The Role of Nitric Oxide and Inflammatory Cytokines in Delayed graft Function of DCD Renal Transplants; The Effect of Induction Immunosuppression

Thesis submitted for the degree of Doctor of Medicine Cardiff University by

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Abstract

Introduction

Ischemia Reperfusion Injury (IRI) has a significant influence on patient outcomes after renal transplantation. The role of Nitric Oxide (NO) in IRI is controversial with evidence for both protective and deleterious effects. The aim of this project was to investigate NO in recipients of Donation After Cardiac Death (DCD) renal transplants, who are expected to have high rates of Delayed Graft Function (DGF) as a result of IRI, and to correlate this with their inflammatory response and induction immunosuppression.

Methods

Plasma samples were collected from 35 recipients of DCD renal transplants around the time of transplantation. NO_2^- and NO_3^- were measured using the Griess reaction and Ozone chemiluminescence and cytokines were measured using Luminex.

Results

At 2 and 8 hours post reperfusion of the transplanted kidney changes in NO_2^- were associate with the age of the recipient ($p = 0.05$ and 0.001 respectively) and at 8 hours very strongly related to the warm ischemic time ($p < 0.0005$). At 8 hours post reperfusion there was a strong negative correlation between the age of the recipient and the change of NO_3^- ($p = 0.002$).

IL-6 and IL-10 were found to increase significantly at 30 minutes and 2 hours after reperfusion ($p < 0.0005$). IL-6, IL-10 and TNF-α were all influenced significantly by induction immunosuppression and at 30 minutes post reperfusion changes in NO_2^- were also affected by the induction.

Conclusion

Changes in NO_2^- were influenced by factors effecting renal IRI and also by induction immunosuppression. Increasing age was associated with dampening of the NO_2^- and NO_3^- response. Cytokines levels rose after reperfusion of the kidney and their changes were modified by induction immunosuppression.

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Awards

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British Transplant Society Annual Congress. 22nd February 2012.

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Changes In Plasma Nitrite Levels In Renal Transplant Recipients Are Associated With Reperfusion Injury And May Be Influenced By Induction Immunosuppression**.**
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Abbreviations

ALG	Anti-lymphocyte globulin
APCs	Antigen presentation cells
ATG	Anti-thymocyte globulin
ATN	Acute tubular necrosis
ATP	Adenosine triphosphate
AUC	Area under curve
BMI	Body mass index
cAMP	Cyclic adenosine monophosphate
CAMs	Cellular adhesion molecules
cGMP	Cyclic guanosine monophosphate
CIT	Cold ischemia time
CNI	Calcineurin inhibitor
cNOS	Constiutive nitric oxide synthase
DBD	Donation after brain death
DCD	Donation after circulatory death
DGF	Delayed graft function
DMA	Dimethylamine
DNA	Deoxyribonucleic acid
ECC	Excitation contraction coupling
ECD	Extended criteria donor
EDRF	Endothelium derived relaxing factor
ELISA	Enzyme-linked immunosorbant assay
eNOS	Endothelial nitric oxide synthase
ET	Endothelin
g	Gram
GFR	Glomerular filtration rate
h	Hours
HLA	Human leucocyte antigen

ICAM	Intracellular adhesion molecule
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IQR	Inter-quartile range
IRI	Ischemia reperfusion injury
IV	Intravenous
kDa	KiloDaltons
LAK	Lymphokine activated killer
LD	Live donor
M	Molar
MAF	Macrophage activating factor
mg	Milligrams
MHC	Major histocompatibility complex
ml	Millilitres
MMF	Mycophenalate mofetil
mTOR	Mammalian target of rapamycin
mV	Millivolts
Myfortic	Mycophenalate sodium
NADPH	Nicotinamide adenine dinucleotide phosphate
NANC	Non-adrenergic, non-cholinergic
NaOH	Sodium hydroxide
NF	Nuclear factor
NGAL	Neutrophil gelatinase-associated lipocalin
NK	Natural killer
nm	Nanometres
nM	Nanomolar
nmol/L	Nanomoles per litre
nNOS	Neuronal nitric oxide synthase

NO	Nitric oxide
NOA	Nitric oxide analyser
NOS	Nitric oxide synthase
PTLD	Post-transplant lymphoproliferative disease
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RRT	Reperfusion ratio
RSNO	Nitrosothiols
SNP	Single nucleotide polymorphism
STAT	Signal transducer and activator of transcription
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNF	Tumour necrosis factor
UHW	University hospital of Wales
VCAM	Vascular cell adhesion molecule
WIT	Warm ischaemia time
XOR	Xanthine oxidoreductase
µL	Microlitres
µmol/L	Micromoles per litre

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CHAPTER 1. INTRODUCTION

1.1. The History of Renal Transplantation

Transplantation is a comparatively new surgical specialty, despite this, in just a few decades it has become the gold standard of treatment for patients with end stage renal failure. The following paragraphs are a summary of the pivotal discoveries that have shaped the field of clinical transplantation.

1.1.1. Surgical technique

The first successful human renal transplant was performed in 1954, however this was preceded by half a century of experimental work. During the first decade of the 20th century experiments were conducted in Austria and France using animal models (1). Alexis Carrel worked particularly on improving methods of vascular anastomosis and is credited with establishing the modern method of vascular suturing. As well as his contribution to the technicalities of transplantation he considered the conditions that the donor kidney should be kept in prior to implantation concluding that; "*the blood supply of the transplanted kidney should not be occluded for more than 50 to 60 minutes without consequences to its subsequent function".* He also suggested that cooling may have some value (2). Today the warm and cold ischemic times, to which he was eluding, are known to be pivotal in transplant outcomes and as we will see later in the text, are particularly important to this project in the context of Ischemia Reperfusion Injury (IRI). By the start of the First World War technical aspects of renal transplantation had been established, however, despite the improvements in surgical technique the grafts all failed. Although it was acknowledged that the recipient was likely to be "rejecting" the organ, the mechanism was uncertain and as a result interest in organ transplantation diminished (1).

1.1.2. The immune system

The next real period of progression in transplantation was the 1950's when Peter Medawar defined the biological features of graft rejection and showed it to be an immunological process (3). Medawar also contributed to the work which lead to the proposal that immunological tolerance may be induced by suppression of the immune system (4). Initially this was attempted using total body irradiation followed by concurrent renal and bone marrow transplantation. However, this practice had an unacceptably high mortality and with the introduction of chemical immunosuppression was abandoned (5). Following the discovery of HLA (Human Leucocyte Antigens) in 1952 by Jean Dausset there was a rapid expansion in knowledge about the human immune system leading to the concepts of tissue typing, histocompatibility and cross-matching (6).

1.1.3. Clinical application

Dr. Joseph Murray performed the first successful renal transplant between identical twins in Boston on December 23rd 1954. It was preceded by years of experimental work on dogs and was the beginning of live donation of organs as well as solid organ transplantation. Murray pioneered the field of transplant immunology and indeed transplant surgery. In 1990 he was awarded the Nobel prize for Medicine for the first successful transplantation of kidneys from a genetically identical twin, from a non identical twin and from a cadaveric donor.

1.1.4. Organ donation

Murray is well known for his work in renal transplant surgery. However, he is less renowned for his contribution to the development of brain death status and organ recovery, which was an essential step for the expansion of deceased donor organ transplantation. In 1967 a committee, including Murray, set out to objectively define criteria for death in the context of an irreversibly comatose

patient; they concluded "*the time honored criteria of stoppage of the heartbeat and circulation are indicative of death only when they persist long enough for the brain to die*". This definition allows the medical profession to legally obtain organs from a deceased donor, in the UK cadaveric organs make up over half of those used for renal transplantation (7).

1.1.5. Immunosuppression

By the 1960's foundations were in place for the topic of transplant immunology to develop into clinical practice; the concept of chemical immunosuppression had been introduced, initially, with 6-mercaptopurine (Purinethol) and its less toxic derivative Azathioprine. Azathioprine later proved to be the more effective of the two drugs by permitting survival of the graft whilst maintaining other protective immune responses in the recipient. It was later discovered that dual therapy with steroids produced a synergistic effect (1). In the first half of the 1960's transplants were performed from cadaveric and live donors with success using dual therapy. In 1966 a triple drug program was introduced by adding antilymphocyte globulin (ALG) (8).

As the specialty grew, so did its infrastructure; societies were formed to share information on evolving HLA typing methods, organ sharing schemes and organ procurement. Governments became involved and public awareness increased; in 1971 the Kidney Donor Card was introduced in Britain to be replaced by the Multi-Organ Donor Card 10 years later. HLA matching as well as the introduction of a new immunosuppressant called Cyclosporine improved results of organ transplantation. It also provided sufficient immunosuppression to permit successful liver, pancreas, heart and lung transplant and remained within the drug regimen until the 1990's when drugs such as Tacrolimus and Mycophenolic acid were introduced (5). Induction therapies with polyclonal antibodies (ALG and antithymocyte globulin (ATG)) were superseded by monoclonal therapies; OKT3, Basiliximab and Alemtuzumab. Although,

depending upon the clinical factors, both polyclonal antibodies are still used today.

1.1.6. Transplantation today

Renal transplantation is firmly established as the gold standard of treatment for end stage renal failure and it is an expanding area of both clinical activity and research in the UK. One-year graft survival rates now range from 92-97%, depending on the type of donation with patient survival at one year between 95-99%. Nationally, a total of 4753 organ transplants were performed in the last year, 3348 of which were kidney transplants. Despite this, the organ waiting list in the UK currently stands at around 6388 patients, with 5233 patients waiting for kidney transplants. In the last year 470 patients died whilst active on the transplant waiting list and a further 875 were removed due to deteriorating health. All of the above figures are taken from the *Organ Donation and Transplantation Activity Report 2016/17* produced by NHSBT.

In order to address the gap between supply and demand of organs, transplant physicians are constantly on the look out for ways to expand the donor pool and improve the longevity of grafts. One approach to this is the use of extended criteria donors (ECD) and increased use of donation after cardiac death (DCD) organs. DCD and ECDs would have previously been thought to be unusable due to donor characteristics and long ischemic times and are now being considered for transplantation into appropriate recipients. The consequence of this for transplant recipients is that these organs are prone to higher rates of delayed graft function (DGF) as a result of increased ischemic injury. Despite the expected difficulties that come with use of marginal organs the prognosis for patients with end stage renal failure who receive a transplant remains preferable to spending time on dialysis. It is therefore important for patient outcomes to understand the mechanisms of injury that these organs are subjected to and to develop means of reducing the injury sustained.

In this project we hope to contribute to this field of research by studying DCD kidneys where we would expect to find rates of delayed graft function around 50%. We will be investigating Nitric Oxide (NO) production, which is one of the pathways which influences ischemic injury in transplanted organs and correlating our findings with clinical outcomes.

