levels ranged from between 0.17-0.20 l/l and 4.9-5.6 g/dl (P = 0.531, 0.535). Levels declined slightly in all groups after 3 hours reperfusion but were within a comparative range (0.16-0.18 l/l and 4.2-5.1 g/dl respectively; P = 0.629, 0.312).

Increasing CS from 2hr to 18hr reduced renal blood flow (RBF) (AUC 444 ± 57 vs. 325 ± 70; P<0.01), but this was restored by NP (563 ± 119; P=0.035 vs. 18hr CS) with no difference seen compared to CP (600 ± 319) (Figure 2). Intra renal vascular resistance (IRR) in groups 1 & 2 were higher at the start of reperfusion (1.1 ± 0.4, 1.39 ± 0.9) compared to group 3 & 4 (0.39 ± 1, 0.56 ± 0.21; P=0.002) and this trend persisted to the end of reperfusion which reflects the pattern of RBF. Overall levels fell with a significantly lower AUC in group 3 and 4 compared to group 2 (P=0.001, Table 10, Figure 3).

Figure 2: Renal blood flow over 3 hours of reperfusion with 2hrs CS (Group 1), 18hrs CS (Group 2), 18 hr CP (Group 3) and 16hrs CS + 2 hrs NP (Group 4)
All values are mean ± SD. **(5.5.3)**

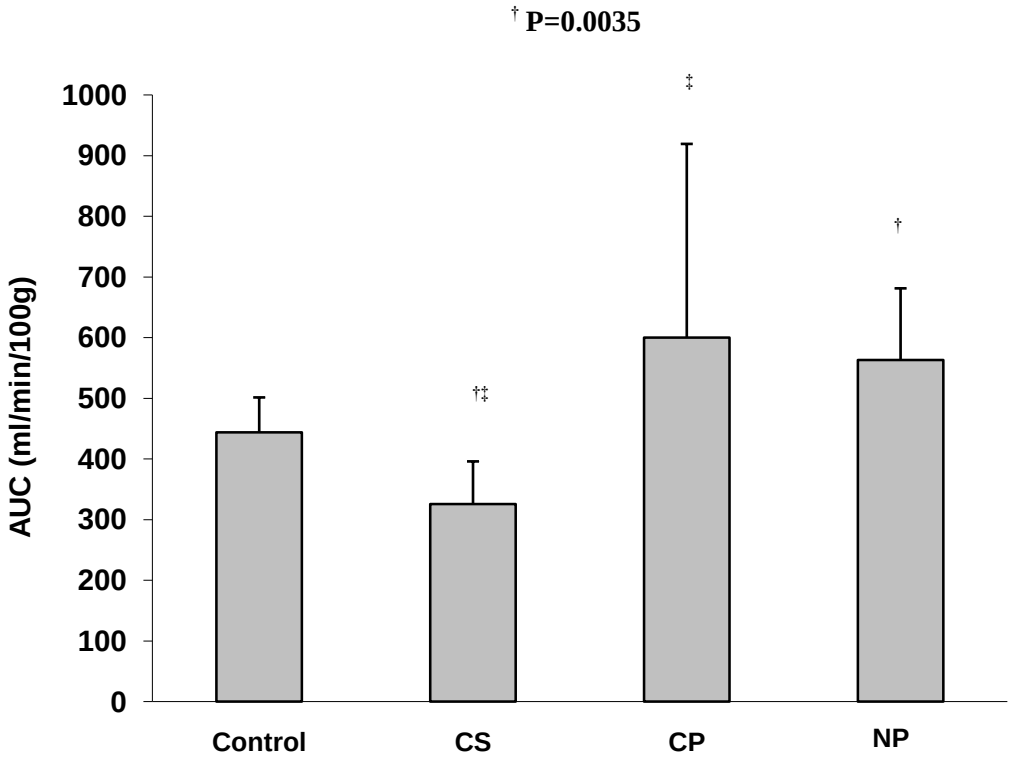


Figure 3: Intra renal resistance over 3 hours of reperfusion with 2hrs CS (Group 1), 18hrs CS (Group 2), 18 hr CP (Group 3) and 16hrs CS + 2 hrs NP (Group 4) All values are mean ± SD. **(5.5.3)**

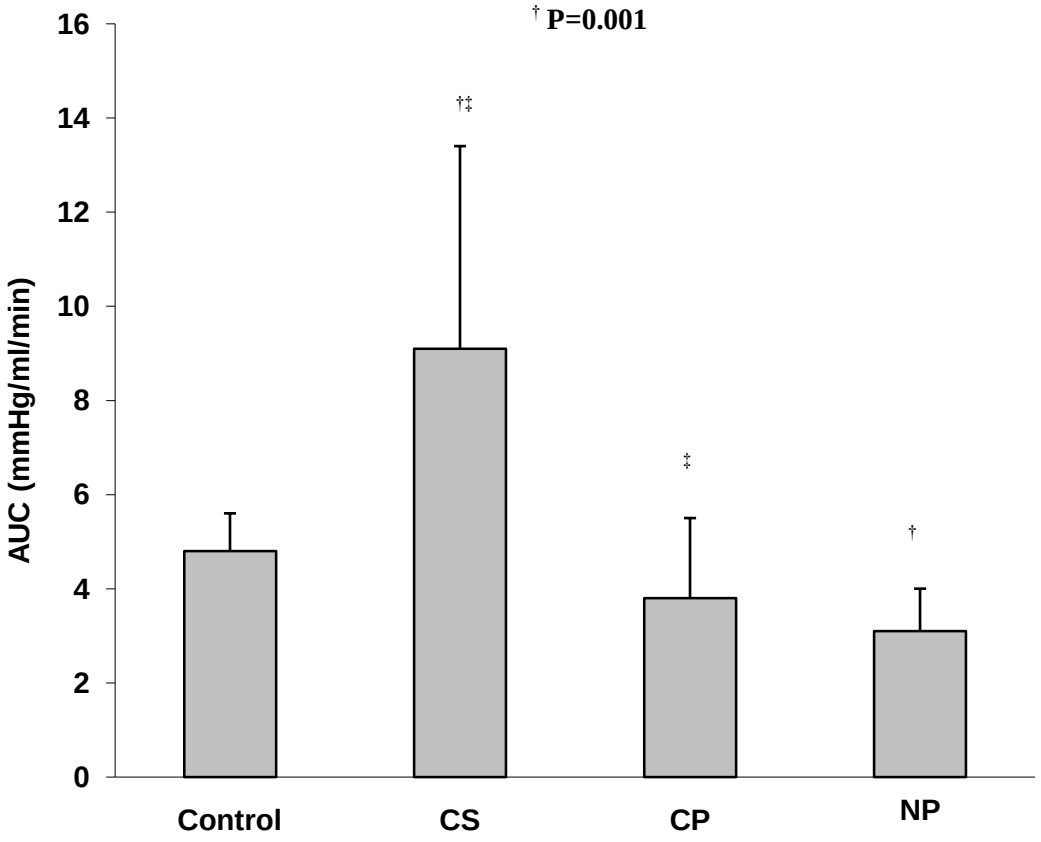


Table 10: Area under curve (AUC) for functional parameters: 3 hr reperfusion (5.5.3)

Parameter (AUC)	2hr CS	18hr CS	CP	NP	P
Serum Creatinine (µmol/L)	1102 ± 260*	2156 ± 401*	1354 ± 300	1756 ± 280	0.013
Creatinine Clearance	36.2 ± 22*¶ Ψ	2.2 ± 1.7 *	9.8 ± 7.3¶	3.8 ± 4.1Ψ	0.0012
RBF (ml/min/100g)hr	444 ± 57	325 ± 70†‡	600 ± 319‡	563 ± 119†	0.0035
IRR (mmHg/min)hr	4.8 ± 0.8	9.1 ± 4.3†‡	3.8 ± 21.7‡	3.1 ± 0.9†	0.001
Serum AST (IU/L)	129 ± 16Ψ	239 ± 70	168 ± 38	596 ± 98Ψ	0.0011
Fractional excretion Na^+ (%)	12.6 ± 5.4*	117 ± 38 *†‡	25 ± 28‡	45 ±39†	0.001

* P <0.05 versus 2hr CS and 18hr CS
¶ P <0.05 versus 2hr CS and CP
Ψ P <0.05 versus 2hr CS and NP
† P<0.05 versus NP and 18hr CS
‡ P<0.05 versus CP and 18hr CS

5.5.4 Renal function

On reperfusion serum creatinine levels fell in all groups over 3 hr. A significant fall was demonstrated in groups 1,3 and 4 vs. group 2 at 3 hr (% serum creatinine fall 92 ± 6, 79 ± 9 and 64 ± 17 vs. 44 ± 13% respectively, P=0.001). AUC creatinine was significantly lower in group 1 compared to group 2 (1102 ± 260 vs. 2156 ± 401 respectively; P = 0.013) but comparable to group 3 & 4 (1354 ± 300 and 1756 ± 280; Figure 4) (Table 10).

Creatinine clearance was significantly better in group 1 throughout the reperfusion period peaking at 2 hours to a level of 20.4 ± 11.6 ml/min/100g compared to levels of 0.9 ± 7.4, 2.5 ± 1.7 and 1.8 ± 1.9 ml/min/100g in groups 2, 3 and 4 respectively (p = 0.0015; Figure 5); (Table 10).

Figure 4: **Serum creatinine** over 3 hours of reperfusion with 2hrs CS (Group 1), 18hrs CS (Group 2), 18 hr CP (Group 3) and 16hrs CS + 2 hrs NP (Group 4) All values are mean ± SD. **(5.5.4)**

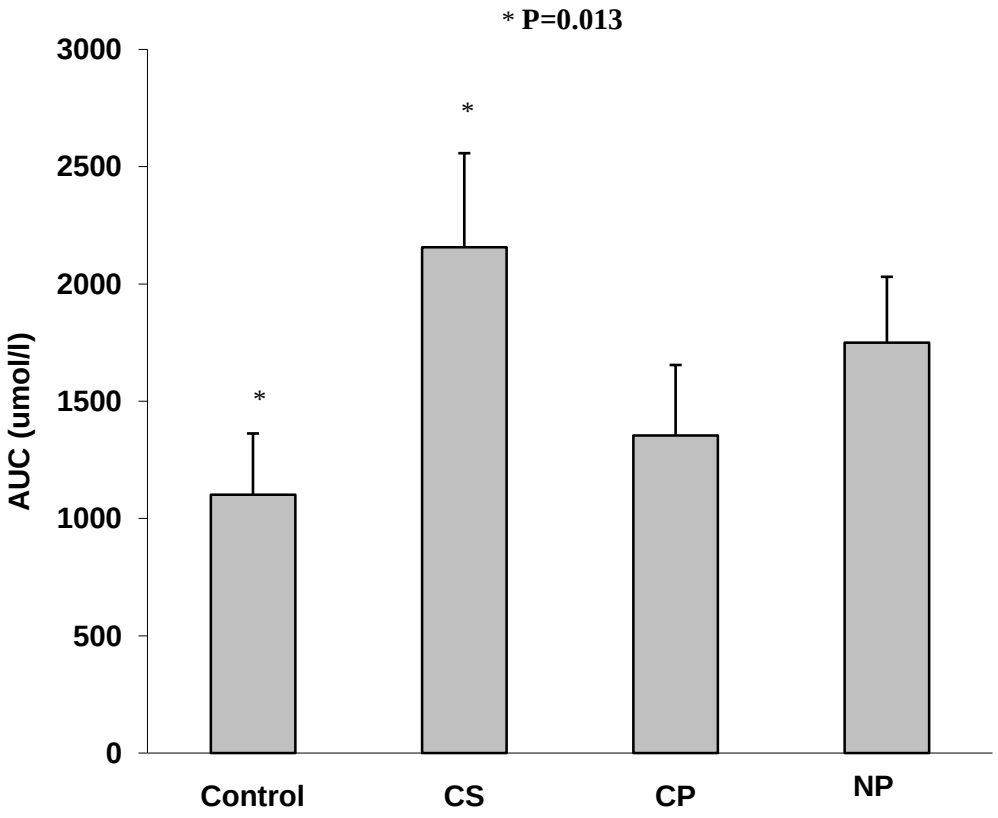
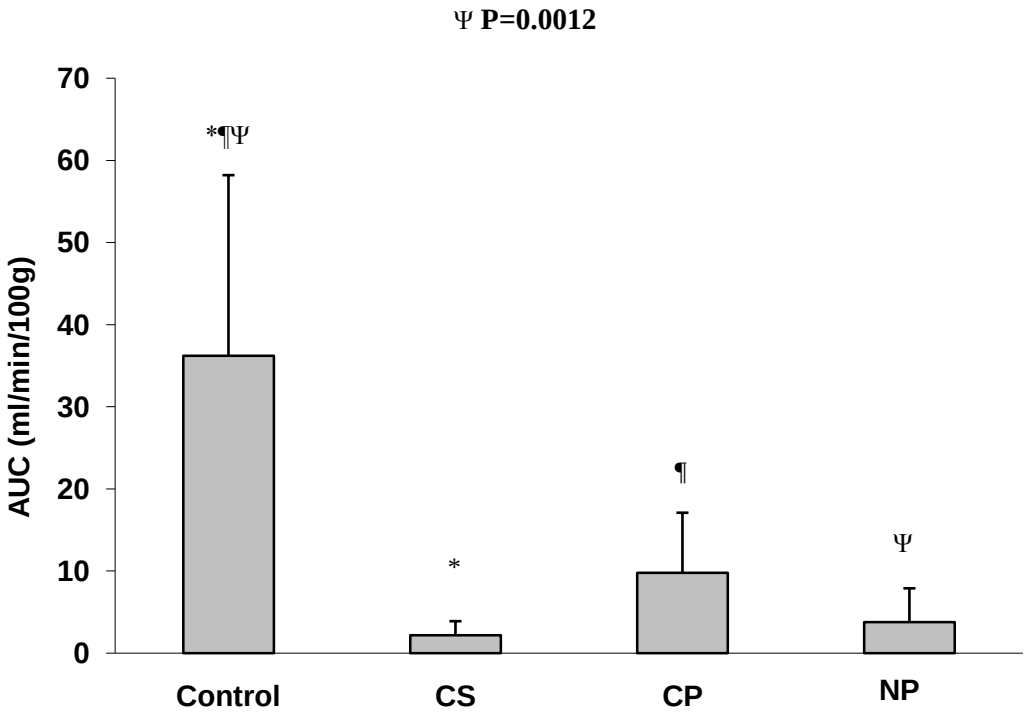


Figure 5: Creatinine clearance over 3 hours of reperfusion with 2hrs CS (Group 1), 18hrs CS (Group 2), 18 hr CP (Group 3) and 16hrs CS + 2 hrs NP (Group 4) All values are mean ± SD. **(5.5.4)**



Oxygen consumption was significantly higher in group 4 to other groups (49 ± 10.4 ml/min/g compared to 31 ± 6.2, 29.2 ± 7.6 and 44.2 ± 27.9, groups 1,2 & 3 respectively; P = 0.049) after the first hour of reperfusion. At the end of 3 hours, oxygen consumption had fallen in groups 2, 3 and 4 (23.7 ± 11.9, 35.1 ± 18.9 & 40 ± 9.4, respectively) but increased in group 1 (40 ± 9.4), levels comparable to group 4 (P = 0.177).

All kidneys maintained near normal acid base homeostasis, although there was an increase in pH and bicarbonate levels in group 4 at 3 hours reperfusion compared to groups 2 and 3 (P = 0.026, 0.008 respectively; Table 11).

Renal tubular function was significantly better in group 1 and 3 than in group 2 throughout reperfusion with lower levels of fractional excretion of sodium (P = 0.001). Though group 4 demonstrated a lower excretion level compared to group 2 (P = 0.009) these excretion levels were comparable to group 3 (P = 0.339); (Table 10, Figure 6).

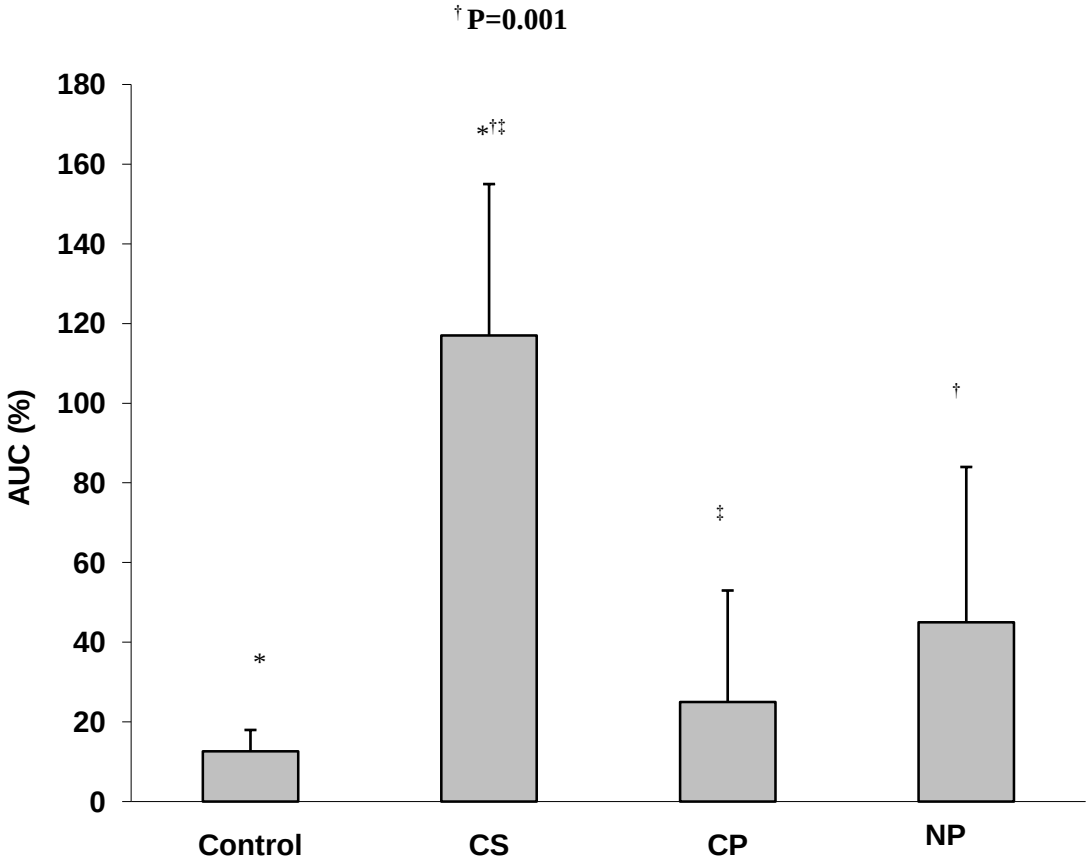
Total urine output was similar in both groups 2 and 4 (302 ± 210, 271 ± 171 ml) significantly lower than group 1 (723 ± 219 ml; P = 0.009). While the total urine output of group 3 (463 ± 176) was not quite significant compared to group 4 (P=0.0845).