1.2. Ischemia Reperfusion Injury

1.2.1. Introduction

Ischemia reperfusion injury is a pathological condition characterized by initial restriction of blood supply to an organ followed by subsequent restoration of perfusion and oxygenation. IRI contributes to a range of pathology including myocardial infarction, cerebrovascular accident (ischemic stroke), acute kidney injury, trauma and circulatory arrest. It also presents a major challenge for surgical specialties including organ transplantation, cardiothoracic, vascular and general surgery. IRI is a mechanism by which damage to solid transplanted organs occurs as a consequence of the process of retrieval and organ implantation and contributes significantly to the morbidity and mortality rates in clinical transplantation (9). As the name suggests it is a biphasic process; the initial hypoxia deprives cells of the energy needed to maintain their ionic gradients and homeostasis. This damage is exacerbated on reperfusion of the organ when an inflammatory reaction is triggered (10). The result of this process is endothelial dysfunction (9).

Renal IRI is a complex phenomenon with many variables but one that may significantly affect patient outcomes following transplantation. In this text we will first explore the clinical consequences for the renal transplant patient of the damage sustained as a result of IRI. We will then consider the pathophysiological mechanisms behind this damage and look at the cellular and molecular reactions behind them. There are many mediators of this process but the focus of this work is the significance and effects of Nitric Oxide (NO) on the endothelium. As part of our investigation we will also look at the effect that

endothelial activation has on the production of NO and the cytokines that mediate this in the context of renal transplantation.

1.2.2. Clinical consequences of renal IRI during kidney transplantation– Delayed Graft Function

IRI is an inevitable consequence of organ transplantation; the clinical sequelae of IRI for the renal transplant recipient include primary non-function of the graft, long-term graft dysfunction and DGF. DGF is a clinical manifestation of acute kidney injury in the immediate postoperative period and may result in the recipient requiring temporary dialysis. The prevalence of DGF after deceased donor transplants is reported with wide variability (between 25 and 75%). Shortage of donors has resulted in increased use of DCD, as well as use of marginal organs from ECDs. As both groups demonstrate higher rates of DGF than donation after brain death (DBD) or living donor kidneys, IRI and DGF are becoming increasingly relevant subjects in the field of transplantation.

The exact definition of DGF is debated with 18 different definitions identifiable in the literature (11)! This is problematic when comparing studies that use DGF as an endpoint and explains, partly, the wide variation in its reported incidence. The most widely used definition is *the use of dialysis within the first week after transplantation* (12). *Failure of a fall in serum creatinine of 10% on two consecutive days in the first postoperative week* is an alternative popular definition as it is a more objectively delineated; the dialysis definition is criticized because it is subjective to clinician or unit practice. Irrespective of how DGF is defined it is significantly more prevalent in DCD transplants with the incidence being double that found among DBD transplants. Interestingly, when DGF does occur, it translates to poorer graft function at one year after transplantation in kidneys from DBD but not from DCD donors (12, 13). This implies a different pathophysiological process between the two groups. To further support this, the duration of cold ischemic time does not appear to have an effect on DBD kidneys however is associated with reduced graft survival in

DCD transplants (14). The consequences of DGF, regardless of donor group, are clinically significant and include an increased potential for early graft loss, acute and chronic rejection and patient mortality (15-18). We can see therefore that IRI carries a significant risk of morbidity and mortality for the recipients of kidney transplants and as such is the focus of multiple avenues of investigation; this study aims to explore the impact of nitric oxide on this injury.

1.2.3. Pathophysiology of renal IRI

Historically DGF was thought to be due to kidney tubular damage secondary to ischemia and hypoxia sustained during organ retrieval. It is now thought to represent the tissue damage sustained from ischemic reperfusion injury. In the preceding discussion we can see that there are differences between donor types in renal transplantation; the patient group studied in this work is the cohort receiving renal transplants from DCD donors; therefore we shall now consider the pathological changes that occur during the ischemic phase and on reperfusion within the kidney as well as the resultant IRI and the differences between donor groups.

1.2.3.1. Ischemia

As part of the process of organ retrieval and storage prior to implantation the oxygen supply to the organ is suspended. This causes a switch from aerobic to anaerobic metabolism and consequentially a fall in intracellular adenosine tri-phosphate (ATP) levels and lactic acidosis. Tissue hypoxia causes destabilization of the cellular structure with leakage of lysosomes, dysfunction of the Na^+ /K^+/ATPase and Ca^{2+} pumps and consequent intracellular oedema (19). These changes are accompanied by opening of the mitochondrial permeability transition pore which disrupts the membrane potential of the mitrochondria and further attenuates ATP production. Within the kidney, afferent arterioles act as baro-receptors and trigger activation of renin synthesis in the macula densa in an attempt to maintain the perfusion pressure. As anaerobic metabolism takes over, oxygen free radicals accumulate, disrupting intracellular metabolic

processes and trans-cellular solute gradients. This ultimately damages the proximal tubular cell structure (20).

1.2.3.2. Reperfusion

On reperfusion with oxygenated blood tissue hypoxia is relieved and pH returns to normal, however cellular injury is exacerbated; increased production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) such as super oxide radicle (O_2^-) and peroxynitirite ($ONOO^-$) occur. These free radicals disrupt cellular membranes and the cytoskeleton and affect signal transduction in post hypoxic cells resulting in the initiation of cell death programs (19-21). Later in the chapter we will see the conflicting interactions of NO with ROS and RNS that forms part of the premise of this project. In the kidney the proximal tubular cells produce ROS and the injury sustained as a result of hypoxia and reperfusion causes acute tubular necrosis (ATN), leading to DGF. These processes are common to both types of deceased donation however there are some differences between DBD and DCD organs.

DBD organs

Upon brain death the sympathetic nervous system induces an inflammatory state and this process may commence long before organ recovery (22). Complement activation is triggered as well as endothelial cell activation with up regulation of cell adhesion molecules (intracellular adhesion molecule-1 (ICAM-1) and P-selectin), which facilitate immune cell localization (23-25). In the kidney, P-selectin in particular has been associated with neutrophil infiltration and DGF (26). Endothelial activation and inflammatory cell accumulation is exacerbated by thrombin (released from injured tissues) binding to the endothelial surface causing enhanced neutrophil localization (27), ultimately leading to endothelial dysfunction and ATN of the kidney. The duration of brain death and the time spent in the intensive care unit (prior to organ retrieval) are both associated with DGF (28, 29).

DCD organs

Although DCDs are not subject to the physiologic effects of a sustained period post brain death prior to organ retrieval, the rates of DGF are highest in patients receiving organs from DCD donors. This is attributed to the perfused ischemic time between withdrawal of treatment and asystole known as 1st warm ischemic time (1st WIT) (30). If the warm ischemic time is prolonged it results in greater risk of primary non-function (31) as well as increased rates of DGF. For this reason there are procurement protocols (variable for each organ) that limit the time of warm ischemia accepted prior to DCD organ retrieval. Although in kidney transplantation the rates of DGF are significantly higher in patients receiving organs from DCD donors, the long-term outcomes are comparable (32) and DCD organs seem to have a better outcome after DGF when compared to DBD organs (33) implying underlying differences in the pathophysiology of DGF between the groups. This study primarily investigates DCD renal transplants due to their higher incidence of DGF.

1.2.3.3. IRI in the transplanted kidney

IRI is a dynamic process that may persist for days after reperfusion (34). Once transplantation is complete the kidney responds to reperfusion in several ways. Damaged vascular endothelium releases endothelin (ET 1) causing pathological vasoconstriction (35). To counteract this effect, renal NO concentration increases. NO, which is produced by Nitric oxide synthases (NOS), at low concentrations causes vasodilation. However, when present in higher concentrations it reacts with superoxide to produce peroxynitrite (36). One of the aims of this study will be to attempt to clarify the role of NO and its metabolites in IRI; currently it is not clear whether it is protective or detrimental. It might be that at different times and concentrations it plays different roles.

The innate immune system propagates the inflammatory reaction in IRI; migration of neutrophils and macrophages into the transplanted kidney is seen within 6 hours of reperfusion (37). Macrophages stimulate chemokine synthesis in dendritic cells which in turn activate T lymphocytes (38). The proximal tubule

cells secrete Tumor necrosis factor alpha (TNF-α), Interleukin–6 (IL-6) and (Interlukin-1 beta) IL-1β (all pro-inflammatory cytokines) and up-regulate the Cxc3 receptor on their basement membranes. This allows inflammatory cells to breach the microvascular cuff (39). Within the kidney, ischemic injury results in expression of toll-like receptors (TLRs), by tubular epithelial and mesangial cells, which are involved in regulating T cell activity and have also been associated with acute rejection(40). Reperfusion activates the compliment cascade causing cell lysis within the graft; in an animal model C3 has been found to prime a T-cell mediated response contributing to acute rejection (41). Part of this project will look at the levels of inflammatory cytokines around the time of transplantation and correlate this data with other markers of injury.

The donors' immune system is also relevant after reperfusion; the donors' adaptive immune response can up-regulate major histocompatibility complexes (MHC) (42) and is involved directly with allorecognition. This response is greater in organs from older donors; it seems that there is an age associated increase in graft immunogenicity with increased inflammation, immune recognition and consequential functional loss (43). Thus donor age is an important predictor of long-term outcome in organ transplantation and a factor we will evaluate in our results

To summarize, IRI causes activation of the endothelium and inflammation that stimulates innate and adaptive immune responses, leading to injury of the graft kidney. The products of these processes are associated with the clinical syndrome of DGF. As we have seen above, in transplantation, these mechanisms are also affected by donor factors.

1.2.4. The cellular changes of IRI

IRI may cause cellular injury through several different processes, some of which occur synchronously, leading to damage of the endothelium and consequently

the transplanted organ. There are two distinct phases to the injury with some pathological processes being attributable primarily to one rather than the other.

The ischemic period is particularly associated with weakening of the barrier function of the endothelium. Hypoxia causes reduced levels of intracellular cyclic adenosine monophosphate (cAMP) and adenylate cyclase activity and this results in increased vascular permeability and leakage of the endothelium (44). The ischemic period is also associated with alterations in the transcriptional control of gene expression (transcriptional reprogramming)(45). Even after successful return of vascular supply to an ischemic organ, perfusion of the tissues may not in fact be restored immediately due to persistent endothelial dysfunction; this is known as *no reflow phenomenon* and is a further extension to the period of ischemic insult (9). It is caused by interstitial inflammation and endothelial swelling leading to obstructive micro-vasculopathy and thus prolonged ischemia (46).

The reperfusion injury phase is characterized by the autoimmune response; it involves antibody recognition of neoantigens (i.e. byproducts of metabolic pathways) and subsequent activation of the complement system (47). In addition to these processes occurring, with the appropriate physiological stimuli ischemia and reperfusion together lead to the activation of cell death programs (apoptosis, nuclear fragmentation, plasma membrane blebbing, cell shrinkage, and loss of mitochondrial membrane potential and integrity), autophagy-associated cell death (cytoplasmic vacuolization, loss of organelles and accumulation of vacuoles) and necrosis (progressive cell and organelle swelling, plasma membrane rupture and leakage of proteases and lysosomes into the extracellular compartment)(48). Ultimately the end result of these injuries is endothelial dysfunction; the endothelium is the primary site of NO production and will be considered in more detail in the following paragraphs.