Table 11: Acid base homeostasis, Pre and 3 hour reperfusion (mean ± SD) (5.5.4)

	Group 1		Group 2		Group 3		Group 4	
Parameter	**Pre**	**3hr**	**Pre**	**3hr**	**Pre**	**3hrs**	**Pre**	**3hrs**
pH	7.46 ± 0.1	7.4 ± 0.1	7.51 ± 0.1	7.37 ± 0.1	7.42 ± 0.1	7.33 ± 0.1¶	7.48 ± 0.1	7.51 ± 0.1¶
Bicarbonate (mmol/L)	23.3 ± 1.1	23.1 ± 4.9*	23.9 ± 2.0	21.6 ± 3.7 Ψ	20.52 ± 1.1	22.02 ± 6.7¶	24.3 ± 4.3	32.7 ± 3.8*Ψ¶
Potassium (mmol/L)	5.0 ± 0.6	4.7 ± 0.7	5.5 ± 0.1	10 ± 0 Ψ	5.62 ± 1.2	5.1 ± 0.23	5.5 ± 0.2	6.3 ± 2.6 Ψ

* P <0.05 Group 4 versus Group 1
Ψ P <0.05 Group 4 versus Group 2
¶ P <0.05 Group 4 versus Group

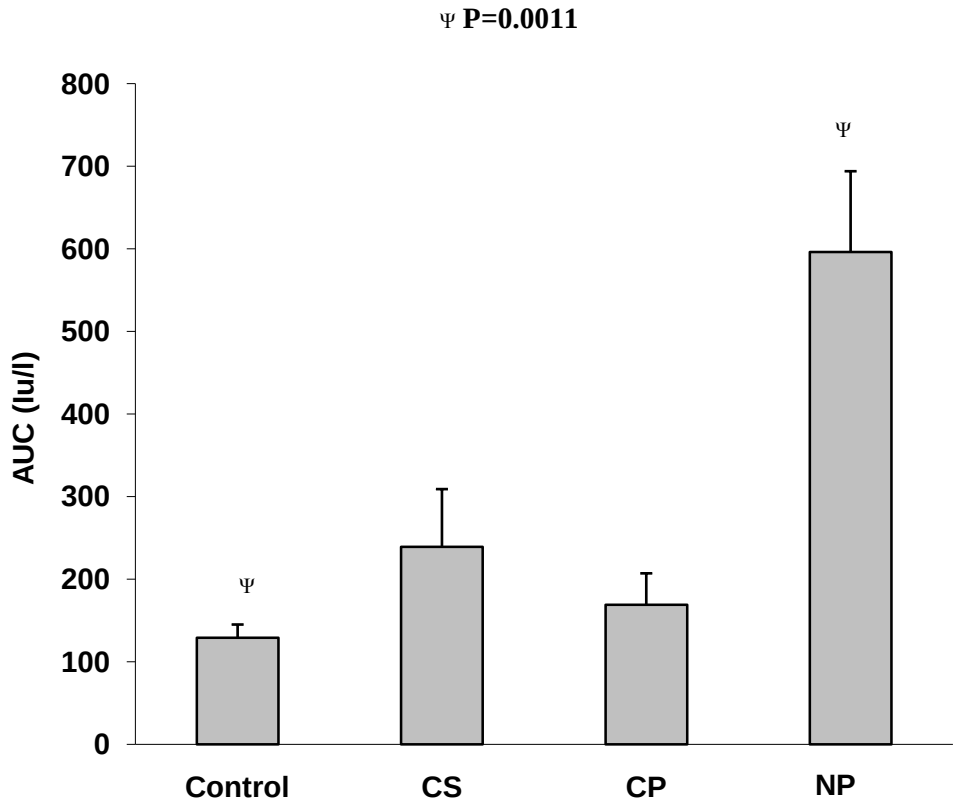
Figure 6: Fractional excretion of sodium over 3 hours of reperfusion with 2hrs CS (Group 1), 18hrs CS (Group 2), 18 hr CP (Group 3) and 16hrs CS + 2 hrs NP (Group 4)
All values are mean ± SD. **(5.5.4)**



5.5.5 Serum AST

The serum AST levels were significantly higher for group 4 versus all the other groups (P= 0.001, Table 10, Figure 7)

Figure 7: Serum AST over 3 hours of reperfusion with 2hrs CS (Group 1), 18hrs CS (Group 2), 18 hr CP (Group 3) and 16hrs CS + 2 hrs NP (Group 4)
All values are mean ± SD. **(5.5.5)**



5.5.6 Histology

An increase in cytoplasmic vacuolation was found in group 4 compared to groups 1, 2 and 3 (1.5 ± 0.5 vs. 0.1 ± 0.1 vs. 0.3 ± 0.6, 0.8 ± 0.8 respectively; $P<0.05$) (Picture 2). Comparisons of all other parameters (tubular dilatation, epithelial flattening, epithelial shedding, tubular debris, condensed tubular nuclei, RBC presence and glomerular shrinkage) showed no statistical significance (data not shown).

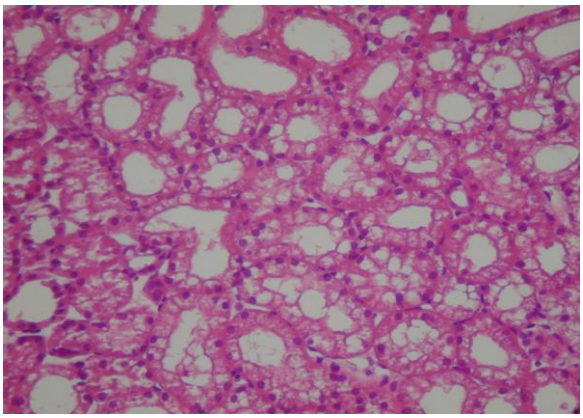
5.5.7 Renal ADP: ATP ratio (Figure 8)

Following 2hr of NP in group 4 reduced the ADP:ATP ratio to a significantly lower level than the pre-perfusion values for groups 1, 2 and 3 ($P=0.046$). Reperfusion during NP did not reduce the already low ADP:ATP ratio any further. ADP:ATP ratio levels after 3 hr reperfusion showed no difference between all four groups ($P=0.0882$).

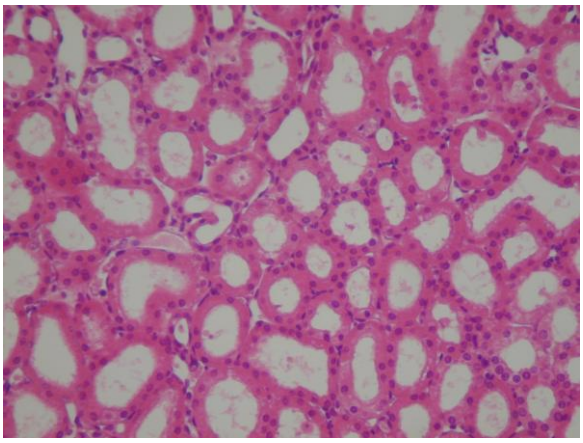
5.5.8 Endothelial damage (Figure 9)

Von Willebrand factor (vWF), a marker of endothelial injury showed no difference in the pre-perfusion values versus the reperfusion values in all the groups ($P=0.1263$).

Picture 3: Histology demonstrating intracytoplasmic vacuolation (5.5.6)



1. Post NP



2. Post 18 CS

Figure 8: ADP-ATP Ratio

Group 1: 2h CS, Group 2: 18h CS, Group 3: CP, Group 4: NP
All values are mean ± SD. **(5.5.5)**

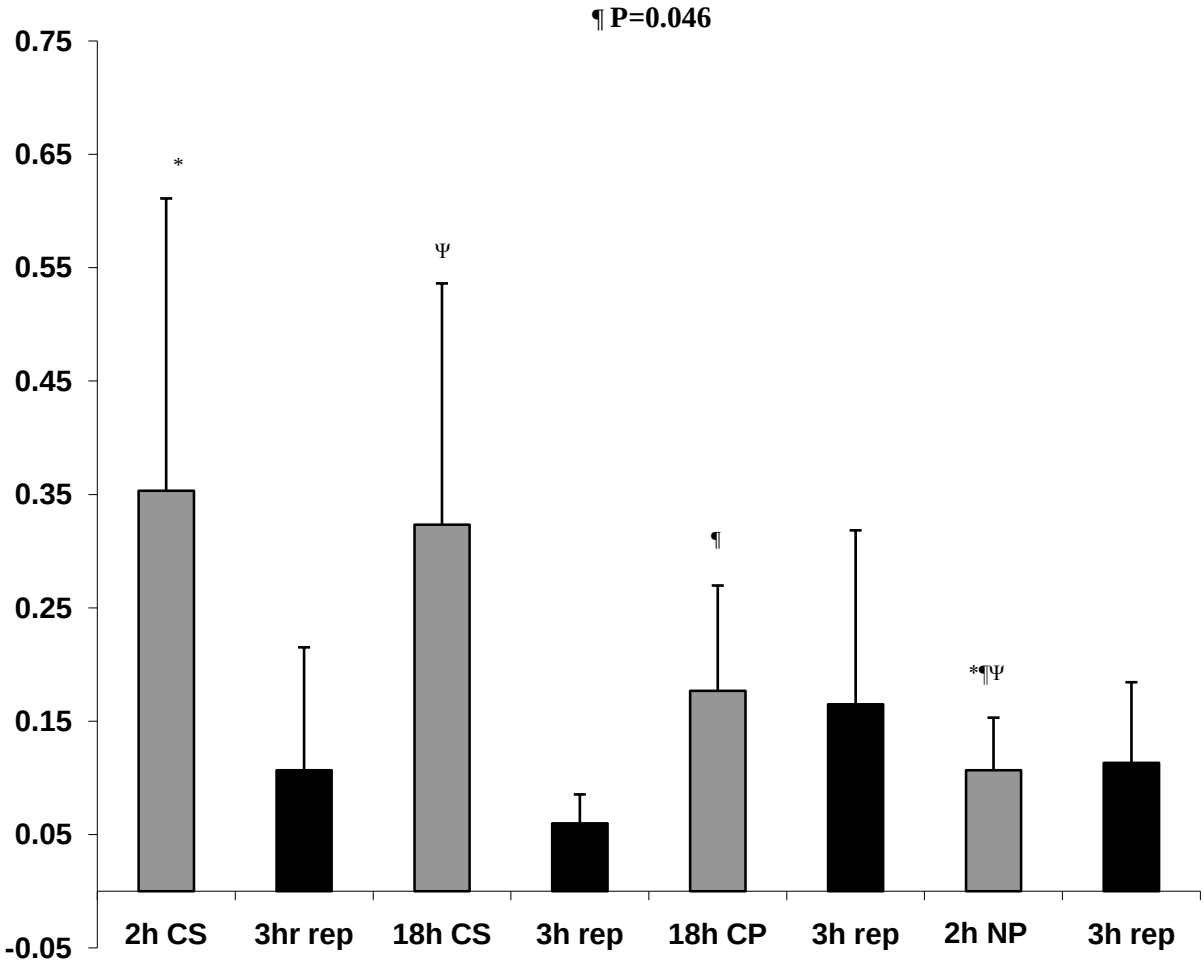
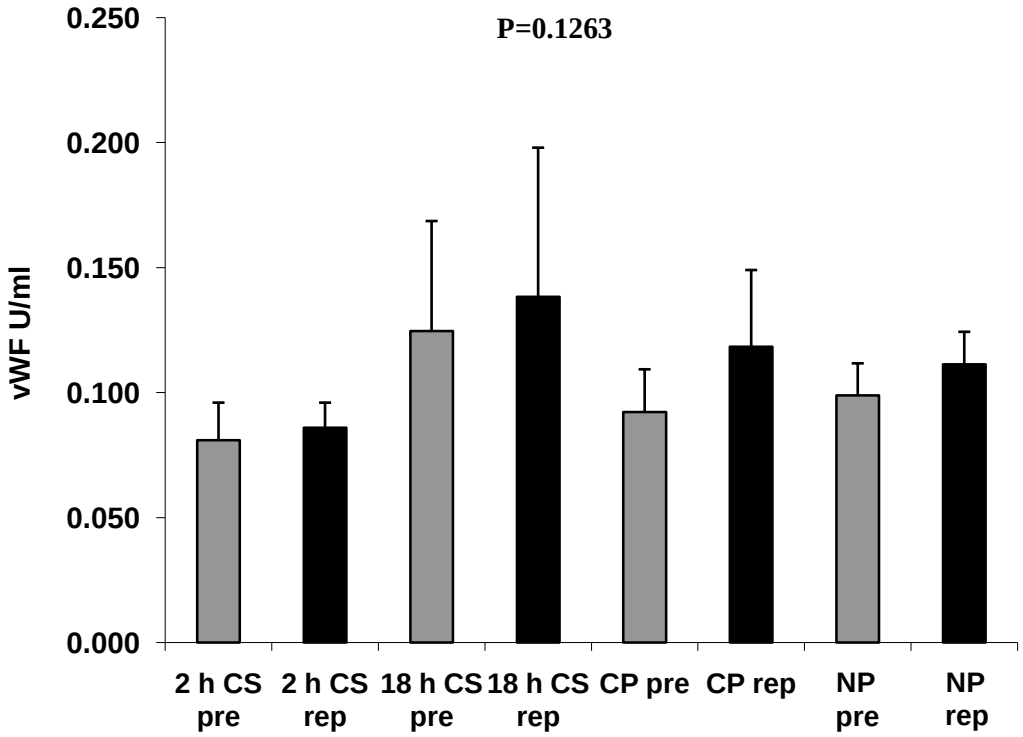


Figure 9: Pre-perfusion and reperfusion levels of vWF
Group 1: 2h CS, Group 2: 18h CS, Group 3: CP, Group 4: NP
All values are mean ± SD. **(5.5.8)**



5.6 Discussion

This study shows that a period of 2 hours normothermic perfusion with autologous blood improves the preservation of porcine kidneys stored for a total period of 18 hours. Kidneys undergoing normothermic resuscitation preservation (NP) demonstrated reperfusion haemodynamics that were equivalent to kidneys undergoing only 2 hours of cold storage. Renal tubular function was also improved, showing that NP was able to reverse effects of a period of cold ischaemia. The protocol used included an initial period of 10 minutes warm ischaemic injury in order to provide a model of controlled NHBD kidneys. This model also demonstrates that a short period of NP adequately resuscitates the organ compared to hypothermic perfusion preservation for 18 hours as seen by a number of various parameters assessed, perhaps superior considering parameters such as oxygen consumption and acid base balance.

The detrimental combination of warm (WI) and cold ischaemic (CI) damage represents a key obstacle for expanding the non heart-beating organ donor pool. Kidneys generally tolerate a period of warm ischaemia but they show markedly reduced viability with a combination of hypothermia and warm ischaemia [54,331].

Historically organs were preserved using hypothermic techniques, which have beneficial effects by diminishing oxygen demand and overall metabolic rate of the organ [59]. Cold preservation also has detrimental effects including altered tissue integrity and a predisposition to subsequent reperfusion injury [315,332,333]. A prolonged duration of CI directly correlates with the severity of the inflammatory process after reperfusion and the associated inhibition of cellular metabolism eliminates the possibility of a

substantial reparative process that could occur with normothermic preservation methods. Hypothermic perfusion preservation in literature portrays a mixed picture where studies show no benefit compared to CS [316,334] to improved viability and functional integrity [317-319, 335]. Lee *et. al.* confirmed most of these findings though showed an increase in endothelial damage during cold machine liver perfusion [322].

Mayfield *et. al.* demonstrated an improvement in the capacity of a normothermic blood perfused kidney to control tissue oedema and ion permeability, suggesting better membrane integrity than kidneys perfused hypothermically[336]. Brasile *et. al.* demonstrated that warm reperfusion using an acellular solution not only prevents further injury but also reverses injury caused during warm ischaemia. This porcine kidney transplantation model showed that kidneys subjected to 120 minutes of warm ischaemia did not function when transplanted immediately without preservation or after cold preservation, but did so after normothermic reperfusion [59]. Schon *et. al.* demonstrated that with *ex-vivo* perfused pig livers, normothermic oxygenated perfusion could restore the metabolic process [337]. Other studies in kidney and liver transplantation have demonstrated the utility of normothermic recirculation to recover adenine nucleotide levels after warm ischaemia [338-342], to restore reduced glutathione levels [339] and to improve post transplant viability [338,341-344].

Net *et. al.* [345] demonstrated the beneficial effects of normothermic recirculation in a model of liver transplantation from NHBD pigs which appeared to have a similar effect to ischaemic preconditioning, the mechanism involving adenosine and reduction of level of hepatic xanthine [345-348].