1.2.5. The Endothelium

The endothelium is the layer of cells that line the blood and lymphatic vessels, it regulates vascular permeability and modulates vasomotor, hemostatic and inflammatory responses (49). Its relevance to this study is as a site of NO production and one that is damaged during IRI. The following paragraphs will explore the normal endothelial physiology and the mechanisms behind its dysfunction following IRI. In the resting state the endothelium is inactive, its primary function being to facilitate blood flow by providing an antithrombotic surface. However, it is also vital for maintenance of vascular homeostasis and therefore is has the capacity to alter its structure and function accordingly (50).

1.2.5.1. Endothelial activation

The vascular endothelium is activated in response to adverse stimuli such as ischemia, reperfusion, sheer stress or inflammation; the process of organ transplantation and therefore IRI encompasses all of these stimuli. Activation of the endothelium initiates a cascade of events leading to increased endothelial permeability and dysfunction. In response to ischemia, endothelial cells express P- selectin, L-selectin, platelet-endothelial and intercellular adhesion molecules which are all involved in leucocyte adhesion. They also secrete cytokines, such as TNF-α and IL-1, 6 and 8 and vasoactive agents endothelin and thromboxane A2. These substances induce other pro-inflammatory cytokines and promote the inflammatory responses that occur on reperfusion (51, 52). On reperfusion aerobic metabolism is re-established and, at a cellular level, the tissue starts to recover. However, the endothelial functions of vascular permeability and vasomotricity remain affected.

Endothelial dysfunction is characterized by impaired release of vasoactive chemicals such as NO, endothelium-derived contracting factors (such as ET-1 and angiotensin), pro-inflammatory, prothombolytic and growth factors. During a period of ischemia, vascular hypoxia leads to increased vascular permeability caused by low cAMP levels (44). Locally, microvascular dysfunction may be exacerbated upon reperfusion by complement activation, leucocyte-endothelial

cell adhesion and platelet-leucocyte aggregation (53). However distant physiological effects may also be seen; an animal model study found that 4-8h of ambient hypoxia (8% oxygen) caused pulmonary oedema, albumin leakage into multiple organs and elevated systemic cytokine levels (9). In humans, micro vascular dysfunction following IRI may lead to distant events such as non-cardiogenic pulmonary oedema and respiratory failure, this is caused again by increased micro-vascular permeability resulting in disruption of the alveolar capillary barrier function (54). We can see that although the injury occurs locally the effects of IRI are systemic and cause significant morbidity for transplant recipients.

As touched upon earlier, dysfunction of the endothelium that persists after reperfusion is known as '*no reflow phenomenon*'. Failure of vascular relaxation after reperfusion causes obstruction of blood flow and is associated with poor clinical outcomes. The reduction in blood flow seen is related directly to the duration of ischemia (55). In animal models sustained contraction of pericytes on micro-vessels even after resolution of hypoxia has been demonstrated. Furthermore, suppression of oxidative/nitrative stress relieves pericyte contraction. This indicates that the micro-vessel wall is a source of ROS and nitrogen radicals and that these radicals are influencing IRI-induced micro-vascular dysfunction (56). One of the aims of this study is to see if these findings in an animal model correlate with clinical outcomes in renal transplant recipients by measuring metabolites of NO in the plasma around the time of transplantation.

1.2.5.2. Endothelium derived relaxing factor

Endothelium derived relaxing factor (EDRF) is now known to be NO and is involved in the regulation of vascular tone in both the arteries and the veins (57). The endothelial cells are mechanosensors; they transduce physical forces exerted on the vessel wall by blood flow into a chemical signal. As is evident from its previous name, NO is one of these biologically active chemical signals (58). NO mediates relaxation of vascular smooth muscle by stimulating soluble guanylate cyclase resulting in the formation of cyclic guanosine monophosphate

(cGMP). Smooth muscle relaxation is seen as a result of protein phosphorylation by cGMP dependent kinases (59). Changes seen in the compliance of the vessel wall are mediated by changes in NO levels that are controlled by the endothelium.

1.2.5.3. Endothelial dysfunction and nitric oxide

At times of endothelial dysfunction, production of NO by endothelial nitric oxide synthase (eNOS) is reduced (60). The fall in NO production is due to "uncoupling" of eNOS; this not only attenuates NO production but also results in increased levels of superoxide and ultimately leads to the formation of peroxynitrite (both free radicals have been discussed previously as propagators of IRI). Uncoupling of NOS occurs when either l-arginine or tetrahdyrobiopterin are not available. Their absence causes electron flow through the enzyme to be disrupted and results in reduction of oxygen rather than formation of NO (61). Animal studies have shown that ROS generation is maximal 15 minutes after reperfusion of tissues (62); this increase in ROS coincides with the development of endothelial dysfunction and is associated with a decreased bioavailability of NO due to eNOS deactivation (63). This has lead to several areas of research into therapeutic possibilities to reduce IRI, including modulation of NOS uncoupling and NO supplementation around the time of reperfusion in an attempt to preserve endothelial function. In rat kidney models, nitrite supplementation has been shown to attenuate IRI when administered locally (64) but not when administered systemically (65). In this respect the kidney is different to both the liver and the heart, which are protected from IRI by systemic nitrite administration (66). It has been further demonstrated that the attenuation effect is mediated by conversion of nitrite to NO as opposed to by the nitrite itself (67).

We know that increased NO leads to elevated peroxynitrite levels (due to its interaction with superoxide) and that peroxynitrite promotes the inflammatory response leading to acute kidney injury. It is therefore surprising that NO appears to exert a protective effect against IRI in some organs. It has been hypothesized that this effect is seen due to the reduction of nitrite to NO at the molybdenum site of the xanthine oxidoreductase enzyme (XOR); this results in

competition for electrons and therefore limits the rate of oxygen reduction at the flavin adenine dinucleotide site of the same enzyme. Consequently superoxide, and therefore harmful peroxynitrite levels, do not rise with NO generation (64) allowing the protective effects of vasodilation and inhibition of leucocyte adhesion and platelet aggregation to prevail.

The seemingly conflicting roles of NO in IRI is a topic which we aim to explore with this study. Our chosen cohort, i.e. patients receiving kidney grafts from donation after circulatory death (DCD) donors, are expected to undergo a more severe IRI than patients receiving grafts from other donor groups for the reasons referred to earlier in this chapter. This is translated clinically into the higher rates of delayed graft function (DGF) seen in this group and is attributable to the warm ischemic and often prolonged cold ischemic times that are inevitable with this mode of organ donation.

1.3. Nitric Oxide

1.3.1. NO – Overview and discovery

Nitric oxide is a free radical gas and the first that was recognized to have signaling properties. It has an unpaired electron and is therefore extremely reactive; in the presence of oxygen it exists only transiently before being oxidized into nitrite (NO_2^-) and nitrate (NO_3^-) (68). The classical production of NO (there is also an alternative pathway) is by conversion of L-arginine into L-citrulline under the control of a group of enzymes known as nitric oxide synthases (69). Unlike the majority of bioactive molecules it is not stored and does not require exocytosis to leave a cell. It is formed endogenously at a basal rate that may be augmented by external stimuli such as cytokines. NO acts intra-cellularly by binding to the iron molecule of haem containing proteins. Many of its effects are as a result of its activation of soluble guanylate cyclase which produces 3'5'-cGMP (59); the contribution of NO to cGMP production explains, in part, its wide spectrum of effects as cGMP is a common physiological signaling molecule.

1.3.2. Production of NO

Until recently it was believed that NO was produced endogenously and that although its end products, nitrite and nitrate, could be acquired from the diet the reaction did not occur in reverse. However, it now appears that there is an alternative to the classical "L arginine NOS" pathway (70). Both sources of NO will be discussed in the following paragraphs.

1.3.2.1. Classical L-arginine pathway

The classical pathway involves production of NO by a five-electron oxidation of a guanidine nitrogen atom of L-arginine under the control of NOS (71). Nicotinamide adenine dinucleotide phosphate (NADPH), tetrahydrobiopterin and oxygen are cofactors (72) and therefore we would expect NO production during times of ischemia or hypoxia to be diminished. The reaction results in the production of L-citrulline and equal amounts of NO and superoxide (O_2^-). The NOS enzymes can be classified into constitutive (cNOS) and inducible (iNOS), with further sub classification of cNOS in to endothelial (eNOS) and neuronal (nNOS). The constitutive forms are responsible for maintaining a continuous basal level of NO and are dependent on calmodulin and calcium binding. They are, therefore, potentially affected by calcineurin inhibitors; although the exact physiology of this is debated it appears that production of NO by eNOS is attenuated by these medications (73). Inducible NOS is a cytosolic enzyme that remains quiescent unless stimulated by cytokines or endotoxins. Once induced it remains active for around 24h and it produces NO in concentrations 1000 times greater than cNOS (69).

cNOS is a dimer with each unit containing two identical subunits. iNOS is a tetrameric complex, again with each unit containing two identical subunits. Each subunit has a flavin adenine dinucleotide, a flavin mononucleotide, a tetrahydrobiopterin and a haem group. Importantly, each unit has a reductase and an oxidase domain linked by a calmodulin binding site; it is this calmodulin binding site that activates the enzyme when a calcium-calmodulin complex is

bound. This process is dependent on an increase in intracellular calcium concentration which may stimulated by a variety of agonists (59).

In whole blood NO is an unstable molecule and exists only transiently before it is metabolized in one of three ways:

- Oxidized to nitrite (NO_2^-) or nitrate (NO_3^-); NO_2^- is produced by oxidization of NO in plasma. This reaction is catalyzed by the plasma copper protein ceruloplasmin (74). NO_3^- is formed when NO reacts with oxyhaemoglobin, a byproduct of which is methaemoglobin.
- Quenched with a superoxide molecule.
- Bound to haem containing proteins such as oxyhaemoglobin (75).

1.3.2.2. Alternative pathway

NO_2^- was once considered to be an inactive end product of NO however this is now thought to be inaccurate. Reduction of NO_2^- to NO under anoxic conditions was proposed by Zhang *et al* in 1998 (76) however, it was believed that this could only take place in conditions incompatible with life. More recently it has become apparent that this reduction can occur in conditions that span the pathophysiological spectrum although still outside the normal physiological oxygen tension and pH (77). This raises the question of whether NO_2^- supplementation could replenish NO signaling and prevent ischemic tissue injury during times of hypoxia when the NOS pathway is diminished (78).

Sources of NO_2^- other than the previously discussed *classical* L arginine NOS pathway include: reduction of salivary nitrate by commensal bacteria in the mouth and gastrointestinal tract and dietary intake from meat, vegetables and drinking water (77). Four different reactions, each of them requiring different physiological conditions and substrates, have described reduction of NO_2^- to NO. These pathways respectively involve the mitochondrial electron transport system, protonation, haemoglobin and xanthine oxidase.

1. NO_2^- reductase activity has been found in the mitochondrial electron transport system resulting in reversible inhibition of mitochondrial respiration (79).

2. Acidic reduction of NO_2^- requires protonation and one-electron reduction, it will only occur in very acidic environments, e.g. in the stomach or during ischemic events when the pH falls (80).
3. Haemoglobin may function as NO_2^- reductase, although the haemoglobin saturation rate must be 40-50%, this contributes to the phenomenon of hypoxic vasodilatation (81) as NO is a potent vasodilator. Interestingly if NO_2^- reacts with oxygenated haemoglobin then oxidation of NO_2^- to NO_3^- occurs (82).
4. The final substrate is xanthine oxidase, which, at low O_2 tensions and a low pH is capable of NO_2^- reduction. This reduction reaction also produces superoxide and therefore to produce meaningful NO concentrations would require an abundance of superoxide dismutase to be present to scavenge the superoxide before it goes on to react with generated NO, forming peroxynitrite (83).

It is expected that each sub-pathway becomes relevant at different times depending on the environment. The NO produced by both the classical and the alternative pathways is identical and both are biologically active.

1.3.2.3. Nitrate – Nitrite – Nitric Oxide axis

The preceding paragraphs have demonstrated the many reactions that may occur as a result of NO production and metabolism. This may be thought of as a dynamic axis between NO_3^-, NO_2^- and NO as, depending on the physiological conditions at the time, Nitrogen and Oxygen may be present in any one of these three forms. The normal plasma concentrations of nitrate are 20-40μM whereas nitrite is much lower at 50-300nM (84) the relevance of this will become apparent when considering the methodology and results chapters later in the text. As discussed previously, NO only exists for a few seconds in the blood before reacting and forming either NO_2^- or NO_3^- therefore in this study we will be measuring both molecules. Blood samples will be taken at various time points throughout the perioperative period from patients receiving DCD renal transplants, the samples will be centrifuged immediately to separate the plasma component of the blood and frozen to -80 degrees Celsius. Although we will be

processing the samples as quickly as possible oxidation of NO will still have taken place and therefore we will need to use both NO_2^- and NO_3^- as indicators of NO production.

1.3.3. NO in physiology

NO affects many biological systems in both normal physiology and pathological conditions. The following text will consider the systems in turn and then review its role in common pathophysiology. The cardiovascular and renal systems are of obvious connection to this study whereas other systems will be referred to only briefly for completion.

Cardiovascular system and thrombus formation

Endothelium derived NO is involved in the regulation of vascular tone in both arteries and veins, although it acts differently on each vessel with veins being affected to a greater extent than arteries (57). As already described in section 1.2.5. (and therefore not repeated in detail) NO mediates the relaxation of vascular smooth muscle by stimulating soluble guanylate cyclase resulting in the formation of cGMP. Protein phosphorylation by cGMP dependent kinases causes relaxation of vascular smooth muscle and therefore vasodilation (59).

Within the myocardium NO influences contractility, it is known to be a key regulator of myocardial excitation-contraction coupling (ECC)(85). NO signaling in the heart may be either cGMP dependent, cGMP independent or as a result of metabolites of NO. The effect on the myocardium appears to be dependent on the concentration of NO seen which in turn is dependent on the isoform of NOS producing it. At low concentrations, such as those produced constitutively by eNOS and nNOS, NO exerts a positive inotropic effect by increasing ECC and therefore contractility. At the higher concentrations produced by iNOS, negative inotropic effects are seen. This is not thought to be due to the NO itself but rather to the toxic peroxynitrite that is formed as a consequence (86, 87). We expect that the contradictory concentration dependent effects of NO may be

relevant to the potential findings of this study, as both IRI and immunosuppression will stimulate the cytokines that cause up-regulation of iNOS.

NO also plays a role in thrombus formation; it causes a reduction in platelet adhesion, activation and recruitment onto the endothelial cell surface. Again, these actions are mediated by the ability of NO to stimulate guanylate cyclase and also by reducing platelet intracellular calcium levels (88). NO, in combination with prostacycline, causes platelet disaggregation from collagen fibers and inhibits platelet adhesion. Platelets produce NO as a negative feedback mechanism via the classical L-arginine pathway, thus regulating their own aggregation (89).

Renal and genitourinary system

Within the kidney NO effects vascular tone, renin release and influences sodium and water excretion (59). As seen elsewhere in the body, the renal vascular endothelium synthesizes NO inducing vasodilation. This leads to increased diuresis and sodium excretion (90). Renin secretion is affected in a time dependent fashion; initially renin release is inhibited, however after prolonged exposure to NO stimulation of renin secretion occurs (91). In chronic renal failure, dimethylamine (DMA), which is produced as a consequence of increasing creatinine, inhibits NO synthesis (92). This reduction in NO synthesis contributes to the hypertension seen in chronic renal impairment (59).

Respiratory system

NO is released within the airways by NOS and it is protective against excessive bronchoconstriction. NO has bronchodilatory effects as a result of increased cGMP production; by facilitating the production of bronchodilatory nitrosothiols (RSNO) and by its action as a neurotransmitter of the non adrenergic non cholinergic nervous system (NANC) causing relaxation of the airway vessels and smooth muscle (93-95).

Neurological system

In the brain, inhibition of NO is associated with impaired learning ability, vision, feeding, nociception and olfaction (96). Its release causes an increase in synaptic activity via increased production of cGMP (97). NOS is found in the cerebellum, hippocampus, medulla, cerebral cortex and corpus striatum (98).

Gastro-intestinal system

In the gastro-intestinal system NO mediates relaxation of longitudinal and circular muscle in the lower oesophageal sphincter, stomach, duodenum, small intestine and internal anal sphincter (99). It affects pancreatic function by regulating sphincter of Oddi tone (100) and release of insulin (101) and also plays a role in mucosal integrity within the gut; NO production increases mucosal blood flow by causing vasodilatation, while inhibition of NO synthesis restricts it. Mucosal integrity is dependent on adequate mucosal perfusion and therefore, NO production (102).

1.3.4. NO in pathophysiology

Atherosclerosis and vascular dysfunction.

The development of atherosclerosis commences with endothelial cell injury and gives rise to occlusive plaques within the arterial wall and lumen. Animal and human studies have shown that early on in this process there is loss of NO mediated vasodilation and that this may occur before any of the structural changes are seen (103). The importance of NO mediated vasodilation has been confirmed in therapeutic studies where L-arginine supplements are associated with a reduction in atherosclerosis in major vessels (104). Dysfunction of the endothelium, associated with a reduction in basal NO production, is found in myocardial infarction, angina, coronary artery stenosis and hypertension, particularly in diabetic patients.(105, 106).

Intimal hyperplasia

Intimal hyperplasia may occur as a result of the therapeutic interventions used to treat occlusive arterial disease i.e. angioplasty or bypass grafting (107). In the early stages post angioplasty the endothelium has an attenuated ability to mediate vasodilation, loss of NO synthesis contributes to this endothelial dysfunction (59) and this is thought to lead to formation of a fibro-cellular lesion in the overlying endothelium (58). Autologous vein bypass grafts can develop stenosis over time, which may lead to failure of the graft (108), dysfunction of NO mediated relaxation is found within the endothelium of the graft and this is believed to contribute to the generation of stenosis (109). In humans, use of the internal mammary artery for coronary artery bypass, as opposed to a reversed vein graft, is associated with better long-term outcomes; these grafts maintain production of NO and endothelial derived vasodilatory function during the grafting process, which is thought, in part, to explain its superior outcome (110).

Inflammation and sepsis

NO is involved in the process of inflammation and in the clinical syndrome of sepsis. During an inflammatory response NO enhances vasodilation, formation of oedema and modulation of sensory nerve endings (111). Inhibition of NO reduces the magnitude of the inflammation as seen when NOS inhibitors or glucocorticoids are given (112). In the context of sepsis, bacterial endotoxins induce iNOS in vascular smooth muscle, cardiomyocytes and the endocardium. At the high concentrations produced by iNOS, NO causes reduced vascular tone leading to venous pooling and cardiac dysfunction (113) and is therefore a negative inotrope. On one hand, inhibition of NO maintains blood pressure and prevents hypotension (114) but on the other it reduces cardiac output and raises pulmonary vascular resistance (115) this is another example of the dichotomous effects of NO when it is produced by iNOS in high concentrations. Macrophages that are stimulated by the pro-inflammatory cytokine Interferon gamma (IFN-γ) also synthesize NO in high concentrations, this time with protective effects; at these concentrations NO interferes with deoxyribonucleic acid (DNA) synthesis by interacting with iron containing enzymes, causing cytotoxicity to bacteria and

therefore assisting the host immune system to eliminate the bacterial infection (116).

NO affects every system within the body and has a broad spectrum of actions. However common themes are seen within each system and they are: vasomotor effects, effects on the immune system and its role in inflammation. Ischemic reperfusion injury is a condition involving all of these processes and it is therefore logical that NO should play a significant role within it. We will be studying the levels of the metabolites of NO found after the IRI that occurs as a result of organ transplantation. In the following text we will look at what is known currently about NO in the context of IRI.

1.4. Nitric Oxide and IRI

As we have already explored, IRI is an important consideration in organ transplantation. However, the consequences of NO release in IRI are debated, as there is evidence of both protective and deleterious effects. The interplay between NO and ROS is thought to be pivotal to clarifying the answer to this question, however, in-vitro and in-vivo studies contradict one another and it remains unresolved. There is robust evidence that NOS activity is increased in the early ischemic phase. On reperfusion, ROS are generated which may react with NO. This results in both molecules either quenching each other or acting as a substrate for further ROS production. NO and the toxic ROS, superoxide (0_2^-) react rapidly, eliminating them both and therefore exerting a cyto-protective effect. The product of this reaction however, is the highly toxic radical, peroxynitrite ($ONOO^-$) (117). The following paragraphs will explore the mechanisms by which NO may be playing a cyto-protective role and illustrate why it is important to study its role within this investigation.

1.4.1. Cyto-protective effects of NO

During the ischemic phase of IRI NO is generated which, in-vitro, has been shown to damage DNA and inhibit its repair (118). In ischemic conditions NO has also been shown to damage the actin cytoskeleton, altering renal tubular epithelial function (119). However in post-ischemic conditions, as those occurring in transplantation, NO does not appear to be purely cytotoxic, as it also seems to have a variety of cyto-protective effects.

Antioxidant

There are two proposed mechanisms by which NO acts as an antioxidant. The first is related to its reaction with superoxide during which peroxynitrite is formed; peroxinitrite then goes on to act as a superoxide scavenger preventing the formation of other free radicals such as hydrogen peroxide (H_2O_2) and hydroxide (120) (despite this seemingly protective effect, peroxynitrite itself, particularly in high levels, is toxic causing DNA damage, cellular injury and death (85, 121)).

Secondly, NO inhibits mitochondrial respiration and therefore reduces the formation of ROS which would otherwise be produced during ischemic injury (122); NO modulates mitochondrial respiration by competing with O_2 for cytochrome-c oxidase binding. During ischemia, as pO_2 falls, a higher proportion of cytochrome-c oxidase is therefore, bound by NO. During reperfusion as O_2 becomes available again, because of its competitive binding, NO prevents abrupt resumption of the electron transport chain and therefore attenuates the formation of ROS (123). Thus in the context of IRI is seems that NO may have a protective effect by reducing ROS production.