To date it has thus been clearly demonstrated that normothermic preservation has a substantial beneficial role to play in the preservation of organs subjected to warm ischaemia. However, in the clinical setting the logistics of organ retrieval, transfer and crossmatching necessitate a substantial period of cold storage and there is less information relating to the combination of warm plus cold ischaemia seen in NHBD kidneys. Reddy *et. al.*[328] in a liver model though demonstrated that even short periods of cold ischaemia can abrogate the benefits of normothermic preservation of ischaemically damaged livers and failed to resuscitate livers subjected to 60 minutes WI and 4 hours of CS. Our study used a more clinically relevant combination of 10 minutes initial warm ischaemia followed by 16 hours cold storage, prior to NP.

There are other advantages of the NP model described here. The porcine kidney is recognised to have anatomical and physiological characteristics that closely resemble the human situation; hence the data is more representative than findings derived from small animal models [349,350]. The pig kidneys used were of a similar size to the adult human kidney as they were taken from animals weighing 60-70 kilograms. This model is reliable, reproducible and makes use of modern cardio-pulmonary bypass technology whereby continuous measurement of physiological parameters is possible. It is a versatile method for investigating the pathophysiology of warm or cold ischaemia and subsequent renal perfusion injury. This model will permit evaluation of different interventions to ameliorate ischaemic reperfusion injury and may also prove to be the basis of pre transplant viability testing for kidneys from marginal donors, thus helping to safely increase the donor pool.

This ex-vivo normothermic perfusion system used a relatively short reperfusion period of 3 hours. Whilst longer haemo-perfusion periods have been described in the liver [330], the necessity for prolonged perfusion was not required to study the acute affects of reperfusion injury in this model. Creatinine clearance was used as an estimate of GFR in this model and it would be possible to measure this even more accurately by inulin clearance; this is however, a more complex method. The warm and cold ischaemic insults were relatively limited and could be extended as a model of uncontrolled NHBD kidneys. The raised levels of serum AST during NP could be related to the kidney being reperfused twice. Considering the various histological parameters, an increase in cytoplasmic vacuolation remains unanswered but to note histological parameters do not always correlate with functional parameters in kidney transplantation. Organ preservation by cold storage has the advantage of being a simple method and clearly, the technology and trained perfusion staff required for warm preservation renders it far more complicated and expensive.

5.7 Conclusion

This study suggests that a short period of normothermic resuscitation perfusion with autologous blood can reverse some of the adverse effects of a period of cold storage in kidneys that have sustained collateral warm ischaemic injury; in addition it can adequately resuscitate kidneys compared to 18 hours cold perfusion. Normothermic resuscitation preservation therefore has potential application in the field of NHBD kidney transplantation, which continues to be an expanding source of transplant kidneys.

CHAPTER 6

EXPERIMENTAL STUDY: EFFECTS OF ERYTHROPOIETIN (EPO) ON ISCHAEMIA/REPERFUSION (I/R) INJURY IN A CONTROLLED NON-HEART BEATING DONOR (NHBD) PORCINE KIDNEY MODEL

6.0 Introduction:

Kidney transplantation remains the best modality of the treatment of end stage renal failure (ESRF) [351]. Due to lack of suitable organs for transplantation from traditional sources [1], renewed interest into other alternatives such as live donors and non-heart beating donors (NHBD) are on the increase [2]. Though the results are encouraging, delayed graft function (DGF) and primary non function (PNF) are a significant problem as a combined consequence of prolonged warm ischaemia (WI) insults [4-8] and cold ischaemic insults from transport and storage [312-315], hence making ongoing research and development important. This insult is aggravated after reperfusion as a result of a complex mechanism, ischaemia/reperfusion (I/R) injury [60,352,353] subjecting the patient to a major cause of morbidity during transplantation [354-355].

Erythropoietin (EPO) is a major regulator of proliferation and differentiation of erythroid progenitor cells through its anti-apoptotic actions up-regulated by hypoxia [356-357] and has also been shown to have a direct action on proximal renal tubular epithelial cells against I/R injury [82]. In addition *in vivo* evidence from rodent models and *in vitro* evidence in human cell lines confirm its protective actions against ischaemic renal damage [204,206,358], brain[358], heart [359-361], liver [354] and bowel [362]. The erythropoietin receptor is known to be expressed not only in the human and rodent renal tubular cell, but also in porcine renal tubular cells [204,363-364]. Current literature review reveals only one porcine model [363] that confirms the potential application of erythropoietin though restricted to warm ischaemia, which clearly address the benefits of a porcine model over a rodent model [363,365-367].

6.1 Aim

The aim of the study was to determine the effects of high dose of erythropoietin (EPO) administered as a bolus in a model of a controlled NHBD porcine kidney.

6.2 Methods:

6.2.1 Retrieval and perfusion

Kidneys were retrieved from large white pigs and haemoperfused as previously described in chapter 4.

6.2.2 Experimental design

Porcine kidneys (n=6) were subjected to 10 min warm ischaemia and preserved after as follows:

Group1: 16hr Cold storage (CS) + 2hr Normothermic resuscitation preservation [368] (NP)

Group 2: 16hr CS+ 2 hr NP (EPO)

Group 3: 18hr CS

Group 4: 18hrs CS (EPO during cold flush)

The kidneys were then reperfused for 3 hr, at pressure of 85 mmHg with autologous blood on the isolated organ preservation system (IOPS) [366-368] so as to assess renal viability and function. The manipulating agent human recombinant erythropoietin

(EPO) was used in a high dose of 5000 units[207]. This was given as a bolus in group 2 into the blood on reperfusion during NP and as bolus flush prior to CS as in group 4.

6.3 Assessment

6.3.1 Functional and biochemical parameters

Hourly serum and urine samples were used for biochemical assays and whole blood for haematology. Creatinine clearance [urinary creatinine (U_{cr}) ×urinary volume (V)/ plasma creatinine (P_{cr})] and fractional excretion of sodium [(U_t × U flow) / (GFR × P_t) ×100] were calculated and serum creatinine levels recorded hourly.

Blood gases were measured for calculation of oxygen consumption in ml/min/g [(PO_2 in arterial blood - PO_2 in venous blood) x (flow rate / weight)], acid-base homeostasis. RBF and IRR were continuously recorded.

Serum AST levels were also measured at pre-reperfusion and 3 hours post reperfusion in all groups.

6.3.2 Histology

Wedge biopsies were taken pre CS, post preservation and post reperfusion and histology analysed as described in chapter 4.

6.4 Statistical Analysis

Values are presented as mean ± SD and analysis was carried out as outlined in chapter 4.

6.5 Results:

6.5.1 Functional Parameters

Pre-perfusion and post reperfusion haematocrit and haemoglobin levels ranged from between 0.17-0.20 l/l and 4.4-5.6 g/dl, though Levels declined slightly in all groups after 3 hours reperfusion but were within a comparable range (P = 0.575, 0.067 respectively).

Renal blood flow improved in groups 1 & 2 vs. group 3 & 4 though no difference was noted between groups 3 & 4 (563±119 vs. 491±95 vs. 325±70 vs. 418±112, groups 1, 2, 3 & 4 respectively; P=0.012) (Table 12, Figure 10).

Similarly the intra-renal resistance (IRR) was significantly lower in group 1 & 2 vs. group 3 & 4 (3.1±0.86 vs.4.1±1.09 vs. 9.12±4.3 vs. 7.3±1.95, groups 1, 2, 3 & 4 respectively; P=0.012) (Table 12, Figure 11).

Table 12: Area under curve (AUC) for functional parameters: 3 hr reperfusion (6.5.1)

Parameter (AUC)	Group 1	Group 2	Group 3	Group 4	P
Serum Creatinine (µmol/L)	1756 ± 280	1726 ± 244	2156 ± 401	1849 ± 184	NS
Creatinine Clearance	3.8 ± 4.1	3.4 ± 2	2.2 ± 1.7	3.2 ± 0.98	NS
RBF (ml/min/100g)hr	563 ± 119*	491 ± 95 ¶	325 ± 70*¶	418 ± 112*¶	0.012
IRR (mmHg/min)hr	3.1 ± 0.86*	4.1 ± 1.0¶9	9.12 ± 4.3*¶	7.3 ± 1.95*¶	0.012
O_2 consumption/3hrs	39 ± 10	46 ± 10¶	24 ± 12 ¶	24 ± 7¶	0.037
Serum AST (IU/L)	596 ± 98*	724 ± 359¶	239 ± 70*¶	200 ± 32 *¶	0.001
Fractional excretion Na^+ (%)	45 ± 39*	46 ± 23¶	117 ± 38*¶	107 ±34 *¶	0.002

* P <0.05 between groups 1 and 3, 4
¶ P <0.01 between groups 2 and 3, 4

Figure 10: Renal blood flow over 3 hours of reperfusion with 16hrs CS +2hrs NP (Group 1), 16hrs CS +2 hrs NP with EPO (Group 2), 18 hr CS (Group 3) and 18hrs CS with EPO (Group 4)
(6.5.1)

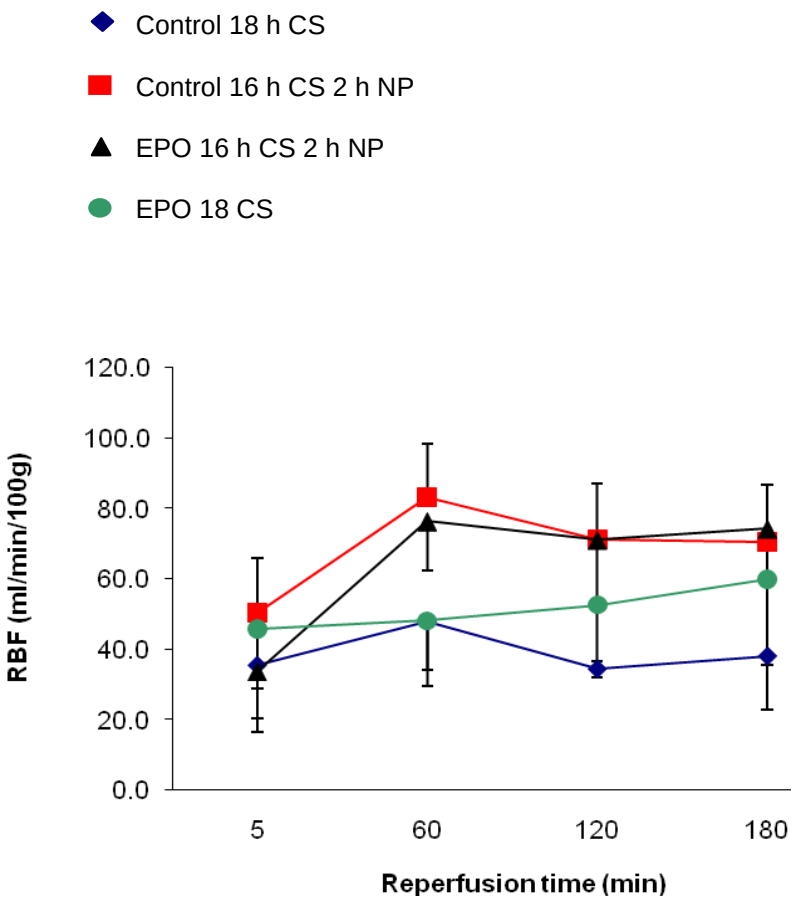
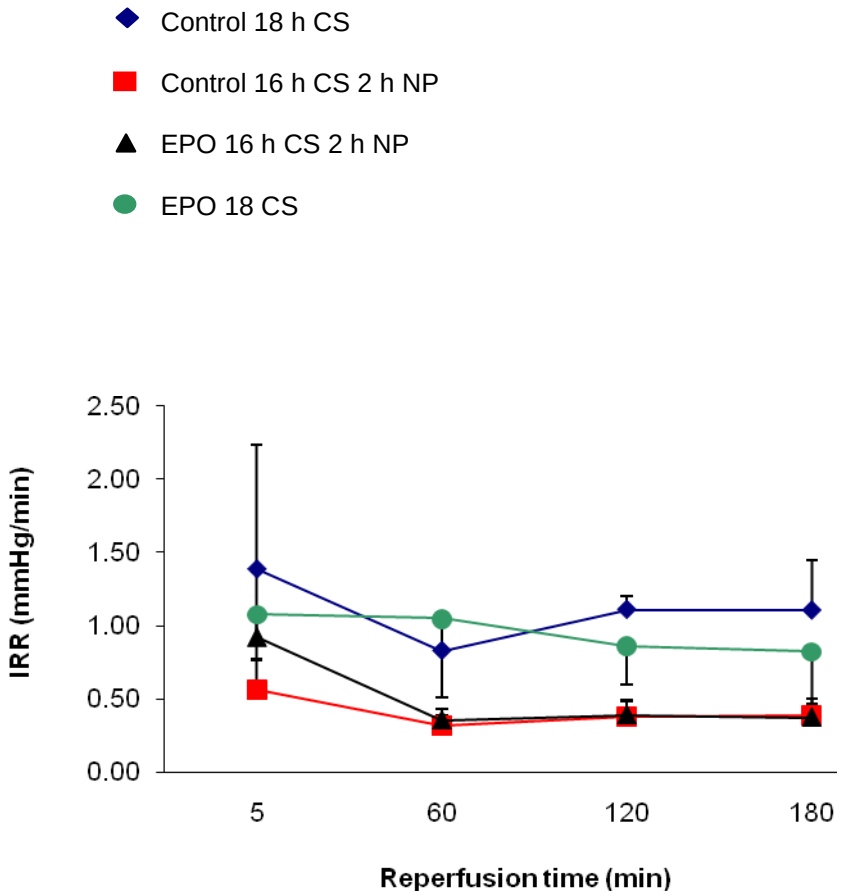


Figure 11: Intra renal resistance over 3 hours of reperfusion with 16hrs CS +2hrs NP (Group 1), 16hrs CS +2 hrs NP with EPO (Group 2), 18 hr CS (Group 3) and 18hrs CS with EPO (Group 4)
(6.5.1)



6.5.2 Renal function

The total urine output showed no difference between groups (271±172 vs. 359±184 vs. 302±211 vs. 421±88; group 1, 2, 3 & 4 respectively; P=0.576). Percentage of serum creatinine fall at 3 hrs was significantly better in groups 1 & 2 vs. group 3, though no difference was seen between groups 1, 2 & 4, (64±17 vs. 60±11 vs. 44±13 vs. 52±8; p= 0.04).

The area under curve (AUC) of creatinine (1756±280 vs. 1726±244 vs. 2156±401 vs. 1849±184; group 1, 2, 3 & 4 respectively) (Table 12, Figure 12) and creatinine clearance (3.8±4.1 vs. 3.4±2 vs. 2.2±1.7 vs. 3.2±0.98; group 1, 2, 3 & 4 respectively) (Table 12, Figure 13) showed no difference among all four groups.

Figure 12: Serum creatinine over 3 hours of reperfusion with 16hrs CS +2hrs NP (Group 1), 16hrs CS +2 hrs NP with EPO (Group 2), 18 hr CS (Group 3) and 18hrs CS with EPO (Group 4)
(6.5.2)

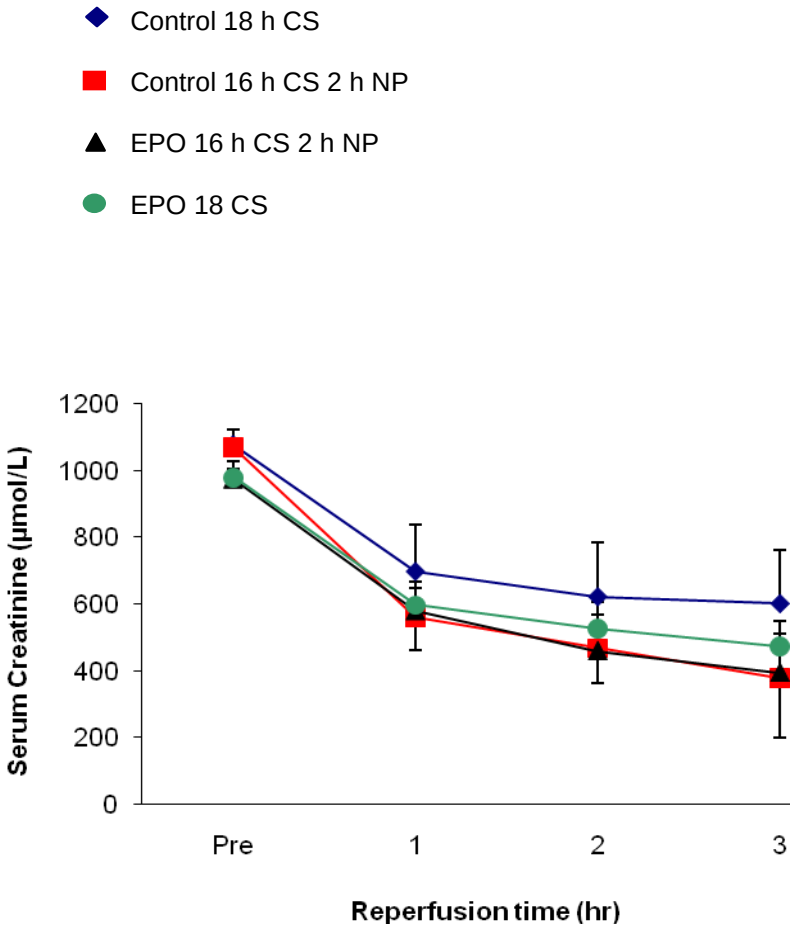
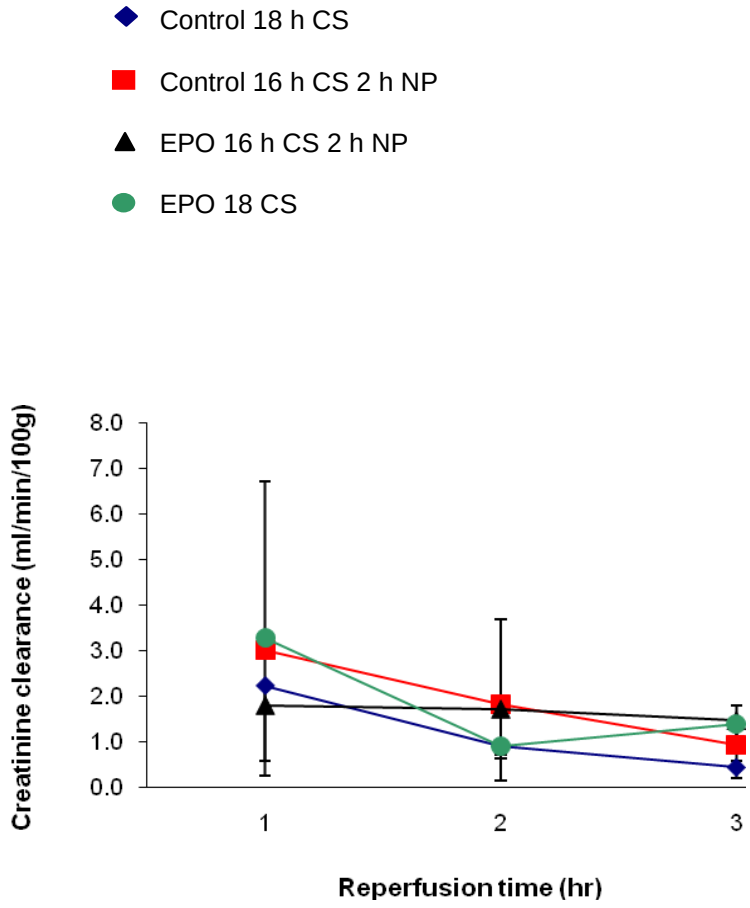


Figure 13: Creatinine clearance over 3 hours of reperfusion with 16hrs CS +2hrs NP (Group 1), 16hrs CS +2 hrs NP with EPO (Group 2), 18 hr CS (Group 3) and 18hrs CS with EPO (Group 4)
(6.5.2)



The fractional excretion of sodium was significantly lower for group 1 & 2 vs. group 3 & 4 (45±39 vs. 46±23 vs. 117±38 vs. 107±34 respectively; p=0.002) (Table 12, Figure 14) suggesting better renal tubular function in these groups.

There was a significant improvement in oxygen consumption in groups 2 vs. group 3 (P=0.037) and a marginal significance between groups 1 vs. group 3 (39±10 vs. 46±10 vs. 24±12 vs. 24±7, groups 1, 2, 3 & 4 respectively) (Table 12).

Kidneys in group 1 & 2 maintained their acid base balance though group 3 kidneys became severely acidotic (P=0.025) following 3 hrs reperfusion, in contrast to group 4 kidneys which showed a trend towards acidosis (P=0.0812) (Table 13). Similarly the post perfusion bicarbonate levels were significantly better for group 1 (P<0.001) & 2 (P<0.05) vs. group 3 & 4 (Table 13).

All these parameters showed no difference between group 1 and 2.

Figure 14: Fractional excretion of sodium over 3 hours of reperfusion with 16hrs CS +2hrs NP (Group 1), 16hrs CS +2 hrs NP with EPO (Group 2), 18 hr CS (Group 3) and 18hrs CS with EPO (Group 4) **(6.5.2)**

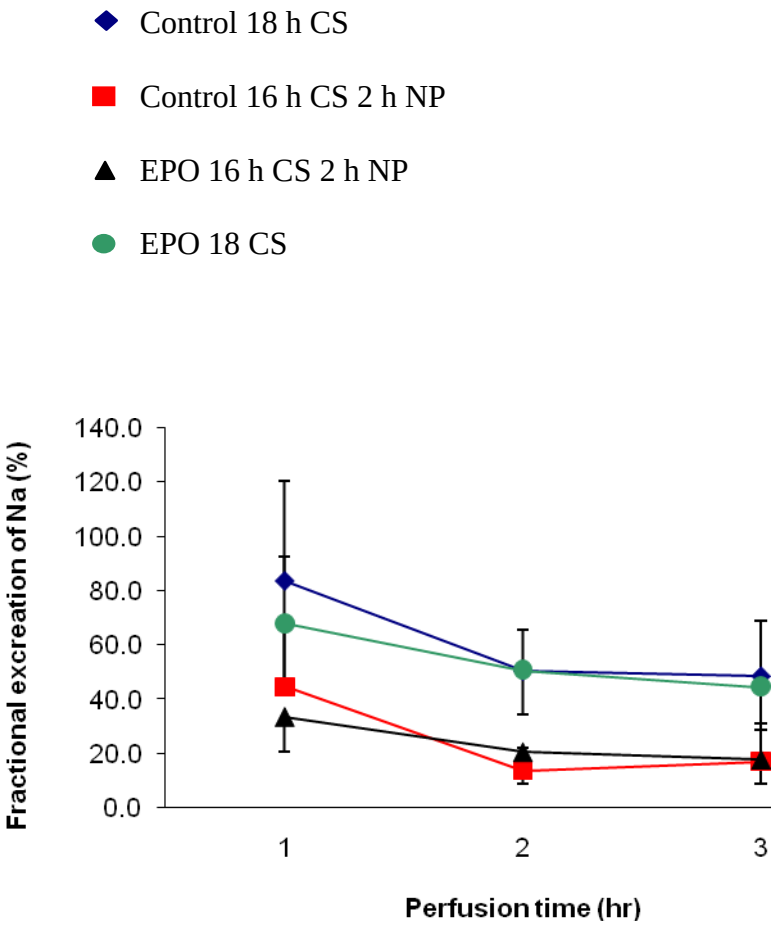


Table 13: Acid base homeostasis, Pre and 3 hour reperfusion (mean ± SD) (6.5.2)

	Group1		Group 2		Group 3		Group 4	
Parameter	**Pre**	**3hr**	**Pre**	**3hr**	**Pre**	**3hrs**	**Pre**	**3hrs**
pH	7.5 ± 0.1	7.5 ± 0.1	7.43 ± 0.04	7.45 ± 0.06	7.51 ± 0.07$^{\Psi}$	7.37 ± 0.07 $^{\Psi}$	7.49 ± 0.11	7.35 ± 0.12
Bicarbonate (mmol/L)	24.4 ± 4.3	32.8 ± 3.8¶	23.5 ± 1.35	28.2 ± 2.43*	24 ± 2.0	21.6 ± 3.7*¶	20.9 ± 1.0	21.2 ± 4.9*¶
Potassium (mmol/L)	5.5 ± 0.2	6.3 ± 2.5¶	5.9 ± 0.15	5.7 ± 1.8*	5.5 ± 0.10	10± 0*¶	5.6 ± 0.15	9.9 ± 0.2*¶

Ψ P <0.05 between Group3 pre-perfusion and 3-h reperfusion
* P <0.05 between Groups 2 and 3, 4
¶ P <0.001 between Groups 1 and 3, 4

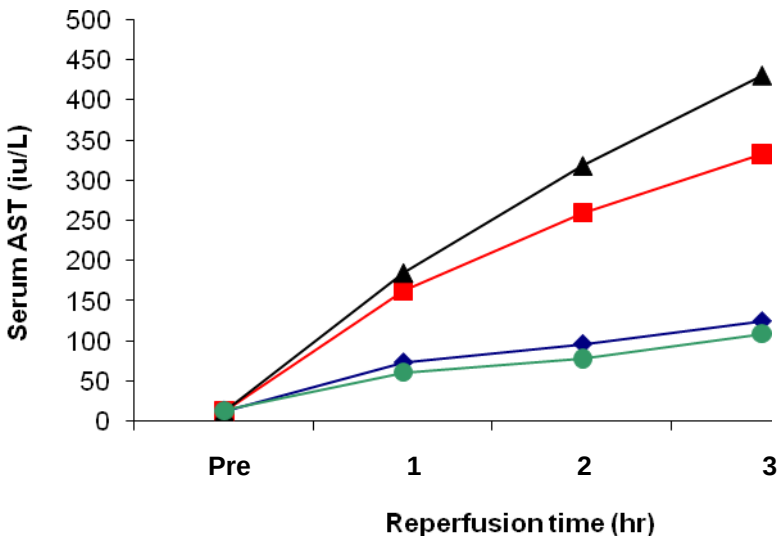
6.5.3 Serum AST

The levels in group 1 & 2 were significantly higher than group 3 & 4; no difference was noted in between groups 3 & 4 (596±98 vs. 724±359 vs. 239±70.4 vs. 200±31.8; groups 1, 2, 3 & 4 respectively, P=0.0007) (Figure 15)

6.5.4 Histology

Intracytoplasmic vacuolation was significantly increased in the 16 hr CS + 2 hr NP group but the addition of EPO reversed this change (P= 0.013). There was no difference in any of the other parameters between the study groups.

Figure 15: Serum AST over 3 hours of reperfusion with 16hrs CS +2hrs NP (Group 1), 16hrs CS +2 hrs NP with EPO (Group 2), 18 hr CS (Group 3) and 18hrs CS with EPO (Group 4)
(6.5.3)



6.6 Discussion

This is the first study assessing the effects of erythropoietin in an experimental NHBD kidney. We simulated a controlled NHBD kidney by subjecting it to 10 min of WI and 18 hrs CS [2]. On reperfusion our study shows that EPO was able to significantly increase the oxygen consumption when added to NP compared to the control NHBD kidney, while NP alone only showed a marginal improvement. In addition the kidney with an EPO flush during CS were able to maintain their acid base balance, while the counterpart control NHBD kidneys became severely acidotic on reperfusion. Thus suggesting EPO as a manipulating agent did not add any major benefits in a model of a controlled NHBD kidney. EPO added to the NP (Group 2) was better than NHBD controls (Group 3) and EPO flush during CS (Group 4), but rendered no benefit in comparison to the control NP [368] (Group1).

Erythropoietin has not shown any toxicity in animal models of ARF with high doses of EPO (5000 iu/kg) [207], though its potential side effect of thrombosis due to raised haematocrit levels cannot be excluded in clinical settings. EPO is currently being used safely as therapy for anaemia in-patient with ESRF [369]. Ehrenreich H *et. al.* [216] in a multicenter randomised controlled trial showed that a dose of 100,000 units (app. 1500 iu/kg) was safe in the acute stroke setting. Considering the logistics of a NHBD kidney and recent advances of EPO in various small animal models [207,354,358,362,363]; a high dose of 5000 units is likely to be relatively safe, as it would be an ideal treatment modality to the organ and not the patient as described in this experimental design. Sharples *et. al.* [82] in a rat model demonstrated that EPO administered as a single intravenous bolus before onset of ischaemia, at reperfusion or as late as 30 min after reperfusion reduced I/R

injury in the rat kidney. They concluded that EPO directly protects the proximal tubular epithelial cells by activating EPOreceptor/JAK-2 kinase, activating PI3K leading to Akt, up-regulation of Bcl-X_L and XIAP and preventing activation of caspase-3 and thereby apoptosis. These findings have been confirmed by a number of other studies in such small animal models for various organs [82,207,358, 370].

However recently, Sepodes *et. al.* [354] has shown that pre treatment with EPO significantly reduced I/R injury in a rat liver model, but when administered 5 min before reperfusion rendered no such protection. Our study results are in line with these findings. There could be a number of explanations, so our model the first in a NHBD organ takes into consideration damage due to prolonged cold ischaemia in addition to WI which no other study addresses. It is evident from the literature that organs recover relatively well when subjected to prolonged WI, though show marked reduction in viability with a combination of hypothermia and warm ischaemia [372]. The porcine kidney model simulates the human kidney when subjected to I/R injury [363, 365-367] and hence is superior to the rodent model. It has been shown that several agents found to protect the rodent kidney fail to do so when translated to porcine and human kidney [365]. Nonetheless Forman JC *et. al.* [363] have shown that in the porcine kidney, EPO given at the time of ischaemia significantly reduced I/R injury compared to controls not receiving EPO. Patel NSA *et. al.* [358] also showed that a pre-treatment regimen of EPO afforded better protection over a single dose administered at the time of reperfusion in a rodent model. To address why they put forward several explanatory hypotheses; 1) Pre-treatment protocols resulted in favourable systemic or renal haemodynamics, though Hung C *et. al.* [373] showed a reduction in cortical blood flow following repetitive pre-treatment with EPO, 2) Higher steady-state plasma concentration resulting from

repetitive pre treatment (1000 iu/Kg x 3 doses), again we showed no real difference to the control NP group when a single large dose of 5000 iu was used, 3) Up-regulation of protective genes due to repetitive pre treatments, though this does not explain the beneficial effects with a pre-reperfusion dose in various other isolated perfusion models; not seen in our model and rat liver model [354] and 4) Bone marrow-derived stem cell contributing to regeneration of proximal tubular epithelial cells [374], again the hypothesis represents the same shortfalls as above.

6.7 Conclusion

EPO did not seem to add any major benefits when used as a manipulating agent during two hours of NP or as a flush prior to CS in a controlled NHBD kidney. However, it increased renal oxygen consumption when added during NP and maintained the acid base balance in kidneys flushed with EPO prior to CS. Thus EPO though safe, may have its shortfall when applied to a NHBD kidney programme.

CHAPTER 7

EXPERIMENTAL STUDY: EFFECTS OF CARBON MONOXIDE RELEASING MOLECULE (CORM-3) ON REPERFUSION IN A CONTROLLED NON-HEART BEATING DONOR (NHBD) HAEMOPERFUSED PORCINE KIDNEY MODEL

7.0 Introduction:

Kidney transplantation continues to be the best modality of renal replacement therapy for end stage renal failure (ESRF) [351]. The main constraints in renal transplantation remain the demand and supply of organs [1]. This has lead to an increase in organs procured from non-heart beating donors (NHBD) [2]. Though results from such transplants are encouraging [4,5], these organs undergo significant damage from ischaemia/reperfusion (I/R) injury due to initial warm ischaemic (WI) injury [5,8,9] and cold ischaemic (CI) injury during storage and transport [10,11,12,13]. On transplantation these kidneys are subjected to further damage during reperfusion with normothermic oxygenated blood as seen by a complex pathophysiological process involving cellular oedema, generation of reactive oxygen species, triggering an acute inflammatory response, which promotes programmed cell death, 'Apoptosis' [60,352,353].

Carbon monoxide (CO), historically a poison is now viewed as an important by product of haem oxygenase-1 (HO-1) that has an important role in many cellular and biological process [305], such as promoting vasodilation [236,264], inhibition of proliferation of smooth muscle [249], transplant rejection [266], inflammation [251,375], platelet aggregation [376], cytokine production [273,377], oxidative stress [274], and apoptosis [274,246,378,379]. CO has been shown to reduce I/R injury in isolated cells [305], livers [117,281], heart [266,232,269], nerve tissue [272], lungs [273,274,275] and kidneys [118,271,352].

Different approaches have been used to study the pleiotropic effects of this diatomic gas such as direct administration of the gas [251], use of pro-drugs (methylene chloride) that are catabolised by hepatic enzymes to generate CO [286], and more recently transport and delivery of CO by means of specific CO carriers (transitional metal carbonyls)

[266,287,288,304,352] eg. Carbon monoxide-releasing molecule-3 (CORM-3). This water-soluble molecule [266] can release CO in a controlled manner both *in-vivo* and *in-vitro* under physiological conditions [288,289].

7.1 Aim

This study was designed to study the effects of CORM-3 as a manipulating agent on reperfusion in a controlled NHBD haemoperfused porcine kidney model. The isolated haemoperfused porcine model [366,367], a system based on paediatric cardio-pulmonary bypass technology was used in this study as previously described in chapter 4.

7.2 Methods:

7.2.1 Retrieval and perfusion

Kidneys were retrieved from large white pigs and perfused as described in chapter 4.

7.2.2 Experimental Design

Porcine kidneys (n=6) were subjected to 10min warm ischaemia and reperfused after 18hr Cold storage (CS) as follows:

Group 1: CORM-3 [tricarbonylchloro(glycinato)ruthenium(II)]

Group 2: iCORM-3 (inactive CO-releasing molecule)

Group 3: Control (no treatment)

The kidneys were then reperfused for 3 hr, with autologous blood on the isolated organ preservation system (IOPS) [366,367] at pressure of 85 mmHg, in order to assess renal viability and function. The manipulating agent was used at a concentration of 50µm, based on a preliminary dose response study [50µm, 100µm, 200µm & 400µm of CORM-3 (n=4), Figure 17, 18, 19] and published literature [352,304]. This was diluted in 60 ml of normal saline and infused 5 minutes before into the circuit prior to organ being attached for reperfusion for a period of 1 hour at a rate of 50 ml/hour. The inactive parent molecule (iCORM-3) was prepared beforehand in 60ml of saline and left at room temperature for 18 hr to allow for complete liberation of its contained CO[305]; 50µm of iCORM was then administered in the same way.

7.3 Assessment

7.3.1 Functional and biochemical parameters

Hourly serum and urine samples were used for biochemical assays and whole blood for haematology. Creatinine clearance [urinary creatinine (U_{cr}) × urinary volume (V)/ plasma creatinine (P_{cr})] and fractional excretion of sodium [(U_t × U flow) / (GFR × P_t) ×100] were calculated and serum creatinine levels recorded hourly. Blood gases were measured for calculation of oxygen consumption in ml/min/g [(PO_2 in arterial blood - PO_2 in venous blood) x (flow rate / weight)], acid-base homeostasis and carboxyhaemoglobulin (COHb) levels. RBF and IRR were continuously recorded. Serum AST levels were also measured at pre-reperfusion and 3 hours post reperfusion in all groups.

7.3.2 Histology

Wedge biopsies taken pre CS, post preservation and post reperfusion and each parameter was scored using the following semi-quantitative scale: 0 none; 1 mild; 2 moderate; 3 severe as described in chapter 4.

7.4 Statistical Analysis

Values are presented as mean ± SD and variables analysed as outlined in chapter 4. A P value of $\leq$ 0.05 was considered significant.