Anti-inflammatory – Anti-TNF-α + Anti-IL-1

NO is known to inhibit neutrophil infiltration and reduce the production of pro-inflammatory cytokines (124) despite its production being promoted by them. TNF-α induces the accumulation of neutrophils and the production of other pro-inflammatory cytokines. Studies have shown that administration of NO donors

such as molsidomine and nitroprusside reduce TNF-α and IL-1 production (125, 126) and therefore inflammation and tissue injury. In an animal model this was associated with improved renal function and a decreased inflammatory response after renal ischemia (125). Both TNF-α and IL-1 may be up-regulated as part of the inflammatory response to IRI in the context of organ transplantation, NO may be attenuating this and therefore providing a protective role by reducing inflammation.

Anti-adhesion molecule

After ischemic reperfusion injury cellular adhesion molecules (CAMs) are up-regulated. CAMs such as selectins are mediators of the initial attachment between polymorphonuclear neutrophils and activated endothelium and therefore play a critical step in reperfusion injury (127). It has been demonstrated that infusion of an NO donor prevents P-selectin expression on endothelial surfaces (128) and that other CAMs, such as vascular cell adhesion molecule-1 (VCAM-1), ICAM-1 and endothelial-leucocyte adhesion molecule-1 (E-selectin) are also inhibited (129). Thus, in this context, NO seems to inhibit binding of neutrophils with activated endothelium thereby preventing progression of IRI.

Apoptotic effect

NO may act in both a pro-apoptotic and anti-apoptotic manner. TNF-α induced apoptosis of endothelial cells caused by oxidative stresses (including IRI) may be reduced by NO administration however this effect is only seen at low concentrations; at higher concentrations necrosis and apoptosis still occur. The same study also looked at the role of cGMP in this pathway; it seems that NO reduced cGMP dependent apoptosis at low concentrations however in higher doses cGMP independent apoptosis was induced (130). This study implies that at low levels, such as those produced constitutively in physiology, NO is protective but at higher concentrations, such as those produced in the context of ischemia or inflammation, it was detrimental. This is contradicted however by more recent work in which an animal model of renal IRI was used; this study found that decreased apoptosis was seen after administration of exogenous NO

and this also correlated with down regulation of the p53 gene (131). Further work on NO and p53 has shown that NO inhibits a family of cysteine proteases called caspases. Caspase activation is required for p53-dependent apoptosis and therefore explains the reduction in apoptosis found when NO is administered (132). NO clearly plays a role in apoptosis however whether that role is pro or anti – apoptotic seems to be dependent on the concentration and also the mechanism of apoptosis that is prevalent i.e. TNF-α, cGMP or p53.

NO as a therapeutic target.

We have seen that NO is important in many biological systems and therefore therapies that either regulate NOS activity or produce NO exogenously have become an expanding area of research. Despite many investigations into their therapeutic role, very few have made it into clinical practice. Organic nitrites are the obvious exception to this; isosorbide mononitrite and glyceryl trinitrite are extensively used for their vasodilatory properties to treat angina, cardiac failure, pulmonary oedema and anal fissure however their use is accompanied by side effects such as hypotension, headaches and evolving tolerance (77).

1.4.2. NO concentration; iNOS and eNOS

Resultant NO concentration is emerging as a recurring theme that appears to influence the consequences of its production. The concentration produced is dependent on the isoform of NOS that is activated; in IRI NO is produced by both constitutive and inducible NOS, however the majority is by iNOS (with concentrations of 1000 times greater then eNOS). The renal expression of eNOS and iNOS occurs in different parts of the kidney and varies in response to ischemia (133); eNOS is found in the vasa recta, inner medullary collecting duct and glomeruli (134) whereas iNOS is expressed by vascular smooth muscle, renal tubular cells and immune cells such as monocytes, macrophages and neutrophils (135, 136). Inducible NOS expression in the nephron has been shown to increase by up to 30 times during IRI, its peak being at one-hour post reperfusion. In contrast, eNOS expression diminishes in response to either

ischemia alone or to IRI, an effect that is not neutralized until 3 hours after reperfusion (60). Although the NO produced by the different isoforms of NOS is identical, its effects on the kidney are contradictory; NO produced by eNOS up-regulation appears to prevent vascular dysfunction (by preventing platelet aggregation and leucocyte activation) (137) and also to promote vasodilation (138), thus having a protective role in IRI. Furthermore, inhibition of eNOS worsens cortical and medullary blood flow and oxygenation (139). INOS-derived NO however, seems to contribute to vascular dysfunction (140) as a result of increased production of peroxynitrite and inhibition of protective eNOS-derived NO. Several studies have reported that inhibition of iNOS attenuates renal injury in IRI (141-145).

The conflicting effects of NO are demonstrated effectively by *Yates et al*; using an ex vivo porcine model and NO supplementation they found a biphasic response to NO donation that initially resulted in increased renal blood flow in the treated group. However, this effect was not sustained and between 30 and 180 minutes the renal blood flow in the control and treatment groups converged. Intra-renal resistance, unsurprisingly, demonstrated a reciprocal relationship. The initial increase in flow is thought to be due to the vasodilatory effects of NO that are well documented. However it is the fall in blood flow between 30-180 min that highlights the conflicting functions of NO. The authors of the study proposed that rising levels of peroxynitrite and superoxide caused endothelial dysfunction thus preventing vasodilation (146). This inconsistent response is not just limited to NO as peroxynitrite also has conflicting properties; in low doses it increases renal blood flow due to indirect vasodilation however at higher doses this effect is not only lost but vasoconstriction occurs. If NOS activation is also inhibited, further vasoconstriction and reduction in renal blood flow occurs (147) it is thought that at low concentrations and in the presence of activated NOS, peroxynitrite may be reduced back to NO and/or NO_2^- hence the vasodilator effects seen.

Concentration appears to be emerging as significant in the interpretation of the NO:superoxide axis. The complex and varied reactions of these ROS that may

occur, depending upon the physiological conditions at the time, make IRI an interesting and challenging environment in which to investigate them. This study will attempt to explore further the questions that remain unanswered with regards to NO and IRI by measuring concentrations of nitrite and nitrate at a various time points during reperfusion of kidney transplants and by relating this to physiological inflammatory reactions occurring concurrently.

1.5. Cytokines

1.5.1. Cytokines – Overview and discovery

The simplest definition of a cytokine this author has encountered is the description of them as a "chemical messengers", however Jan Vilcek proposes a more comprehensive definition: (148) "*Cytokines are regulatory proteins secreted by white blood cells and a variety of other cells in the body; the pleiotropic actions of cytokines include numerous effects on cells of the immune system and modulation of inflammatory responses".* No short description exists to encompass all of the defining features of a cytokine however there are characteristic properties that can be explained to help understand their function and purpose. It should be noted that these properties are not exclusive to cytokines.

Features of cytokines include:

- They are soluble proteins or glycoproteins, most being ≤ 30 kDa in size.
- Their production and release are often described as 'burst like', i.e. transient and in response to a stimulus, rather than at a constant background rate.
- Their actions are mediated by binding to specific cell surface receptors.
- Their actions are usually caused by modification of gene expression in the target cell and may lead to changes in the rate of cell proliferation or differentiation of the cell.
- Their actions are varied and diverse but each one has some effect on cells within the haematopoietic system (148).

1.5.2. Structure, function and family groups

Cytokines may be grouped into six distinct "families" according to the structure of their receptors (149), see appendix 1. The question of "*What does an individual cytokine do?"* is a difficult one to answer succinctly. Cytokine function is highly contextual; its actions on a cell depend upon the conditions that the cell is exposed to at the time. The interactions of cytokines are described as synergistic and antagonistic; cells exposed to different combinations of multiple cytokines may have completely different responses and those responses may be greater than those produced by an individual cytokine. An example of this may be seen in the reaction of damaged epithelial cells in the colons of patients with inflammatory bowel disease; stimulation of HT-29 cells with IFN-γ or TNF-α alone induces production of IL-8. However in combination, they cause apoptosis (150). Transforming growth factor (TGF-β) in the liver provides a good example of another cytokine property known as *pleiotropy.* In chronic liver disease TGF-β is involved with regulation of proliferation of hepatic progenitor cells (thereby preventing hepatocellular carcinoma). It also plays a role in blunting inflammatory responses within the liver as well as mediating wound healing and immune responses (151). Pleiotropy describes the multiple actions and multiple cell targets that individual cytokines may influence. Conversely different cytokines may have similar actions and this is termed *redundancy*.

One cytokine may stimulate or inhibit the production of another. When this action is stimulatory it may involve multiple cytokines and is known as a *cytokine cascade.* A well described example of this is IL-1, which, exerts its action on T cell proliferation by stimulating IL-2 (152). Two further important functions of cytokines are their ability modulate cytokine receptor activity; this may be through *receptor transmodulation* (when a cytokine influences receptor expression of a different cytokine) or *receptor trans-signaling,* where a cytokine may interfere with the signaling properties of a receptor.

The clinical effects of cytokines include; regulation of the immune system and inflammation by their influence on leucocyte migration; haematopoietic cell

proliferation; the systemic inflammatory response; temperature regulation; tissue remodeling and cell survival. As previously discussed these actions are the result of the pattern of release of multiple cytokines and rarely attributable to a single cytokine (149). As part of this study on NO and IRI we will measure some of the cytokines we believe may be influential around the time of kidney transplantation. We will also look for relationships between cytokines and induction immunosuppression as this may cause an overriding effect in the context of this project. The following paragraphs will review what is already known about cytokines in transplantation and specifically that which is relevant to this study.

1.5.3. Cytokines in transplantation

T-cells play a crucial role in transplant immunology; CD4+ helper T-cells secrete IL-2, IL-4, IL-5 and IFN-γ. Graft damage is caused by cytotoxic T-cell responses, antibody mediated responses and delayed-type hypersensitivity reactions; all of which are modulated by these cytokines (153). IL-2, released directly by CD4+ cells, causes CD8+ T-cells (in turn activated by recognition of donor MHC class 1 antigens) to differentiate further into cytotoxic T-cells (154). CD4+ T-cells initiate a delayed-type hypersensitivity reaction and release INF-γ and TNF-α, which causes recruitment of macrophages and monocytes (155). The patterns of cytokine release in relation to CD4+ (T-helper) cells have been simplified into two groups; T-helper 1 and T-helper 2 (156). T-helper 1 lymphocytes produce IL-2, IFN-γ and TNFβ; the differentiation of T-cells into T-helper 1 lymphocytes is controlled by IL-12 and IFN-γ. In contrast, IL-4 and IL-13 promote the generation of T-helper 2 lymphocytes (157), which then produce IL-5, IL-6, IL-9, IL-10 and IL-13. Broadly speaking, T-helper 1 cytokines are thought to promote rejection (158), where as T-helper 2 cytokines are believed to be protective against it and to encourage allograft acceptance or tolerance (159). They also stimulate antibody production by B-cells. Multiple cytokines regulate B-cell behavior including IL-4, IL-5, IL-6, IL-10 and IL-13 (160).