7.5 Results

7.5.1 Dose Response Studies

The renal blood flow deteriorated with increasing doses of CORM-3, though no difference was seen between the 50µm and 100µm groups (774 ± 18 vs.643 ± 139 vs. 481 ± 182 vs. 140 ± 51 for 50, 100, 200, 400 µm respectively, P=0.002) (Figure 16).

Similarly the creatinine clearance was significantly better in the 50 µm and 100 µm groups compared to the other two groups (14 ± 5.8 vs. 13 ± 1.6 vs. 2.8 ± 1.1 vs. 0.42 ± 0.2 for 50, 100, 200, 400 µm respectively, P=0.0013) (Figure 17).

While renal tubular injury was significantly less in 50μm and 100μm groups as demonstrated by the fractional excretion of sodium (51 ± 27 vs. 30 ± 6.8 vs.100 ± 30 vs. 206 ± 37.4, P=0.002 for 50, 100, 200, 400 μm respectively) (Figure 18).

The carboxyhaemoglobulin levels remained less than 1 percent as analysed on the blood gases for these two groups. This suggested that 50μm was the most suitable dose of CORM-3.

Figure 16: Dose Response Studies: Renal blood flow comparing groups receiving 50µm, 100µm , 200µm, 400µm concentrations of CORM-3. **(7.5.1)**

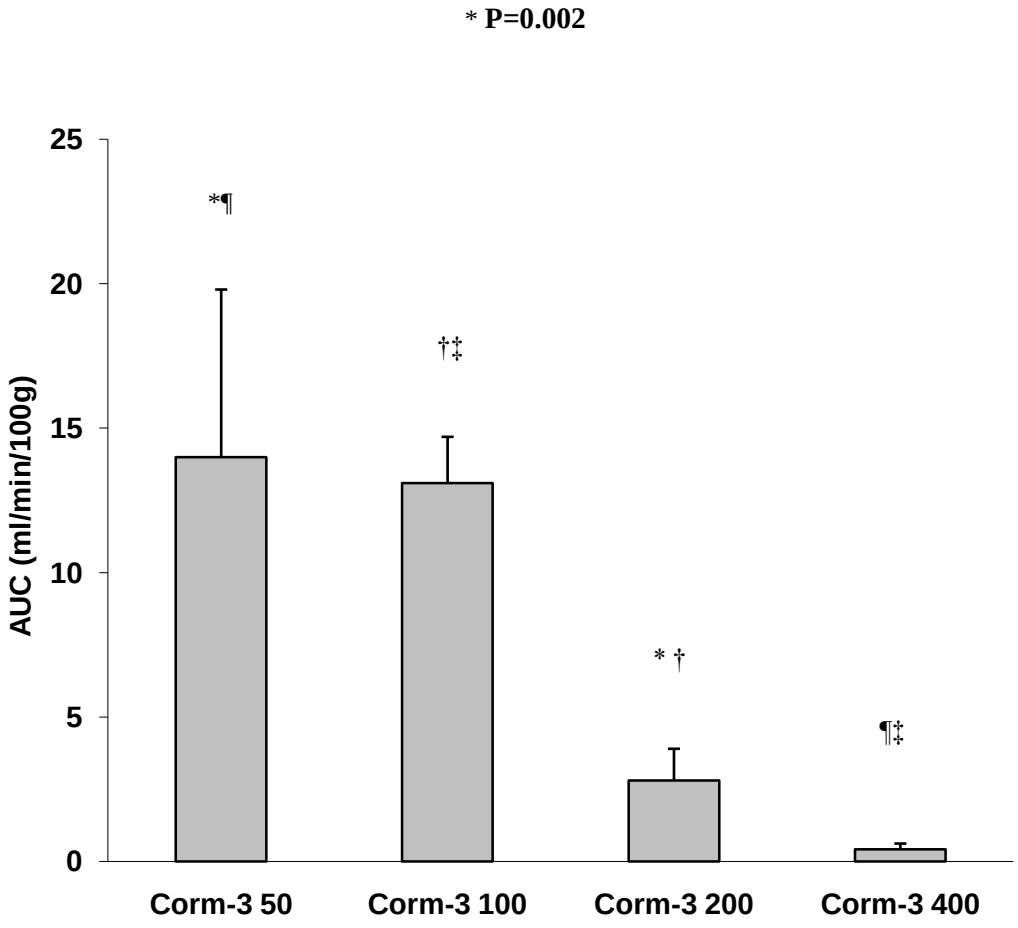


Figure 17: Dose Response Studies: Creatinine clearance comparing groups receiving 50µm, 100µm, 200µm, 400µm concentrations of CORM-3. **(7.5.1)**

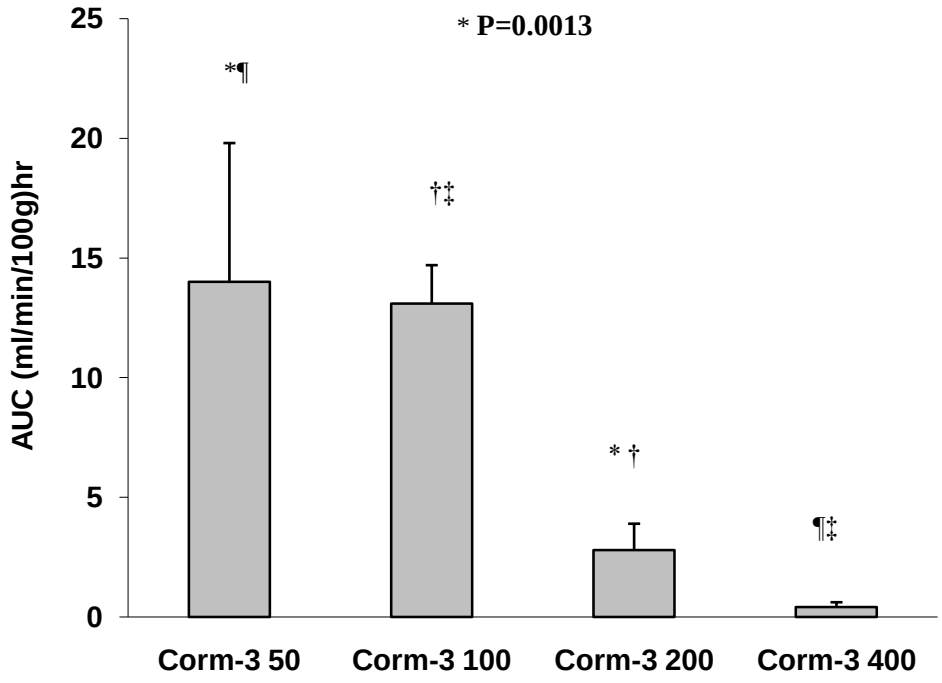
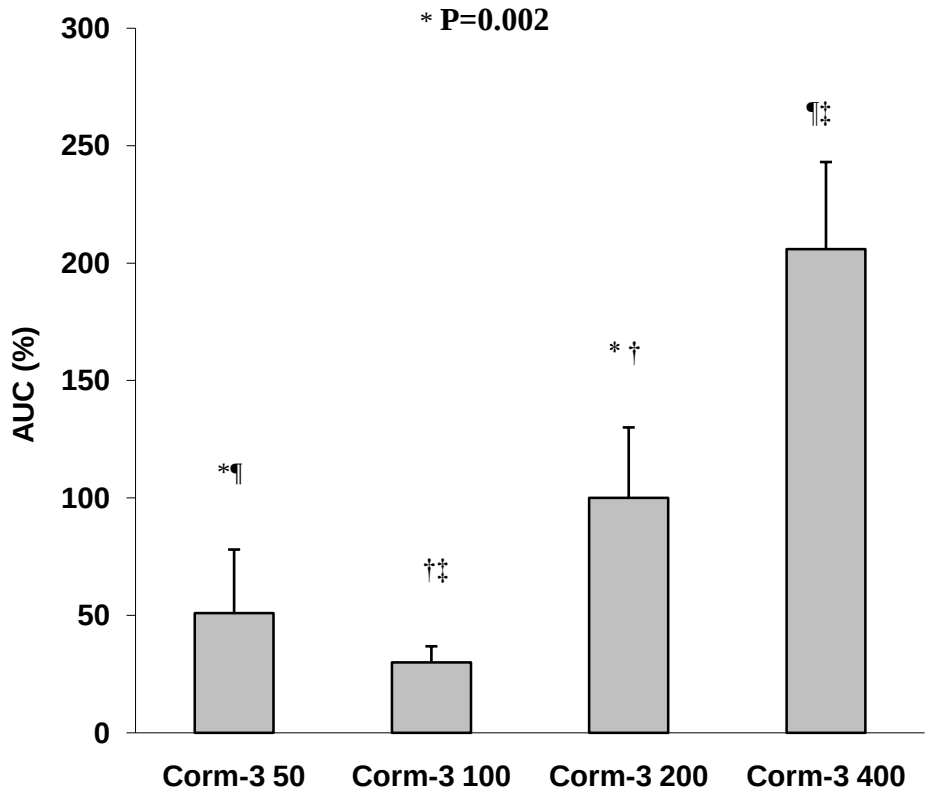


Figure 18: Dose Response Studies: Fractional excretion of sodium comparing groups receiving 50µm, 100µm, 200µm, 400µm of CORM-3. **(7.5.1)**



7.5.2 Reperfusion data

Pre-perfusion and post reperfusion haematocrit and haemoglobin levels ranged from 0.17-0.20 l/l and 4.67-5.68 g/dl, although levels declined slightly in all groups after 3 hours reperfusion but there was no significant difference between groups. COHb levels remained below 1% when concentration dose of 50µm of CORM-3 was used.

Renal blood flow (RBF) was significantly higher in 50µm CORM-3 group vs. 50µm iCORM and control group (774 ± 19 vs. 448 ± 88 vs. 325 ± 70 respectively; P=0.002) (Table 14; Figure 19), this correlated to a significantly lower IRR for 50µm CORM-3 vs. 50µm iCORM-3 and control group (3.9 ± 0.1 vs. 7.9 ± 1.2 vs. 9.1 ± 4.3 respectively; P=0.007) (Table 14; Figure 20).

Table 14: Area under curve (AUC) for functional parameters: 3 hr reperfusion

Parameter (AUC)	CORM-3	iCORM-3	Control	P
Serum Creatinine (µmol/L)	1450 ± 335*	1933 ± 55	2156 ± 401*	0.046
Creatinine Clearance	14 ± 5.8*¶	3.3 ± 0.1¶	2.2 ± 1.7*	0.006
Total Urine output (ml)	793 ± 212*	368 ± 72	302 ± 211*	0.01
RBF (ml/min/100g)hr	774 ± 19*¶	448 ± 88¶	325 ± 70*	0.002
IRR (mm Hg/min)hr	3.9 ± 0.1*¶	7.9 ± 1.2*¶	9.1 ± 4.3*	0.007
O_2 consumption (ml/min/g)hr	57 ± 25*¶	32 ± 15¶	24 ± 12*	0.033
Serum AST (IU/L)hr	355 ± 158¶	131 ± 15¶	240 ± 71	0.0037
Fractional excretion Na^+ (%)hr	51 ± 27*¶	105 ± 6¶	117 ± 38*	0.04

* P <0.05 between CORM-3 and Control group
¶ P <0.05 between CORM-3 and iCORM-3 group

Figure 19: Renal blood flow over 3 hours of reperfusion with CORM-3 (Group 1), iCORM (Group 2), 18 hr CS (Group 3).
All values are mean ± SD
(7.5.2)

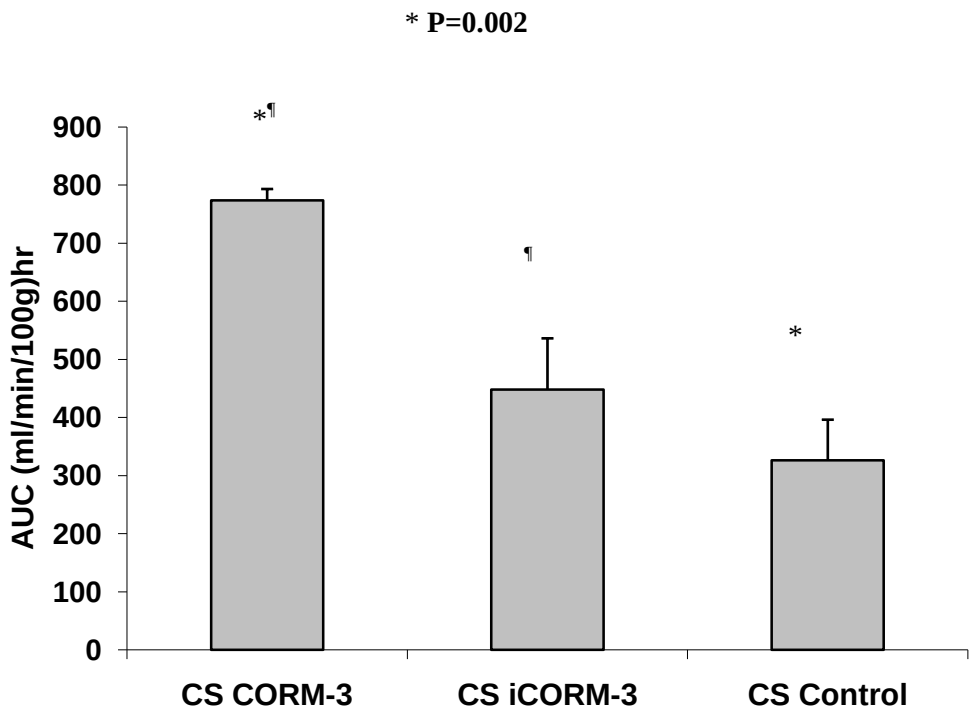
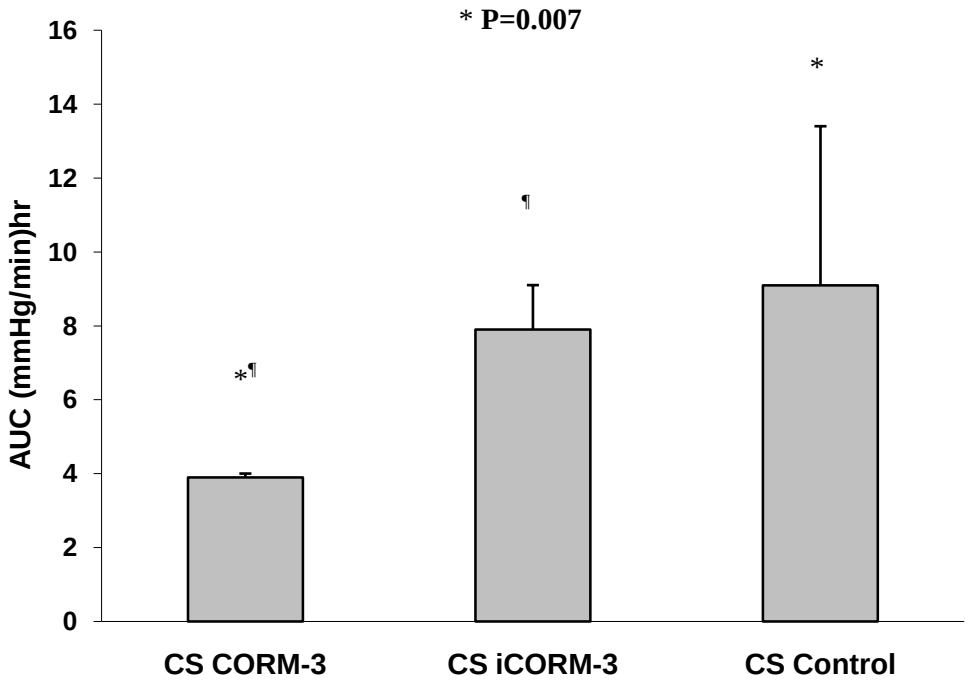


Figure 20: Intra renal resistance over 3 hours of reperfusion with CORM-3 (Group 1), iCORM (Group 2), 18 hr CS (Group 3).
All values are mean ± SD
(7.5.2)



7.5.3 Renal Function

On reperfusion the total urine output was significantly higher for 50µm CORM-3 vs. 50µm iCORM-3 & control group (793 ± 212 vs. 368 ± 72 vs. 302 ± 211 respectively; P=0.01) (Table 14; Figure 21). While the serum creatinine levels fell in all groups over 3 hr, a significant fall was demonstrated in 50µm CORM-3 vs. 50µm iCORM-3 & control group at 3 hr (% serum creatinine fall 79 ± 10.1, 48 ± 1.5 and 44 ± 13 respectively, P=0.017).

AUC creatinine was significantly lower in 50µm CORM-3 compared to 50µm iCORM-3 and control group (1450 ± 335 vs. 1933 ± 55 vs. 2156 ± 401 respectively; P = 0.046) (Table 14; Figure 22).

Creatinine clearance was significantly better in 50µm CORM-3 throughout the reperfusion period, a level of 14 ± 5.8 ml/min/100g compared to levels of 3.3 ± 0.1 and 2.2 ± 1.7 ml/min/100g in 50µm iCORM-3 and control group respectively (p = 0.006) (Table 14; Figure 23).

Figure 21: Total urine output over 3 hours of reperfusion with CORM-3 (Group 1), iCORM (Group 2), 18 hr CS (Group 3).
All values are mean ± SD
(7.5.3)

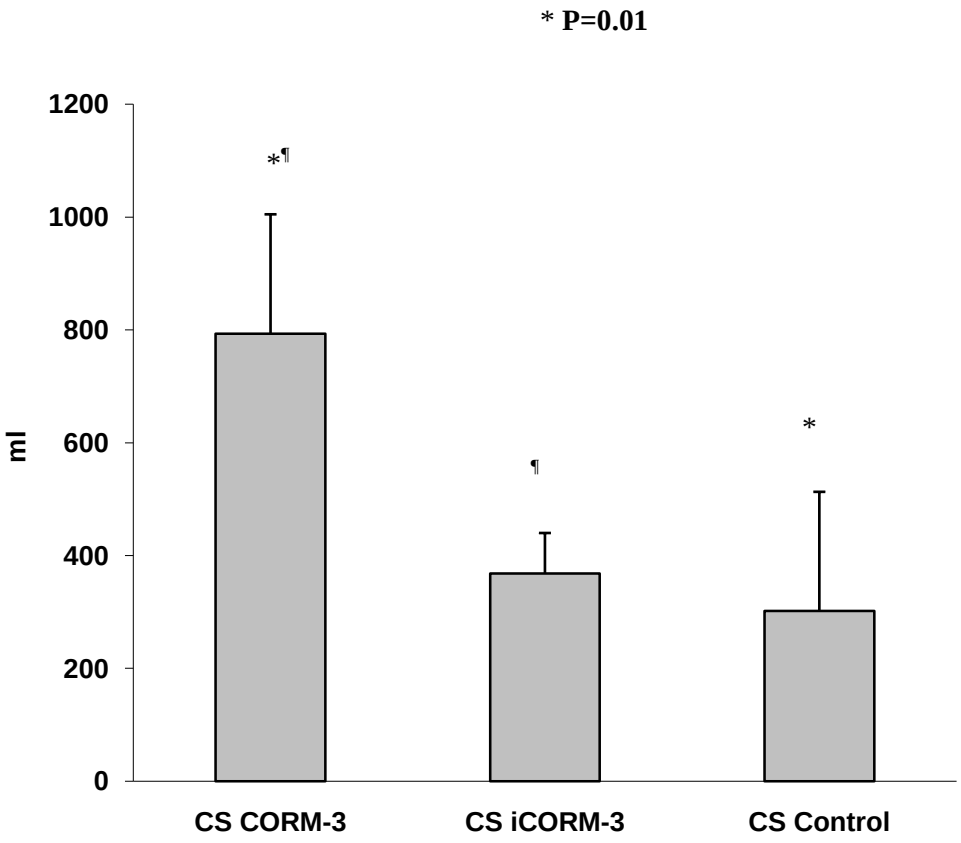


Figure 22: Serum creatinine over 3 hours of reperfusion with CORM-3 (Group 1), iCORM (Group 2), 18 hr CS (Group 3).
All values are mean ± SD
(7.5.3)

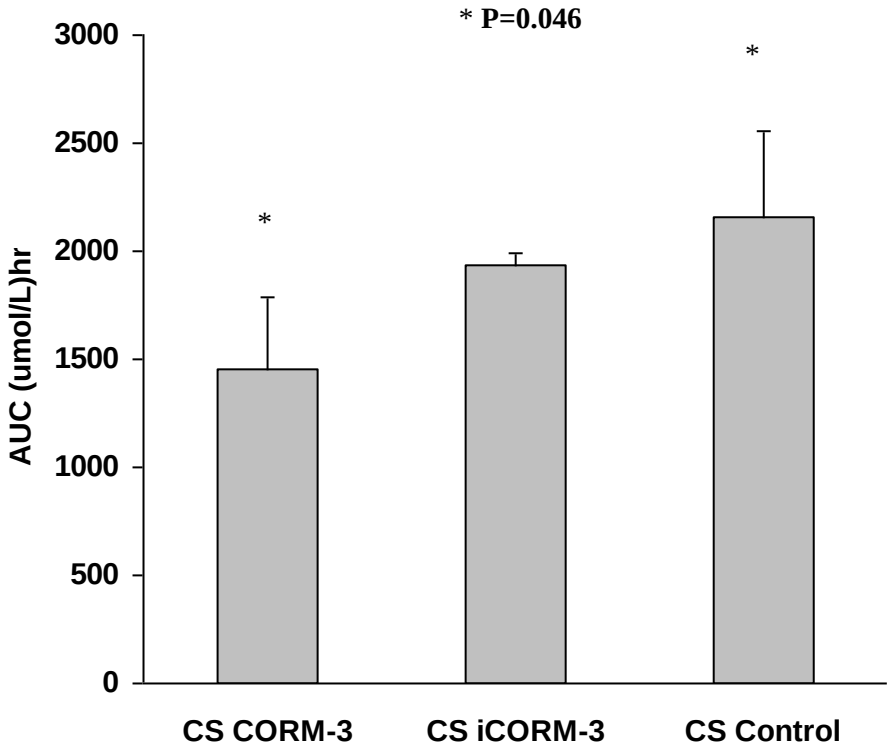
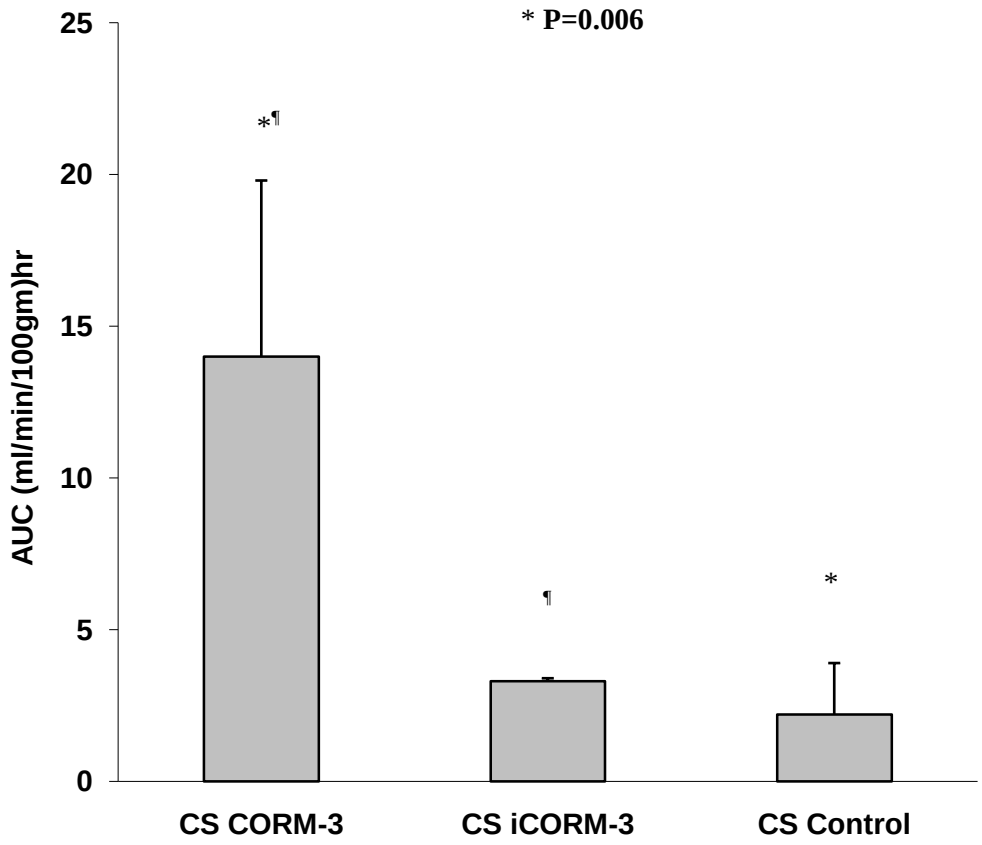


Figure 23: Creatinine clearance over 3 hours of reperfusion with CORM-3 (Group 1), iCORM (Group 2), 18 hr CS (Group 3).
All values are mean ± SD
(7.5.3)



Oxygen consumption was significantly improved in CORM-3 to iCORM-3 and control group following 3 hours of reperfusion (57 ± 25 ml/min/g compared to 32 ± 15 and 24 ± 12) (Table 14; P = 0.033).

All kidneys maintained near normal acid base homeostasis, although there was a decrease in pH and bicarbonate levels in control group at 3 hours reperfusion compared to CORM-3 and iCORM-3 groups, not reaching any significant difference (Table 15). While there was a significant rise in the serum potassium levels in iCORM-3 and control group compared to CORM-3 (P=0.0044, Table 15)

Renal tubular function was significantly better in CORM-3 than in iCORM-3 and control group throughout reperfusion with lower levels of fractional excretion of sodium (51 ± 27 vs. 105 ± 6 vs. 117 ± 38 respectively, P = 0.04) (Table 14; Figure 24).

7.5.4 Serum AST

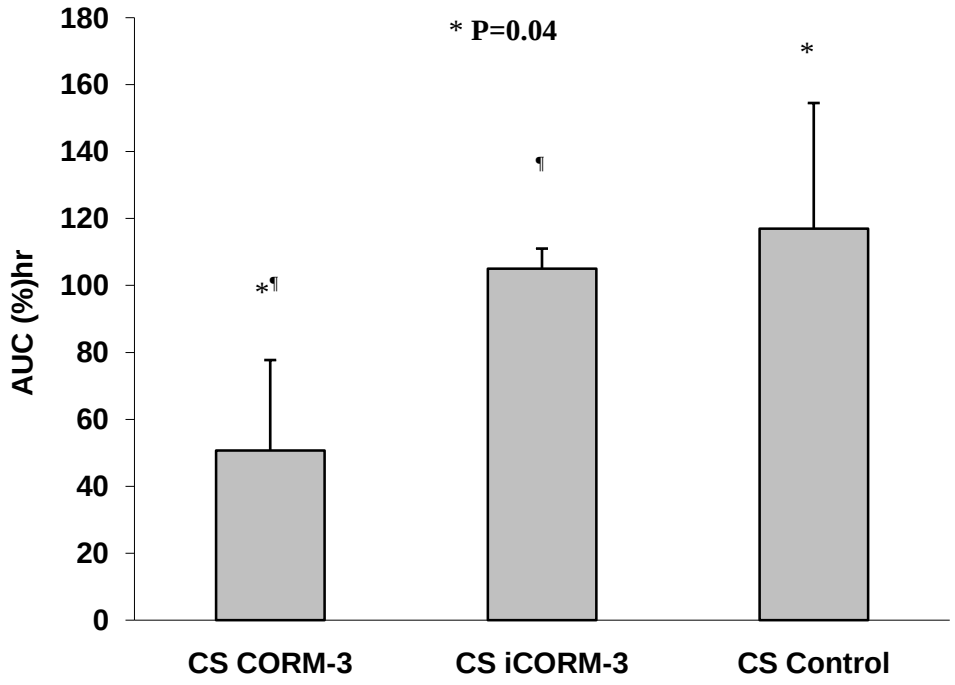
The serum aspartate aminotransferase (AST) levels were significantly higher for CORM-3 versus iCORM-3 (P<0.05), [355 ± 158 vs.131 ± 15 vs. 240 ± 71, (Table 14)].

Table 15: Acid base homeostasis, Pre and 3 hour reperfusion (mean ± SD) (7.5.3)

	CORM-3		iCORM-3		Control	
Parameter	**Pre**	**3hr**	**Pre**	**3hr**	**Pre**	**3hrs**
pH	7.41± 0.03	7.42 ± 0.07	7.38 ± 0.03	7.38 ± 0.02	7.51 ± 0.07	7.37 ± 0.07
Bicarbonate (mmol/L)	18.75 ± 0.53	24.95 ± 2.62	18.1 ± 0.72	19.83 ± 1.13	23.97 ± 2.02	23.97 ± 3.66
Potassium (mmol/L)	5.4 ± 0.1	5.2 ± 0.85*	5.2 ± 0.17	9.5 ± 0.55¶	5.5 ±0.10	10.0 ± 0.00*

* P <0.05 between CORM-3 and control group
¶ P <0.05 between CORM-3 and iCORM-3 group

Figure 24: Fractional excretion of sodium over 3 hours of reperfusion with CORM-3 (Group 1), iCORM (Group 2), 18 hr CS (Group 3, control).
All values are mean ± SD
(7.5.3)



7.5.5 Histology

There was no difference in any of the histological parameters (tubular dilation, epithelial shedding, tubular debris, condensed tubular nuclei, RBC presence, intracytoplasmic vacuolation and glomerular shrinkage) between the study groups.

7.6 Discussion:

This study shows that normothermic perfusion with CORM-3 can be used to ameliorate I/R injury in a porcine kidney that has been subjected to 18 hours of cold storage. The protocol used included an initial period of 10 minutes of warm ischaemic injury in order to simulate a model of controlled NHBD kidney transplantation. Kidneys generally can tolerate a period of warm ischaemia, although with a combination of warm ischaemia and hypothermia they show a marked reduction in viability [331]. This represents a key obstacle in the expansion of the non heart-beating organ donor pool. The continuous infusion of CORM-3 did not substantially raise the COHb levels (<1%) in blood. Kidneys undergoing reperfusion with CORM-3 demonstrated significant improvement in a number of functional and biochemical parameters, showing that CORM-3 was able to reverse some of the effects a period of cold ischaemia, as well as a short period of warm ischaemia.

Recent advances in the application of the pharmacological properties of CORMs in various pathophysiological circumstances are on the increase [276,297,303,304,305,331,352,380]. Sandouka *et. al*. [352] showed that CORMs used during CS at 4ºC were adequate to limit damage to the donor organ. This effect was not seen at 37ºC as CO is very rapidly released in physiological buffers and plasma at a normothermic temperature with a half-

life from 1 to 5 min [289,297]. In the porcine model described here, which used a continuous infusion of the manipulating agent CORM-3 we did not encounter such a problem, but rather showed a significant improvement in haemodynamic as well as biochemical parameters, with no substantial increase in carboxyhaemoglobulin levels. This is highly advantageous as it is the first model in kidney where blood is used to perfuse the kidney instead of a physiological buffered solution. In addition this model demonstrates that just a small period of 1-hour infusion of CORM-3 starting at reperfusion was adequate. Thus this is the first study in a kidney model that takes warm ischaemia into consideration hence addressing a major issue in NHBD transplantation.

As the kinetics of CO release from the parent molecule (CORM-3); play a crucial role in determining and maximizing the beneficial effects of these CO carriers, the current IOPS system can clearly address an effective delivery and thereby limit the CO lost from the system a downfall in the Sandouka *et. al.* study [352] where they partially address it by the use of a slow release CO donating molecule CORM-A1. Vera *et. al.* [276] reported that CORM-3 decreased plasma creatinine levels and limited renal damage in a mouse model of ischaemia induced renal failure. Sandouka *et. al.* [352] and Arregui *et. al.* [296] showed marked improvement in GFR and sodium reabsorption, thus suggesting CO also protects renal tubular function. Collectively these findings are similar to our findings though the model we describe also extends to WI, hence beneficial effects in a NHBD programme. Regan *et. al.* [381] and Kim *et. al.* [382] has shown that during I/R injury there is a significant exacerbation to tissue injury in several kidney diseases due to impairment of renal blood flow. The persistent reduction in renal blood flow is directly proportional to a decrease in GFR that is associated with increased production of endothelin and reactive oxygen species, which in turn are responsible for increased

renovascular resistance after I/R injury [383,384,385]. The use of CORM-3 in our study significantly improved both these parameters, proportional to an increase in intra-renal resistance (IRR) associated with a poor GFR as seen in the control group. As the inactive form (iCORM) did not cause any of these effects we can clearly demonstrate that they are related to CO, as seen in other published data. Hosgood *et. al.* [367] has clearly shown that a high mean perfusion pressure is associated with better renal function. Compared to Sandouka *et. al.* [352] we showed that a lower, more physiological mean perfusion pressure of 85 mmHg is adequate compared to the high pressure of 100 mmHg, which may have independently effected the results in their study.

Vera *et. al.*[276] showed that CORM administered 1 hr before the onset of ischaemia significantly decreased the levels of plasma creatinine, though this was not seen when administered 1 hour after reperfusion. Our findings suggest that CORM administered at the time of reperfusion produce significant amelioration in renal function, these findings are in line with those of Guo *el. al.* [304] using a myocardial I/R injury model.

There are other advantages of the porcine model described here. The porcine kidney is recognised to have anatomical and physiological characteristics that closely resemble the human situation; hence the data is more representative than findings derived from small animal models [349,386]. The kidneys used were of similar size to the adult human kidney as they were taken from culled animals weighing 60-70 kilograms. This model is reliable, reproducible and allows continuous measurement of physiological parameters.

This *ex-vivo* normothermic perfusion system used a relatively short reperfusion period of 3 hours while Vera *et. al.* [276] used longer periods of 24 hours. The warm ischaemic

insults were relatively limited and could be extended as a model of uncontrolled NHBD kidneys. Technically the model is rather complicated and requires trained perfusion personnel. This study did not address the various mechanisms underlying the protection induced by CORM-3. Further studies could compare CORM-3 with slow CO releasers, which could be used during static hypothermic storage or hypothermic machine perfusion. Nonetheless, current literature has shown a trend of organ preservation moving towards normothermic preservation.

7.7 Conclusion

This study provides further evidence that CO may be protective in renal perfusion injury and supports the use of low-dose CO releasing molecules as a method of CO delivery. CO-releasing molecule-3 was effective when administered at the time of reperfusion, and this increases the likelihood of the findings being translated to the clinical setting. Thus CORM-3 has potential application in the field of NHBD kidney transplantation, which continues to be an expanding source of transplant kidneys.

CHAPTER 8

EXPERIMENTAL STUDY: EFFECTS OF CARBON MONOXIDE (CO) IN A CONTROLLED NON- HEART BEATING DONOR (NHBD) NORMOTHERMIC HAEMOPERFUSED PORCINE KIDNEY MODEL

8.0 Introduction

Kidney transplantation is the best modality in treatment of end stage renal failure (ESRF) [351]. The steady rise in patients needing transplants cannot be matched by the lack of suitable organs procured via traditional methods of organ retrieval and this has lead to a renewed interest in non-heart beating donors (NHBD) [2].Transplant from these organs have encouraging results, but the combined effects of prolonged warm ischaemia (WI) [4-8] and cold ischaemia [10,12] are reflected in higher rates of primary non-function (PNF) and delayed graft function (DGF) [5,387,388]. This is due to a complex patho-physiological process ischemia/reperfusion (I/R) injury [60,353] subjecting the recipient to a major cause of morbidity following a transplant [354,355].