1.5.4. Cytokines effecting NO production

IFN-γ, in combination with either TNF-α or IL-1, activates the transcription factor nuclear factor - κβ (NF-κβ). NF-κβ results in up-regulation of iNOS in activated macrophages, therefore causing increased NO production (161). However as we have previously discussed, the nature of cytokines, the immune system and IRI are such that in order to account for the multiple actions and reactions occurring simultaneously, it is prudent to consider a panel of cytokines to look for changes and associations. For the purposes of this study we will be looking at IFN-γ, IL-2, IL-6, IL-10, IL-17a and TNF-α; all of which are involved in the immune response to organ transplantation. Each will be discussed in the following paragraphs.

1.5.4.1. IFN-γ

IFN-γ (also known as Macrophage Activating Factor (MAF)) is produced by CD8+ and CD4+ T-cells as well as Natural Killer (NK) T-cells. IFN-γ is involved in many of the process of the inflammatory and immune responses including proliferation and differentiation of T-cells, B-cells, macrophages, NK-cells, endothelial cells and fibroblasts (162). It acts on antigen presentation cells (APCs) to enhance HLA expression and also has antiviral and anti-proliferative actions (163, 164). As mentioned previously, IFN-γ is involved in the up regulation of iNOS (and therefore NO production) and also the host's T-cell response to the graft, it is also likely to be affected by immunosuppressive agents (as described later in this chapter). By measuring it at specific time points we hope to see what effects are most influential in the context of renal transplantation; is its concentration in plasma linked to NO production, or are the known responses overshadowed by the effect of induction immunosuppression?

1.5.4.2. IL-2

Initially known as T-cell growth factor, it is not surprising that IL-2 stimulates growth and differentiation of T cells. It is produced by T-cells and also influences the behavior of many other immunological cell lines including B-cells, NK-cells,

Lymphokine-activated killer (LAK) cells, monocytes, macrophages and oligodendrocytes (165). It can induce production of other cytokines including TNF-α and IFN-γ and broadly may be considered a pro-inflammatory cytokine. In the context of this study, Simulect, one of the induction immunosuppressive therapies, is an antibody that specifically targets the IL-2 receptor. We will therefore be looking to see of this translates into a detectable effect in the plasma samples.

1.5.4.3. IL-6

IL-6 has also been known by the name IFN-β2 and B-cell stimulatory factor 2; it is produced by both T and B-cells, as well as macrophages, bone marrow stromal cells, fibroblasts, keratinocytes and endothelial cells. It acts to regulate function of T and B-cells, haematopoiesis and acute phase responses (149). In the kidney, IL-6 appears to have both protective and detrimental effects; it is released as a pro-inflammatory mediator as part of the T-cell immune response and is found to be elevated early after reperfusion (166). However it also plays a role in the repair of the tissues via a trans-signaling mechanism that attenuates oxidative stress, limiting further tissue damage and activating signal transducer and activator of transcription (STAT) 3 in the epithelial cells to promote resolution of the injury (167, 168).

Once again we see that the mediators and products of IRI have dichotomous functions in renal transplantation and in the context of this study we may find that precise roles are difficult to define. In order to correct for this we are measuring multiple cytokines and will be looking for changes as opposed to absolute values.

1.5.4.4. IL-10

Although IL-10 has both inhibitory and stimulatory effects, on the whole in the context of transplantation it is considered an *immunosuppressive* cytokine; it inhibits the maturation of dendritic cells (preventing T-cell activation and recruitment of NK cells (169)) and has an inhibitory action on T-helper 1 cells where it blocks cytokine synthesis (including IFN-γ and TNF- α (170)). Its

stimulatory effects include roles in B-cells, thymocyte and mast cell proliferation (149). Its relevance to this study is to see if IL-10 production differs in any way from the other *pro-inflammatory* cytokines and also to see if it has any detectable relationship with IFN-γ or TNF-α.

1.5.4.5. IL-17

IL-17 is a stimulatory and pro-inflammatory cytokine; it is produced by CD4+ T-cells, mast cells, neutrophils and macrophages and its effects include recruitment of neutrophils and monocytes and production of IL-6, IL-8 and TNF-α (171-173). In the kidney, IL-17 producing T-cells have been found to promote inflammation and induce production of IFN-γ and TNF-α (174). Furthermore IL-17, in combination with IFN-γ, is thought to be important in renal IRI (175). IL-17 is intimately linked with the cytokines we are measuring, in particular IFN-γ and TNF-α, which are important for NO production.

1.5.4.6. TNF-α

TNF-α has a varied role in inflammation and immunity regulating growth and differentiation of many cell lines. It acts synergistically with IFN-γ and many other cytokines. Secretion is primarily by activated monocytes and macrophages although it may also be produced by T-cells, B-cells and fibroblasts. Its receptors are found on most cell types with the exceptions of erythrocytes and inactive T-cells (149). TNF-α is released from the kidney in response to IRI; it causes a reduction in glomerular filtration rate (GFR) via glomerular fibrin deposition, cellular infiltration and vasoconstriction. In the kidney, binding at the TNF receptor may induce cell apoptosis (176, 177), which is one of the hallmarks of IRI. As previously mentioned TNF-α and IFN-γ act synergistically to cause up-regulation of iNOS and are therefore linked directly to NO production.

1.5.5. Cytokines – NO; Inflammatory axis

As we have already discussed, the production of NO by iNOS is stimulated by pro-inflammatory cytokines via their effect on NF-κβ. The resultant increase in

NO concentration further perpetuates the inflammatory process resulting in ongoing production of pro-inflammatory cytokines and increased inflammation. This effect is seen due to the production of free radicals (peroxynitrite and superoxide) which occurs as a result of the reactivity of NO and the high concentrations of NO that are produced by iNOS (178). One of the challenges of interpreting the results of this study will be the many concurrent process and influences that may affect our analytes; we anticipate that the induction immunosuppressant may be particularly influential. The following chapter will consider the immunosuppressive drugs that are of relevance to this project and discuss their mechanisms of action, with particular reference to their effect on cytokines, which may be important in the interpretation of the results of this study.

1.6. Immunosuppression

The introduction and development of immunosuppressive agents for transplantation was a breakthrough in the field of transplant surgery and it is therefore reasonable to say that without immune manipulation, organ transplantation would not be possible. Today, immunosuppressive agents are used around the day of transplantation (induction), for maintenance and in the event of episodes of rejection (rescue therapy). The exact regimen will vary between transplant units depending upon local policy but the purpose of each is set out below. For the purposes of this project we will only explore in detail the medications which may exert an effect on our results i.e. those administered around the time of surgery; other medications will be mentioned but not described in detail.

Induction immunosuppression is an intense, upfront treatment that aims to inhibit the immune response at the time of transplantation; it might affect various aspects of the immune response not necessarily targeting rejection. It is important to note that its prolonged use is unsustainable due to its toxicity. Maintenance immunosuppression is used for the life of the graft and is often

comprised of two or three agents; it must therefore be tolerable for sustained use. Rescue therapy is comparable to induction therapy and indeed, the agents used may be the same. It is an intensive regimen designed to treat allograft rejection. Figure 1.1 below depicts the cellular mechanism of both the induction and the maintenance agents used by our unit.

Immunosuppression – Mechanism of action

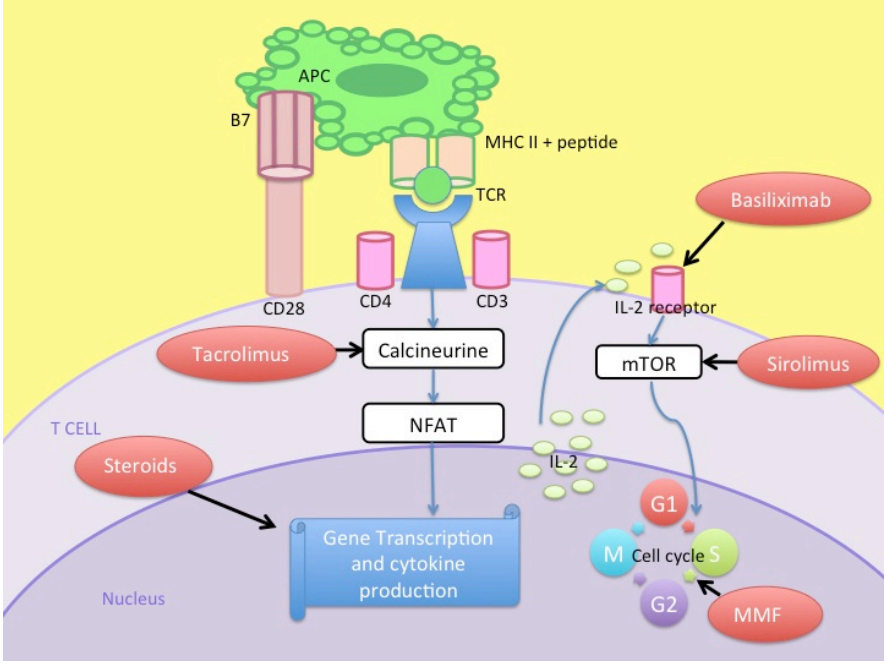


Figure 1.1: Immunosuppression – Mechanism of action. *Demonstrating the sites of action of commonly used immunosuppressants in their prevention of T-cell activation and proliferation.*
(APC: Antigen presentation cell. TCR: T-cell receptor. NFAT: Nuclear factor of activated T cells. MMF: Mycophenalate mofetil) (179).

1.6.1. Induction therapy

On the day of transplantation, both pre and intra-operatively, an intense prophylactic immunosuppressive therapy is administered to reduce the risk of early acute rejection and graft loss. Regimens vary between transplant centers but broadly they consist of a combination of biological agents and corticosteroids.

1.6.1.1. Biological agents

Biological agents act to target particular cell surface receptors (including antibodies) resulting in a clinical effect. Within the field of organ transplantation biologics are used in the form of either polyclonal or monoclonal antibodies for induction immunosuppression.

Polyclonal antibodies; ATG

Polyclonal antibodies are derived from animals, usually rabbits or horses. The preparation used by the Cardiff Transplant Unit and many others, particularly in USA and France, is ATG; when administered to the patient it causes profound lymphopoenia via a variety of mechanisms. T-cell depletion is caused primarily by complement mediated cell lysis although antibody mediated cytotoxicity and activation of cell death programs both contribute (180). B-cell depletion occurs by induction of apoptosis in immature B-cells, activated B-cells and mature plasma cells within the bone marrow. As well as its effects on T and B-cells, ATG may also cause alteration in function of dendritic and NK T-cells (181).

Side effects of polyclonal antibodies may be acute or delayed. Acutely, cytokine release syndrome (a dose dependent effect, experienced most commonly after the first exposure) may cause febrile episodes, rigors, nausea, vomiting, diarrhoea, dyspnea, headaches, skin rashes, hypotension and even anaphalaxis. Cytokine release occurs as a result of cell surface receptor engagement, in particular TNF-α, IL-1 and IL-6 (182); stopping the infusion and administering steroids is used to treat the syndrome. More severe reactions have been reported with ATG use, including myocardial infarctions, pulmonary oedema, tachy-arrhythmias and death (54). In our unit because of the risk of severe reactions, anti-histamines and steroids are given routinely prior to infusion of polyclonal antibody.