Carbon monoxide (CO), a by-product of haemoxygenase-1 (HO-1) mediated breakdown of haem is involved in many biological processes which reduce (I/R) injury [117,118,246,249,264,266,272,273-275,305,352,375,376]. The pleiotropic effects of this simple diatom have been studied using either direct administration of gas [251] , use of pro-drugs (methyl chloride) [286] and use of specific CO carriers (transitional metal carbonyls) [266,287,288,304,352,389]. One of these carbon-monoxide releasing molecule (CORM-3) is a water soluble molecule [266] and can release CO in a controlled manner under physiological conditions [288,289,304,389].

Normothermic resuscitation perfusion (NP) in its self is promising as it restores metabolism and allows reversal of some of the detrimental effects of ischaemia prior to transplant [330,368]. In addition to this it allows an opportunity to create the required physiological conditions so as to target the organ with manipulating agents [389]. This was

investigated using isolated haemoperfused porcine kidneys in a well established pathophysiological model [366,367,390].

8.1 Aims

This study was thus designed to investigate the effects of CO gas and CORM-3, as manipulating agents administered in blood at the time of NP, on reperfusion injury in a model of controlled NHBD Kidneys.

8.2 Methods

8.2.1 Retrieval

Kidneys were retrieved from large white pigs as previously described in chapter 4.

8.2.2 Experimental design

Porcine kidneys (n=6) were subjected to 10 minutes warm ischaemia and 16 hours of cold storage to simulate the clinical situation of the controlled NHBD. After this they were subjected to 2 hours NP as follows:

Group 1: Control, 16hr Cold storage (CS) + 2hr Normothermic perfusion (NP) [368]

Group 2: CO gas (300ppm)

Group 3: CORM-3 [tricarbonylchloro(glycinato)ruthenium](II)

Group 4: iCORM-3 (Inactive CO-releasing molecule)

8.2.3 Normothermic perfusion (NP) circuit [368]

The mean arterial pressure (MAP) was set at 75 mmHg and kidneys underwent NP for a period of 2 hours as outlined in chapter 4.

Solutions of the carbon monoxide releasing molecule were prepared immediately before perfusion, at a concentration of 50µm CO-releasing molecule-3 [tricarbonylchloro(glycinato)ruthenium(II)] [288,304,352,389] in 60ml of saline at 4°C. Infusion of CORM-3 solution began 5 minutes before kidneys were connected to the IOPS and continued for the first hour of the NP. The inactive parent molecule (iCORM-3) was prepared beforehand in 60ml of saline and left at room temperature for 18 hr to allow for complete liberation of its contained CO[305]; 50µm of iCORM was then administered in the same way. CO gas (300ppm)[251] was bubbled through the IOPS via the arterial line during 1st hour of NP. Thus NP was used as the preservation arm to deliver the carbon monoxide.

8.2.4 Reperfusion circuit

The kidneys were then reperfused for 3 hr, at MAP 85 mmHg with autologous blood on the isolated organ preservation system (IOPS) [366,367,368,390] in order to assess renal viability and function. For this second stage of perfusion a white cell filter was not included in the circuit in order to more accurately reflect the clinical situation at the time of transplant reperfusion, hence the viability testing arm.

8.3 Assessment

8.3.1 Functional and biochemical parameters

Hourly serum and urine samples were used for biochemical assays and whole blood for haematology. Creatinine clearance [urinary creatinine (U_{cr}) × urinary volume (V)/ plasma creatinine (P_{cr})] and fractional excretion of sodium [(U_t × U flow) / (GFR × P_t) × 100] were calculated and serum creatinine levels recorded hourly. Blood gases were measured for calculation of oxygen consumption in ml/min/g [(PO_2 in arterial blood - PO_2 in venous blood) x (flow rate / weight)], acid-base homeostasis and carboxyhaemoglobin (COHb) levels. RBF and IRR were continuously recorded. Serum aspartate aminotransferase (AST) levels were also measured at pre-reperfusion and 3 hours post reperfusion in all groups.

8.3.2 Histology

Wedge biopsies taken pre CS, post preservation and post perfusion and each parameter was scored using the following semi-quantitative scale as outlined in chapter 4.

8.4 Statistical Analysis

Values are presented as mean ± SD and variables analysed as outline in previously in chapter 4. A P value of ≤ 0.05 was considered significant.

8.5 Results

8.5.1 Reperfusion data

Preperfusion and postreperfusion hematocrit and haemoglobin levels showed no significant difference between groups. The COHb levels measured in all groups remained below 1 %.

Renal blood flow (RBF) significantly improved when CORM-3 was added to NP compared to the control and CO groups, but no difference was observed against the inactive compound (iCORM-3).[AUC (ml/min/100g) hr; 435±95 (control) vs. 439±194 (CO) vs. 751±222 (CORM-3) vs. 558±165 (iCORM); P=0.024; Table 16 and Figure 25]. The intra-renal resistance (IRR) reflected the same pattern [AUC (mm Hg/min) hr; 5.51±1.6 vs. 6.9±3 vs. 3.3±0.8 vs. 4.6±2.7; P=0.033; Table 16 and Figure 26].

Table 16: Area under curve (AUC) for functional parameters: 3 hr reperfusion on IOPS (8.5.1 & 8.5.2)

Parameter (AUC)	Control	CO	CORM-3	iCORM	P
Serum Creatinine (µmol/L)h	1892 ± 215	1895 ± 247	1662 ± 538	2255 ± 169	0.053
% Serum Creatinine fall	51.3 ± 10.1	57 ± 9.7*	59 ± 20^{+}	31.1 ± 7.6*$^{+}$	0.0131
Creatinine Cl (ml/min/100g)h	3.73 ± 1.4	4.8 ± 1.8*	7.44 ± 7.34^{+}	1.32 ± 0.56*$^{+}$	0.0076
Total Urine output (ml)	556 ± 202	698 ± 80*	596 ± 367	270 ± 89*	0.005
RBF (ml/min/100g)h	435 ± 95^	439 ± 194$^{@}$	751 ± 222^$^{@}$	558 ± 164	0.024
IRR (mmHg/min)h	5.51 ± 1.6^	6.9 ± 2.98$^{@}$	3.3 ± 0.81^$^{@}$	4.6 ± 2.7	0.033
O_2 consumption (ml/min/g)h	32 ± 9^	44 ± 15	72 ± 18^	54 ± 13	0.0036
Serum AST (iu/L)h	434 ± 275 $^{\Psi}$	488 ± 209*	495 ± 372^{+}	1507 ± 1174 $^{\Psi}$*$^{+}$	0.032
Fractional excretion Na^{+} (%)h	117 ± 56	111 ± 36	65 ± 35^{+}	149 ± 25^{+}	0.02

* P <0.05 between groups: CO and iCORM-3
$^{+}$ P <0.05 between groups: CORM-3 and iCORM-3
$^{\wedge}$ P <0.05 between groups: CORM-3 and control
$^{@}$ P <0.05 between groups: CORM-3 and CO
$^{\Psi}$ P <0.05 between groups: control and iCORM-3

Figure 25: Renal blood flow over 3 hours of reperfusion with Control (Group 1), CO (Group 2), CORM-3 (Group 3) and iCORM-3 (Group 4).
All values are mean ± SD
(8.5.1)

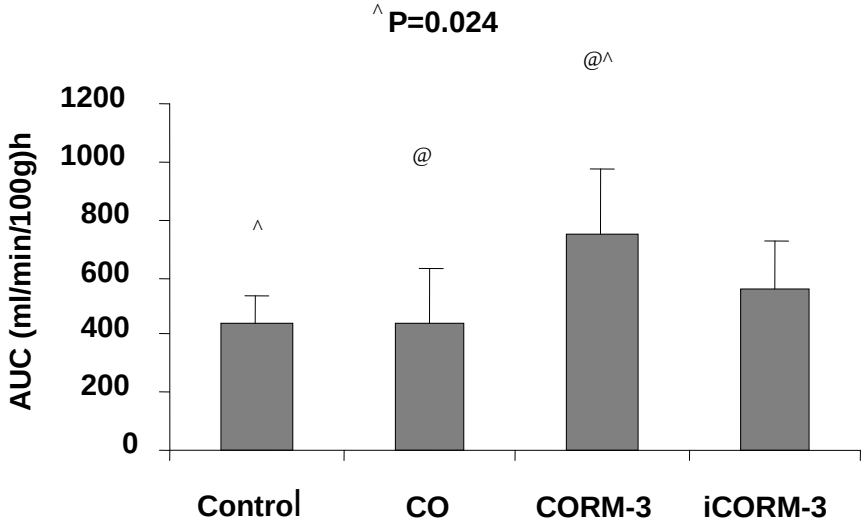
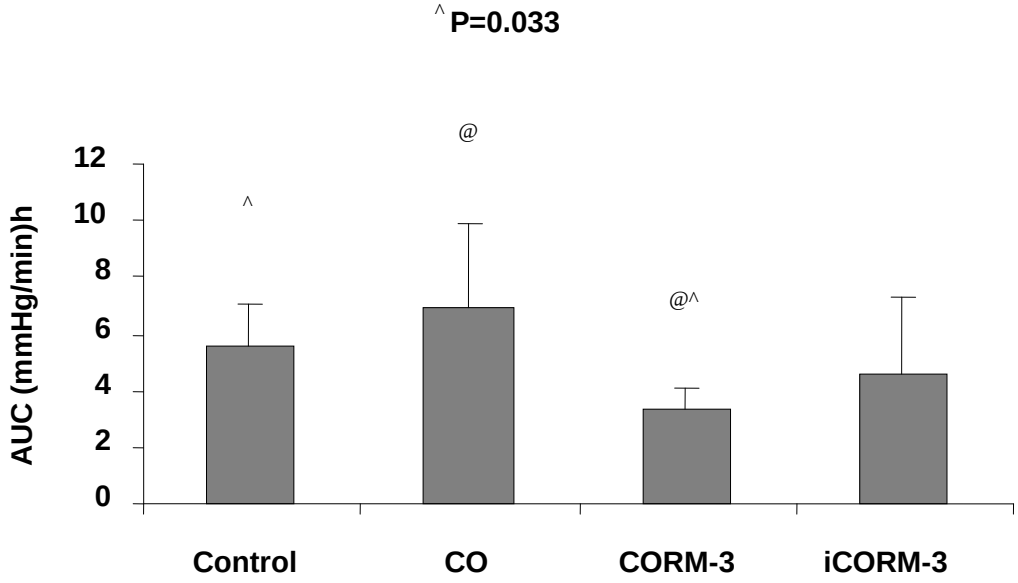


Figure 26: Intrarenal resistance over 3 hours of reperfusion with Control (Group 1), CO (Group 2), CORM-3 (Group 3) and iCORM-3 (Group 4).
All values are mean ± SD
(8.5.1)



8.5.2 Renal function

Total urine output was numerically higher for CO compared to CORM-3 and control groups [556±202 (control) vs. 698±80 (CO) vs. 596±367 (CORM-3) vs. 270±89 (iCORM) ml; P=0.005; Table 16, Figure 27].

The renal functional parameters from CO and CORM-3 groups did not show any difference to the control group, though they were significantly higher when compared to the iCORM-3 group, with a greater fall in the serum creatinine [% serum creatinine fall; 51.3±10 vs. 57±9.7 vs. 59±20 vs. 31.1±7.6; P=0.013; Table 16]. Similarly AUC of creatinine was marginally higher in CORM-3 vs. iCORM with no significant difference compared to the control [AUC (μmol/L)hr; 1892±215 (control) vs. 1895±247 (CO) vs. 1662±538 (CORM-3)vs. 2255±169 (iCORM-3); p= 0.053; Table 16, Figure 28]. The creatinine clearance reflected the same pattern [(ml/min/100g)hr; 3.73±1.4 vs. 4.8±1.8 vs. 7.44±7.3 vs. 1.32±0.56; p= 0.0076; Table 16, Figure 29].

The oxygen consumption was significantly improved in group 3, group treated with CORM-3 [ml/min/g; 31.7±9 vs. 43.5±15 vs. 72.2±18 vs. 54.3±13; P=0.0036; Table 16, Figure 30).

Figure 27: Total urine output over 3 hours of reperfusion with Control (Group 1), CO (Group 2), CORM-3 (Group 3) and iCORM-3 (Group 4).
All values are mean ± SD
(8.5.2)

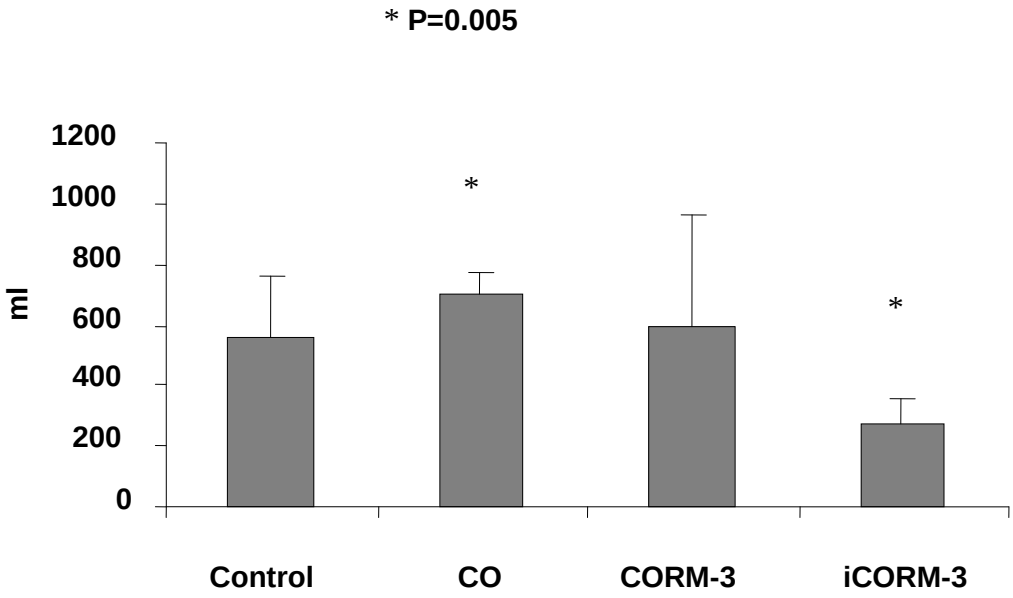


Figure 28: Serum creatinine over 3 hours of reperfusion with Control (Group 1), CO (Group 2), CORM-3 (Group 3) and iCORM-3 (Group 4).
All values are mean ± SD
(8.5.2)

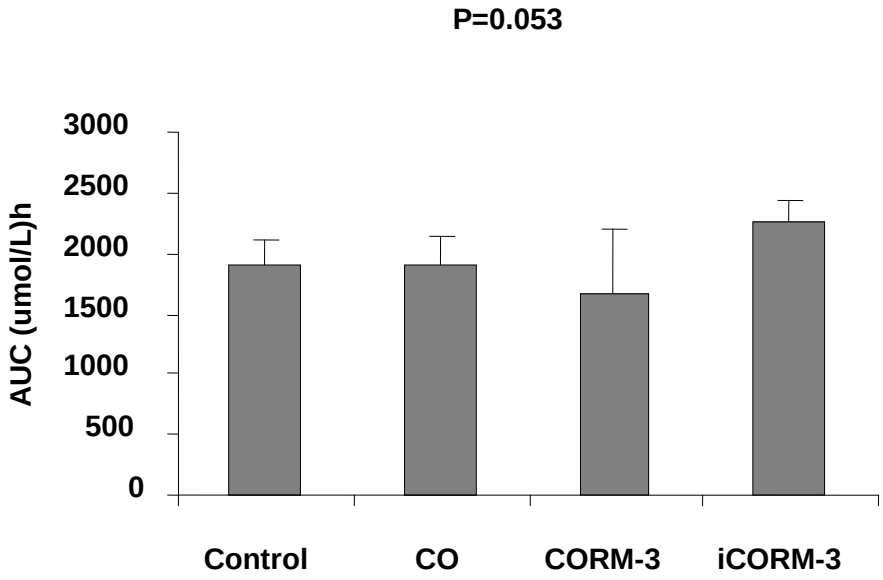


Figure 29: Creatinine clearance over 3 hours of reperfusion with Control (Group 1), CO (Group 2), CORM-3 (Group 3) and iCORM-3 (Group 4).
All values are mean ± SD
(8.5.2)

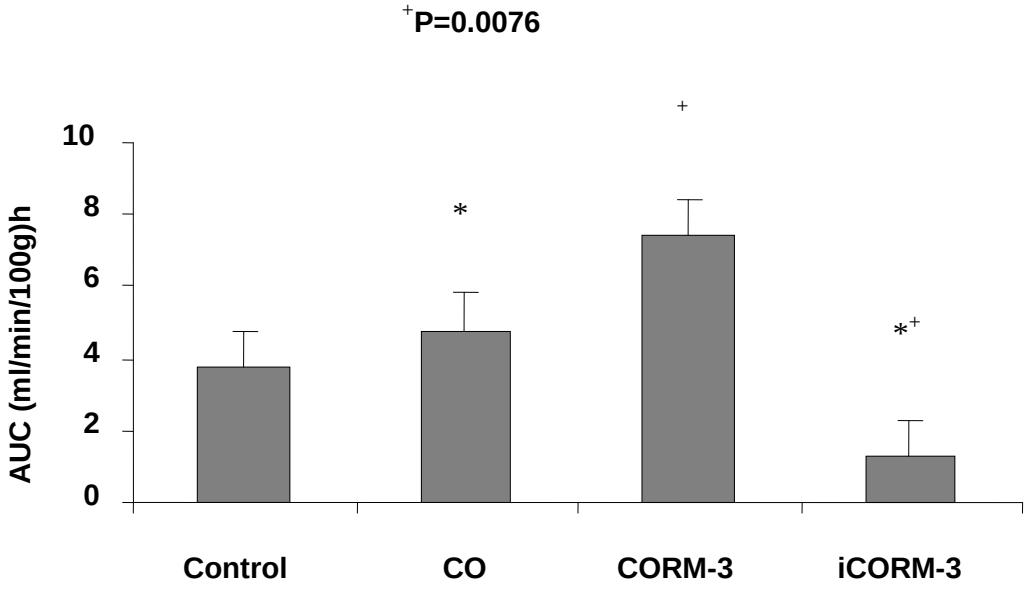
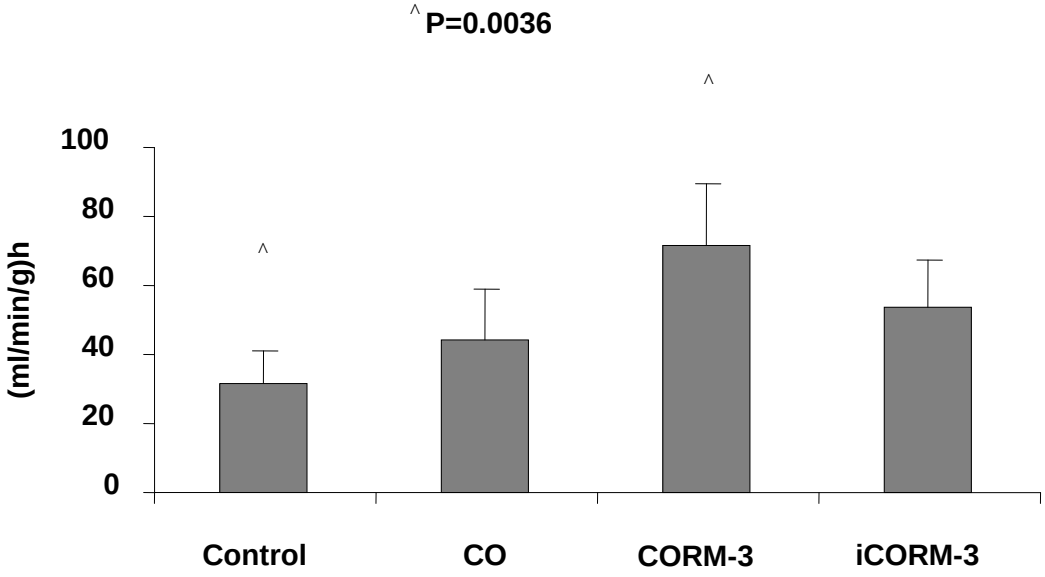


Figure 30: Oxygen consumption over 3 hours of reperfusion with Control (Group 1), CO (Group 2), CORM-3 (Group 3) and iCORM-3 (Group 4).
All values are mean ± SD
(8.5.2)



All kidneys maintained near normal acid-base balance homeostasis at 3 hours of reperfusion (Table 17).