Delayed effects include pancytopenia, serum sickness and infections. Another important side effect of biological therapies is the significantly increased risk of developing post transplant lymphoproliferative disease (PTLD). This is due to a combined increased intensity of immunosuppression as opposed to use of a sole

agent (1). Despite the extensive list of side effects, ATG is still used routinely in transplant recipients as studies show significantly reduced rates of acute rejection when compared with a group receiving no induction immunosuppression (183). Long-term graft and patient survival rates and renal function are not found to be significantly different between the two groups (184).

Monoclonal antibodies

Monoclonal antibody preparations contain a single antibody type that binds to a single cell surface antigen, thereby limiting their spectrum of action but increasing their specificity and reducing the incidence of unwanted effects. There are many different monoclonal antibodies available and they are used to treat a variety of conditions including malignancies and autoimmune diseases. We will be considering two such antibodies that are used for induction immunosuppression in solid organ transplantation.

Monoclonal antibodies bind to specific cell surface epitopes; Campath (Alemtuzumab) is a humanized monoclonal antibody against CD52. Binding causes complement fixation and destruction of the target cell by apoptosis. CD52 is predominantly expressed on T and B-lymphocytes although it is also found to a lesser extent on NK T-cells, monocytes and macrophages (185). Studies on Campath have found that it reprograms the immune system, suppressing production of *pro-inflammatory* IFN-γ and IL-17 and increasing *anti-inflammatory* cytokines such as IL-4 and IL-10 (186). It was initially licensed for use in lymphoma, however it has been investigated in renal transplantation with satisfactory short-term outcomes; long-term results are still unknown. It is not routinely used in our unit, however as part of a concurrently running multi-center randomized control trial, some of the subjects of this study did receive Campath. Side effects include cytokine release syndrome although this is usually mild, particularly when compared with the effects of ATG. Rare anaphylactic reactions have been reported and other effects include neutropenia, anemia, thrombocytopenia and autoimmune thyroid disease (187).

The use of Simulect (Basiliximab) is more wide spread. Simulect is an anti CD25 antibody *(Fig. 1)*, CD25 is found on the IL-2 receptor and is expressed by activated T-cells and mature dendritic cells. The clinical effects of Simulect are caused by inhibition of T-cell activation and proliferation. The antibodies remain in the patient's system for up to 3 months, causing destruction of activated T-cells during this period. Unlike ATG, Simulect does not typically produce a first dose reaction (although it is possible to have anaphylactic responses) and is relatively free of other side effects. In renal transplantation Simulect induction significantly reduced episodes of acute rejection when compared with no induction immunosuppression. However, although historically the occurrence of rejection predicts graft loss, no benefit in five-year graft survival has been demonstrated (188-190). These findings have been echoed in the field of liver transplantation in a recent systematic review (191).

A number of studies have compared ATG with Simulect; the evidence is slightly conflicted, but suggests overall that ATG is of benefit in moderate to high immunological risk deceased donor recipients (192-194); this is the strategy adopted by our unit. No demonstrable benefit of ATG has been detected in the low immunological risk group (195) and therefore Simulect is used in this group by our unit due to its favourable side-effect profile. During part of the subject recruitment and sample collection phases for this project, our unit was participating in a national multi-center trial that involved randomization to receive either Campath or Simulect induction. Therefore when we come to explore the results we have three different induction immunosuppression groups to consider (those who were not enrolled in the national study received the standard departmental induction, which at that time was ATG).

1.6.1.2. Corticosteroids

Corticosteroids are used for induction, maintenance and rescue therapy in renal transplantation. They are anti-inflammatory as well as immunosuppressive drugs and have a therapeutic role in a variety of conditions. In renal transplantation, intravenous methylprednisolone is used at a high dose at induction and may also be used in the treatment of acute rejection. Oral

prednisolone is used for maintenance; the dose starts initially at 20mg daily and is weaned over a period of time depending upon clinical and patient factors. The effects of prednisolone are predominantly glucocorticoid although it does have some mineralocorticoid activity.

Corticosteroids influence the gene transcription of pro-inflammatory cytokines, particularly IL-1 IL-2, IL-6, TNF-α and IFN-γ. A reduction in the production of these cytokines *(Fig. 1)* results in a blunting of the inflammatory response but perhaps more importantly, impairs the function of circulating macrophages and CD4+ T-cells. Other important effects of corticosteroids include a reduction in prostaglandin, histamine and bradykinin release and decreased capillary permeability (196).

1.6.2. Maintenance therapy

Maintenance immunosuppression is used for the life of the graft to prevent rejection; it is comprised of a combination of two or three agents and is taken daily. Maintenance therapy is usually started on the first post-operative day; it is therefore not relevant to the main focus of this study, which is in the few hours following reperfusion of the graft. The agents used include Cyclosporine and Tacrolimus (both calcineurin inhibitors); mycophenolate mofetil (MMF) and mycophelolate sodium (Myfortic) (both inhibitors of nucleotide synthesis) and Sirolimus, which is a mammalian target of rapamycin (mTOR) inhibitor.

The 3C trial (which was recruiting at the same time as this study and in which several of our subjects were also participating) comprised 2 treatment arms. The first group received Campath induction, Myfortic and calcineurin inhibitor (CNI) maintenance, switching to Sirolimus at 6 months. The second group received Simulect induction and standard maintenance (CNI and Prednisolone). Initial results have a reduction in acute rejection in the group receiving Campath induction followed by low dose CNI, Myfortic and steroid avoidance (197). The

long-term results of the trial, including those from the group who switched to mTOR inhibitors at 6 months, are awaited.

The preceding text has highlighted the likely importance of the immunosuppression on the results of this study via their effects on cytokine release. One of the aims of this project is to look at the effect different immunosuppression regimens have on cytokines and how this may affect NO levels.

1.7. Therapeutic Approach to IRI and Clinical Implications of DGF

We have seen how IRI results in damage to grafts used for organ transplantation as well as to other organs in vivo after ischemic events such cerebrovascular accident or myocardial infarction. Therapeutic approaches to attenuate the injury have therefore been studied widely. In organ transplantation, the clinical manifestation of IRI is DGF and this may be reviewed by considering the risk factors that predict injury as well as interventions in the recipient, organ preservation and interventions in the donor.

1.7.1. Risk factors for DGF

The most important epidemiological associations for DGF are cold ischemic time; DCD kidneys; donor age; donor body mass index (BMI) and donor creatinine levels (198). Many attempts have been made to develop scoring systems that quantify an individual's risk for DGF, however they have thus far been unreliable or impractical. Experimentally, levels of urinary IL-18 and NGAL (neutrophil gelatinase-associated lipocalin) at day zero may be predictors of DGF (C statistic = 0.9) (199). Biomarkers in machine perfusate (taken from hypothermic machine perfusion) have also showed associations with DGF (200). Our group has a strong interest in this area.

Recipient characteristics

Certain patient characteristics in the recipient may also place them at increased risk of DGF, with maintenance dialysis prior to transplant being the single greatest influence (201). Other factors including high BMI, diabetes, age > 55 years, male sex, African American race, prolonged waiting period, immune sensitizing events and small for size organs (198).

1.7.2. Prevention and treatment targets

1.7.2.1. Recipient

Identification of those at high risk of DGF through the use of biomarkers and genomic studies preoperatively may facilitate targeted interventions with pharmacological agents. Single nucleotide polymorphisms (SNP) for TLR3 have been identified, as well as a polymorphism in the TNF gene; these polymorphisms have both been found in a higher than expected frequency in patients with DGF (202, 203). Other polymorphisms in IFN-γ might also be cytoprotective and we hope to be able to explore this by measuring these cytokines in our subject group.

Pharmacological interventions in the recipient

Possible pharmacological approaches for attenuating DGF in the recipient include vasodilatory agents, anti-inflammatory agents and induction immunosuppression. Agents that prevent vasoconstriction, such as endothelin receptor antagonists, calcium channel blockers and adenosine A1 receptor antagonists, have been studied with inconclusive results (204, 205).

NO itself has been investigated in animal models of heart, lung, liver and kidney IRI. Its administration has been either by inhalation, (i.e. a systemic treatment) or by topical application of L-arginine, the primary substrate for NOS. Several studies have found that inhaled NO reduces infarct size and improves cardiac function in mouse models of IRI (206, 207). In the lungs, both inhaled NO and NO augmentation has shown promising results in animal models of IRI (208). In

liver transplantation, supplementation with NO exerted a protective effect on the graft (209, 210) and similar findings have been reported in the kidney (211, 212).

It is important to note that the studies referenced above which are reporting on NO augmentation are describing indirect effects of NO, i.e. therapeutic agent is administered which modulates the NO axis in some way to increase NO concentration. The authors are therefore hypothesizing that the effects seen are directly attributable to NO; from what we know about the NO axis there are likely to be several other molecules generated that may also be influential.

Anti-inflammatory agents that prohibit migration of cells involved in either the innate or adaptive immune system have been considered with varying degrees of success in the treatment of IRI. These agents include recombinant P-selectin glycoprotein ligand IgG fusion protein; QPI-1002, which inhibits p53 activation, Erythropoietin and CXCR inhibitors. Although the theoretical benefits are discernible, none have shown conclusive evidence in clinical application (213).

Maintenance immunosuppression is usually commenced as triple therapy involving a steroid, mycophenolic acid and a calcineurin inhibitor. Calcineurin inhibitors (such as Tacrolimus) mediate vasoconstriction. A recent meta-analysis showed calcineurin inhibitor avoidance in the immediate post operative period was associated with significantly less DGF. However, unsurprisingly, it also observed significantly higher rates of acute rejection (214). Although all of the therapies above have been trialed, to mitigate DGF in various organ systems, none of them have been incorporated into routine clinical practice. It would therefore be reasonable to conclude that therapeutic agents to prevent or treat DGF remain at an experimental stage.

1.7.2.2. Organ preservation techniques

Once the organ has been retrieved there is a window of time available to transport and implant it without occurring permanent tissue damage; this is known as the 'cold ischemic time' (CIT) and as mentioned previously is and

important factor in the development of DGF. Cooling the organ (to 4°C) decreases the cellular metabolic rate and therefore attenuates injury (215). Preservation solutions are designed to maintain osmotic gradients and to delay acidosis with H+ buffer systems; they are used along with cooling to transport the organ in a standard method of *cold static storage*.

Hypothermic machine perfusion is now becoming a widely used practice within renal transplantation; studies have found it reduces rates of DGF particularly in ECD and DCD organs (216, 217). Normothermic machine perfusion has also been investigated although it is still at an experimental stage. This is a warm oxygenated perfusion, the aim of which is to maintain cell metabolism but may also allow the delivery of pharmacological agents or gene therapy (218-220).

1.7.2.3. Interventions in the donor

Use of dopamine in stable DBD donors, up to the point of cross clamping during the donation, has been investigated; rates of DGF were found to be reduced in the treated group (24.7% vs 35.4%; $p = 0.01$), subgroup analysis found that this effect was greatest in organs whose cold ischemic times were 17.1-34.4 hours (221). Calcium channel blockers have also been used in the donor, in the recipient and in the perfusion fluid, with reduced rates of DGF reported in the treatment groups (222). Administration of Diannexin (which blocks a pro-inflammatory pathway) to marginal donors prior to organ retrieval has been associated with reduced incidence and duration of dialysis in recipients (223). Once again none of these interventions are used routinely in clinical practice and can largely be considered experimental only.