The fractional excretion of sodium showed a trend to improve in CORM-3 [AUC (%) hr; 117±56 vs. 111±36 vs. 65±33 vs. 149±25; p=0.02; Table 16, Figure 31] suggesting an improvement in renal tubular function.

Serum potassium levels were maintained within the normal levels for CORM-3 group compared to the other groups (p=0.004, Table 17).

The serum AST levels were significantly better for CORM-3 vs. iCORM-3, though no difference was seen in comparison to the control or CO groups [AUC (IU/L) hr; 434±275 (control) vs. 488±209 (CO) vs. 495±372 (CORM-3) vs. 1507±1174 (iCORM); P=0.0036; Table 16, Figure 32].

Table 17: Acid base homeostasis, Pre and 3 hour reperfusion (mean ± SD) (8.5.2)

	Control		CO		CORM-3		iCORM	
Parameter	**Pre**	**3hr**	**Pre**	**3hr**	**Pre**	**3hrs**	**Pre**	**3hrs**
pH	7.41± 0.01	7.47 ± 0.10	7.39 ± 0.02	7.42 ± 0.07	7.34 ± 0.02	7.40 ± 0.06	7.40 ± 0.01	7.42 ± 0.05
Bicarbonate (mmol/L)	20.6 ± 0.5	27.0 ± 5.7	20.5 ± 1.2	24.1 ± 4.6	17.9 ± 0.7	24.3 ± 3.1	20.9 ± 1.2	24.8 ± 4.9
Potassium (mmol/L)	5.7 ± 0.19	7.4 ± 1.4*	5.8 ± 0.25	6.6 ± 1.5*	5.1 ±0.1	4.1 ± 1.2*$^{+}$	5.2 ±0.2	7.8 ± 1.1^{+}

* P <0.05 between groups: CORM-3 and Control, CO
$^{+}$ P <0.01 between groups: CORM-3 and iCORM

Figure 31: Fractional excretion of sodium over 3 hours of reperfusion with Control (Group 1), CO (Group 2), CORM-3 (Group 3) and iCORM-3 (Group 4).
All values are mean ± SD
(8.5.2)

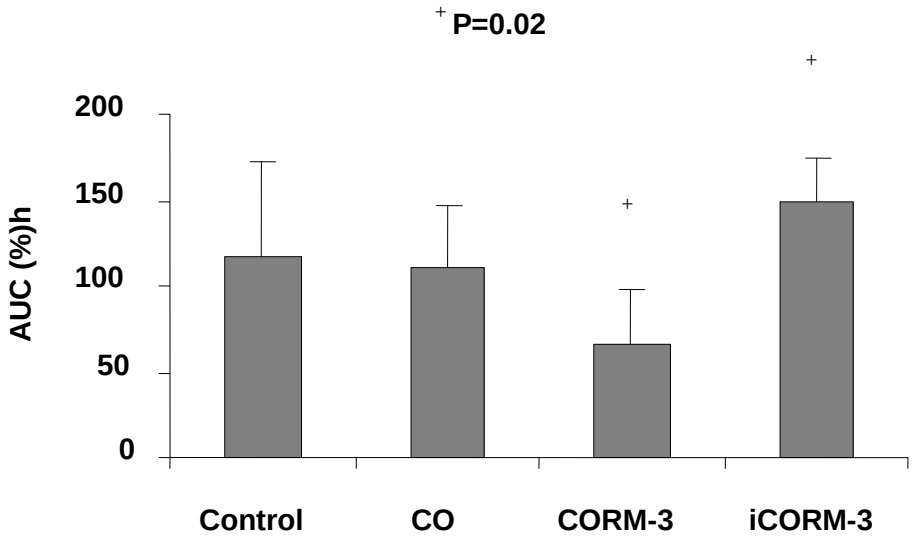
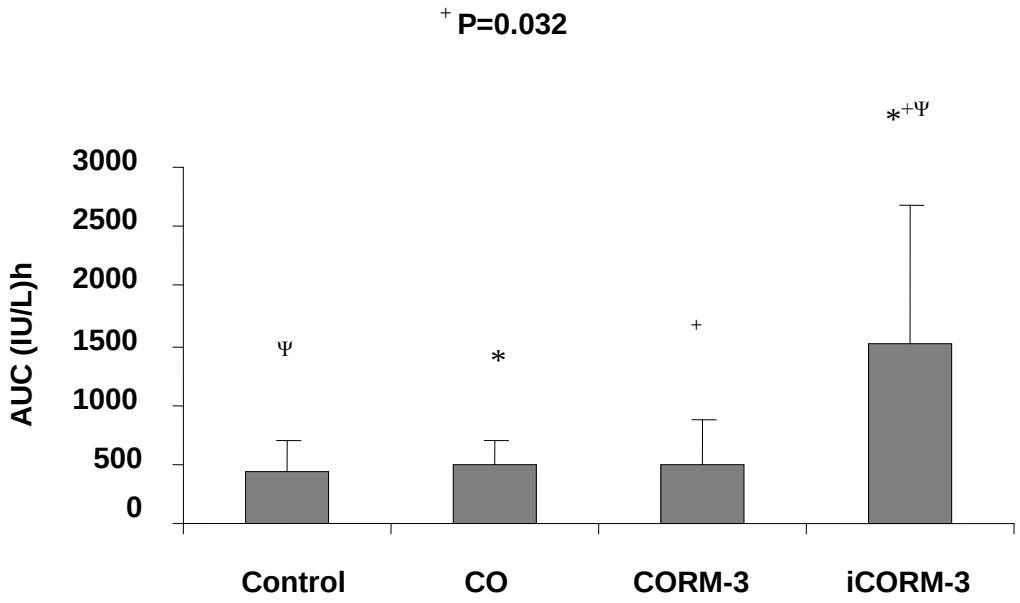


Figure 32: Serum AST over 3 hours of reperfusion with Control (Group 1), CO (Group 2), CORM-3 (Group 3) and iCORM-3 (Group 4). All values are mean ± SD
(8.5.2)



8.5.3 Histology

There were no differences in the histological parameters (tubular dilatation, tubular debris, epithelial flattening, epithelial shedding, intracytoplasmic vacuolation, condensed tubular nuclei and glomerular shrinkage) between the groups.

8.6 Discussion

Normothermic resuscitation perfusion in a NHBD kidney model logistically allowed the application of the manipulating diatom, either as CO gas itself or using a CO donor molecule (CORM-3). On reperfusion, the study confirmed additional benefits with CORM-3 when added to NP [368]. In contrast CO (the presumed active agent) when directly applied showed no additional benefit to NP, when used directly as a gas; the only exception, a trend in improvement of total urine output.

Carbon monoxide has been shown to play a role in many biological processes that reduce I/R injury, such as vasodilatation [264,305], inhibition of proliferation of smooth muscle [249], transplant rejection [266], inflammation [375], platelet aggregation [376], cytokine production [273], oxidative stress [274] and apoptosis [274,276]. Most of these findings were confirmed in various studies using isolated cells [305], livers [117], heart [266,269], nerve tissue [272], lungs [246,273,274] and kidneys [118,352]. Using CORM-3 we showed additional benefit as it increases renal blood flow (RBF), proportionally reduces intra-renal resistance (IRR) [improved haemodynamics], significantly improves oxygen consumption and suggested a trend towards reducing renal tubular injury. Jassem W. *et. al.* [391] and McCord JM *et. al.* [392], have previously shown that rapid restoration of blood flow and maintenance of oxygenation during reperfusion play a vital role in optimising cellular

function by reducing ischemia and generation of reactive oxygen species, thereby down regulating apoptosis and inflammatory mediators. Our study was not designed to address the mechanisms of action of CO, but the functional outcomes are in line with the above studies. These effects were not seen with the inactive molecule (iCORM-3), which implies that the benefits seen are due to released CO rather than the ruthenium molecule. These findings are consistent with our previous work in a large animal model [389] and other small animal models [352,276,296].

A possible explanation for failure of the diatom molecule to induce these protective changes when applied directly as a gas is the fact that CO so readily combines with haemoglobin to form inactive COHb, that it never reaches the target organ. The dose of 300ppm [251] produced COHb levels of less than 1%, but as we did not measured free gas levels in the venous limb this raises the question of concentrations reaching the target organ. Thus the dose may have been too small to achieve enough free CO to reach the target level and ameliorate I/R injury. This would warrant further studies to check the benefit of higher concentrations of CO gas. Now relying on the facts, CORM-3 added further functional benefit during NP without deleterious effects and not iCORM-3; COHb levels less than 1% and no associated histopathological changes, suggest lack of CO reaching the target when used in gas form. There is also further scope to assess low levels of CO gas during NP using an acellular perfusate where this problem can be avoided.

As all kidneys maintained acid-base balance especially where CO and iCORM-3 showed no advantage, the possible explanation supports the beneficial role of NP [368]. CORM-3 had a further advantage as the kidneys maintained potassium (K^+) levels in the normal range compared to the other groups.

There are advantages with this model as porcine kidneys simulate human kidneys and the data obtained is more translatable compared to small animal models [393,394]. The model favours the logistics of kidney transplantation (providing adequate warm and cold ischemic times) and also the manipulating agent can be used to target the organ at the onset of reperfusion (without treating the donor and recipient). Hence clinically applicable in NHBD transplantation; as I/R injury is inevitable and occurs before the treatment can be administered to the donor.

The study does have limitations. The reperfusion period of 3 hours was relatively short so that only very early functional outcome can be assessed. More importantly, long-term effects should be studied with a transplant model rather than increasing the reperfusion time. Slow releasing molecules such as CORM-A1 [352], may exert further additional benefit as the active agent can be readily available to act longer at the target site, a problem we seem to have encountered when using CO as a gas. The warm ischaemic period was also limited; given the benefit, a further study arm to assess an uncontrolled NHBD kidney should be carried out.

8.7 Conclusions

This study not only provides evidence of low dose CO-releasing molecules (CORM-3) as a superior method of CO delivery, but suggests additional benefit from carbon monoxide (delivered as CORM-3) in the amelioration of ischemia reperfusion injury targeted at a transplant organ during normothermic resuscitation perfusion (NP). However, no additional benefits were observed with CO gas when added to NP during reperfusion in a model of controlled NHBD kidneys.

CHAPTER 9

CONCLUSIONS

9.0 Advantages

This research helped develop a reliable model where benefits of normothermic perfusion (NP) could be tested, in addition to it be used to test various manipulating agents. The logistics of a controlled non-heart beating donor programme; which are complex involving organ retrieval, transfer and crossmatching, thus involving varying periods of warm and cold ischaemia could thus be replicated in this experimental setup simulating the clinical day to day practice.

In addition the model has other advantages; porcine kidneys are recognised as having anatomical and physiological characteristic that closely resemble humans so provides data that is more representative than findings from smaller animal studies. The kidneys used in this model were similar to adult human kidney as they were taken from 60-70 kg pigs. This model is reliable, reproducible and makes use of modern cardio-pulmonary bypass technology, whereby continuous measurement of physiological parameters is possible in addition to direct access to serial blood, urine and tissue samples.

The controlled nature of this *ex-vivo* system allows precise manipulation and thus permits evaluation of different interventions to ameliorate ischaemia reperfusion injury. It is a very useful surrogate model in comparison to *in-vivo* transplantation, offering distinct advantages in terms of reproducibility, versatility. This model may thus allow for minimisation or even replacement of live animal experimentation hence have significant benefits in terms of animal welfare and research costs.

9.1 Disadvantages:

This *ex-vivo* normothermic resuscitation perfusion system uses a relatively short reperfusion interval (validity testing arm) of 3 hours. The need for prolonged perfusion needs to be tested, although this aspect was not considered to be a major shortfall in this research thesis, as it was designed ideally for the study of the acute effects of reperfusion injury.

The warm and cold ischaemic insults were also relatively limited, so as to simulate the logistics of a controlled NHBD programme; they need to be extended to a model of uncontrolled NHBD programmes, as the uncontrolled programme would probably eventually represent the major bulk of a NHBD programme. Finally organ preservation by CS has the advantages of being a simple method, where as the technology and trained perfusion staff required for normothermic preservation renders it far more complicated, technically demanding and expensive.

9.2 Experimental studies

9.2.1 Normothermic resuscitation perfusion (NP)

The NP model has two useful arms, (a) a preservation arm and (b) a viability testing arm. This study confirmed that normothermic resuscitation perfusion with autologus blood can restore some of the adverse effects of a period of cold storage in kidneys sustaining collateral warm ischaemic injury. Two hours of NP adequately resuscitated

kidneys, similar to kidneys undergoing 18 hours of cold perfusion (CP) as confirmed after 3 hours of reperfusion in the viability testing arm.

Normothermic resuscitation perfusion thus has potential application in the field of NHBD kidney transplantation and may ultimately allow safer expansion of the donor pool.

9.2.2 Erythropoietin (EPO)

EPO is probably safe and although it did not have any benefits in the current model, further investigation, perhaps with a longer period of viability assessment is warranted. EPO did not seem to add any major benefit, other than improving oxygen consumption when added during normothermic perfusion. The one clear advantage seen from this study was the easy and safe application of this manipulatory agent at the time of onset of reperfusion, confirming the models versatility.

9.2.3 Carbon Monoxide (CO)

The application of carbon monoxide releasing molecule-3 (CORM-3) shows some evidence that CO is protective in renal reperfusion injury, as well as supports the use of low dose carbon monoxide releasing molecules as the method of choice for CO delivery in this model. CORM-3 was effective when administered at the time of reperfusion, supporting the use of this manipulating agent at the time of reperfusion during normothermic resuscitation reperfusion (NP). This technique only targets the transplant organ thus protecting the recipient from possible toxicity of the manipulating agent.

Additional benefits were not seen with the diatomic gas molecule, possibly due to the high affinity of CO for haemoglobin. Further work could assess the same system using an acellular normothermic perfusate. Thus CO in the form of CORM-3 shows further benefit in the amelioration of I/R injury, targeting the transplant organ during NP. Thus a combination of NP and CO may have an important potential application in the field of NHBD kidney transplantation.

9.3 Future directions

1. Most importantly the model should be tested and its safety assessed in an animal transplant model; only then it can be translated to a human NHBD programme.

2. The effects of NP and CO should be extended to an uncontrolled NHBD kidney model. In a realistic day to day practice longer periods of warm ischaemia are anticipated as majority of organs would probably be procured from uncontrolled NHBD kidneys. The kidneys can be easily subjected to longer WIT (30-45 min) so as to simulate an uncontrolled NHBD.

3. The safety of the manipulator agent such as CORM-3 has to be validated in an animal model. The model used to resuscitate the kidney here is *ex-vivo,* which theoretically targets the transplant organ and spares the donor and recipient from possible toxic effects of heavy metal ruthenium. This metal will get deposited in the transplant organ and may lead to organ damage, thus necessitating long term evaluation in an animal model.

4. Study of the effects of manipulator agents like CO where problems are encountered due to high affinity of the agent with haemoglobin rendering it an inactive compound before it reaches the target site. This could be studied using an acellular normothermic (perfluorocarbons) perfusate using the same model.

5. Longer perfusion times are possible and should be evaluated in further studies. It is important to assess the effects of reperfusion in terms of longer renal function and histological data in comparison to hypothermic preservation techniques, as these techniques of organ preservation are much cheaper and do not involve a complex perfusion system and trained perfusion personnel. The model is reliable, easily reproducible, cost effective in terms animal welfare and research costs.

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