Ischemic preconditioning

Ischemic preconditioning is an experimental strategy in which exposure to short, sub-lethal episodes of ischemia results in attenuated tissue injury following subsequent prolonged ischemia and reperfusion. The intervention can be performed in both the donor and the recipient and may be at a site remote to the graft. This phenomenon is profound in animal studies and clinical trials are now being published showing the same effects (224-227)

1.7.3. Management of DGF

Management of DGF is largely supportive; other diagnoses should be excluded and serial biopsies should be taken of the implanted organ to exclude acute rejection and confirm ATN. Haemodialysis may be clinically necessary post operatively to treat hyperkalemia, fluid overload or symptomatic uremia; its influence on duration of DGF is not known. Haemodynamic instability and nephrotoxins should be avoided in any patient with acute kidney injury (228).

In the last decade, our improved understanding of the mechanisms of oxidative stress, vasospasm, cytokine signaling, endothelial cell injury and the immune system has offered potential therapeutic targets to treat IRI and DGF, but they still remain significant contributors to morbidity and mortality in the transplant recipient. We know that the detrimental effects of DGF on both the graft life and patient survival are significant for the renal transplant recipient, this study aims to look at nitric oxide and its metabolites in patients at highest risk of DGF.

Conclusion

Ischemia and reperfusion are inevitable consequences of organ transplantation. Unlike tissue IRI resulting from arterial occlusion (such as myocardial infarction in the heart), transplantation is unique in that there is complete cessation of blood flow, removal and cooling of the organ followed by re-implantation after a period of time. In this time, ischemia deprives cells of the energy needed to maintain homeostasis leading to cellular injury, oedema and release of free radicals. On reperfusion, release of pro inflammatory cytokines activate immune responses in order to allow repair and regeneration processes to begin. The fate of the graft is therefore dependent on regeneration, as opposed to cell death, prevailing.

In transplantation the clinical manifestation of IRI is delayed graft function (DGF) (229). We know that DGF results in poorer clinical outcomes for grafts and

recipients. The greater the extent of the IRI, the greater the immune response and the potential for worse allograft outcome. Long CIT times are associated with higher levels of circulating anti-donor antibodies as well as with complement binding with immunoglobulin complexes (230) in the immediate post operative period. Longer term, biopsy proven chronic allograft changes including interstitial fibrosis, tubular atrophy and endothelial and tubular basal membrane thickening are also linked with the duration of cold ischemia (231).

In this study we investigate the relationship of NO production within this pathway; the dichotomous role of NO in IRI provides an interesting platform for this. We hypothesise that at low concentrations NO is protective, causing vasodilation thereby potentially reducing the no reflow phenomenon. However at higher concentrations, the inevitable production of toxic free radicals has a deleterious effect. We will be looking at concentrations of NO metabolites around the time of renal transplantation in the context of donation after cardiac death organs. We selected those we expected to have greater IRI and therefore higher rates of DGF. We will also investigate the concentrations of pro-inflammatory cytokines responsible for stimulation of NO production and look to see if the induction immunosuppression modifies the pro-inflammatory milieu and consequently, the NO response.

1.8. Previously Published Work

Previously our unit has investigated topics relevant to this project that have helped in the development of this study design. This includes work on the polymorphic expression of the cytokine IFN-γ and also on nitric oxide (52).

Polymorphic expression of IFN-γ

There have been many studies considering the possibility that a genetic predisposition to produce a higher or lower amount of various cytokines may contribute to outcomes in transplantation. This is a poignant question as, if this paradigm could be mapped, it may allow the immunosuppression regimen to be

customized to the patients' genetic predisposition to reject their transplanted organ.

The level of IFN-γ production in the UK population has been shown to be associated with polymorphic expression of a microsatellite sequence on the first intron of the human IFN-γ gene (232) (located on chromosome 12q24.1). The microsatellite itself is a non-functioning part of the gene, however, in the case of IFN-γ, microsatellite polymorphisms have been associated absolutely with a single nucleotide polymorphism (SNP) of T to A at +874 (233). To date, five different alleles of the previously discussed microsatellite have been described in the UK population. Patients may be homo or heterozygotes for any combination of the alleles; however, it has been shown that patients who express allele #2 produce more IFN-γ compared to those that do not. Furthermore, patients who are homozygotes for allele #2 produce more IFN-γ than any of the other combinations (232). It has been shown conclusively that allele #2 correlates absolutely with the functional SNP +874T. Patients may therefore be genetically sequenced to determine which one of three possible groups they fall into:

- T/T are homozygotes for allele #2 (highest IFN-γ producer)
- T/A are heterozygotes for allele #2 (high IFN-γ producer)
- A/A, allele #2 is absent from their genome (low IFN-γ producer)

The clinical significance of this polymorphism has been investigated; in renal transplant recipients the T/T homozygote genotype is associated with higher incidence of acute rejection episodes and chronic allograft nephropathy (234, 235). In lung transplant recipients the presence of allele #2 is associated with graft fibrosis (236), further more, in the general population it has been found to be protective against arthritis and systemic lupus erythematosus (237). As previously discussed, the microsatellite is in itself non-functional, however the +874T polymorphism, with which allele #2 is associated, encodes the NF-κB binding site (238). It is hypothesized that this accounts, at least in part, for the functional consequences of the polymorphism.

NO

Our group has previously studied NO and its metabolites in the context of renal transplantation. We have found that there is a significant increase in NO during episodes of acute rejection; this was studied in all types donor kidney (239). We have also found that NO levels differ between donor types; to account for individual variability in baseline production this was calculated using a reperfusion ratio, i.e. the difference between pre operative concentration and the concentration post reperfusion of the transplanted organ, expressed as a ratio. This reperfusion ratio was significantly higher in the recipients of DCD renal transplants when compared to DBD kidneys and also that there was a trend of increase in the ratio when moving from Living donor to DBD transplants.

To summarize previous work on this topic, we know that cytokines are produced in variable amounts by members of the UK population depending upon their genome, and in the case of IFN-γ this is determined by the SNP +874T. IFN-γ induces up regulation of iNOS on activated macrophages (via its activation of NF-κβ) and so is contemporaneous with the immune response to organ transplantation. NO levels differ between donor groups and this maybe related to the varying levels of ischemic insult.

1.9. Project Objectives

1.9.1. Hypothesis

In DCD renal transplant recipients we hypothesize that we will find changes in the levels of NO metabolites during the early phase of organ transplantation and that these changes may be associated with known risk factors for delayed graft function in the transplanted organ. We expect to find a relationship between the levels of NO metabolites and the induction immunosuppression agent given via the effects of immunosuppression on cytokine production. We anticipate finding variability in the production of cytokines depending on the induction immunosuppressive agent given.

1.9.2. Aims

The experimental aims of this study are: -

1. To investigate the levels of NO metabolites in the early post-operative period in patients undergoing renal transplantation using two different methods.
2. To explore the role of NO in renal transplantation by investigating it's relationship with known risk factors for DGF.
3. To explore the role cytokines play in NO production.

We intend to study levels of NO metabolites and pro-inflammatory cytokines in patients undergoing DCD kidney transplantation who are given different induction immunosuppression. Results will be correlated with the rate of delayed graft function as well as donor and recipient characteristics.

CHAPTER 2: METHODOLOGY

The following chapter will describe the patient selection, sample collection and experiments performed during this project. This study consisted of two parts; the first part incorporated data from work done previously at Cardiff Transplant Unit, which looked at nitrate levels in living donor (LD), DBD, and DCD renal transplants. The main purpose of this previous study was association of nitrate levels with acute rejection and DGF. However, we were able to use some of this data, together with our own early samples, to conduct our pilot study. The second part of the study concentrates on DCD renal transplants and is the main focus of this work.

2.1. Part 1 Method

2.1.1. Experimental subjects

Fifty-eight consecutive renal transplant recipients, who provided informed consent, were recruited at the University Hospital of Wales between July 2002 and May 2003. Three recipients had early transplant nephrectomy and were excluded from further analysis (1 for bleeding secondary to previously undiagnosed acute myeloid leukaemia and 2 for renal vein thrombosis). We added to this cohort of patients with samples taken from our first eleven patients (methods for which are described in section 2.2). The total population of part 1 of the study therefore comprised of 9 LD, 31 DBD and 29 DCD renal transplant recipients. The departmental protocol for immunosuppression was followed for all of the patients in the study, however this changed between the two periods of recruitment; the first 58 patients were given 500 milligrams (mg) intravenous (IV) of methyl prednisolone intra-operatively. The remainder of the patients were treated in accordance with the policy at the time of their recruitment and this is described in detail in section 2.2.

2.1.2. Sample preparation

Peripheral blood samples were collected pre-operatively and at 2 hours post-reperfusion of the kidney in EDTA tubes. The specimens were centrifuged at 800

G for 20 minutes and supernatant collected and stored at -80 degrees Celsius (°C) for analysis at a later date. On the day of the analysis, the specimens were thawed and vortexed.

2.1.3. Sample analysis

All of the samples were analysed in duplicate using the Griess reaction; nitrate and nitrite levels were determined using the *Parameter, total NO/Nitrite/Nitrate* kit from R&D Systems.

2.1.3.1. Scientific principle

The principle of the assay relies on colorimetric detection of nitrite as an azo-dye product of the Griess reaction. The first step is enzymatic conversion of nitrate to nitrite by nitrate reductase. The nitrite then reacts with sulfanilic acid to produce diazonium ion, which, in-turn, couples with *N*-ethylenediamine to form an azo-derivative. The azo-dye absorbs light and so a spectrophotometer is used at 540 nanometer (nm) to determine levels of nitrite. This gives the total concentration of nitrate and nitrite in a sample. To establish their individual values endogenous nitrite is measured first by omission of the nitrate reductase enzyme step. This value is subtracted from the converted nitrite concentration obtained by carrying out enzymatic conversion of nitrate to nitrite, to give the endogenous nitrate value.

We determined the nitrite and nitrate levels using the Griess reaction in all samples; the amount of nitrite in the samples was negligible and so the nitrate only was used for analysis.

2.1.3.2. Griess reaction - methods

The testing took place in the laboratory at the Institute of Nephrology, Cardiff. Good laboratory practice was followed at all times during these experiments. The procedure is detailed in appendix 2.

Calculation of results

We calculated average duplicate reading for each standard and sample and subtracted the average blank standard optical density. A standard curve was created by plotting mean absorbance for each standard on the y-axis against the total nitrite concentration on the x-axis. The nitrite concentrations were taken from the corresponding mean absorbance and the dilution factor was corrected to leave a value in µmol/l. Nitrate values were calculated by subtracting the endogenous nitrite concentration from the total nitrite concentration (after nitrate reductase) using the plate reader *(OPTIMA, BMG LABTECH)*.

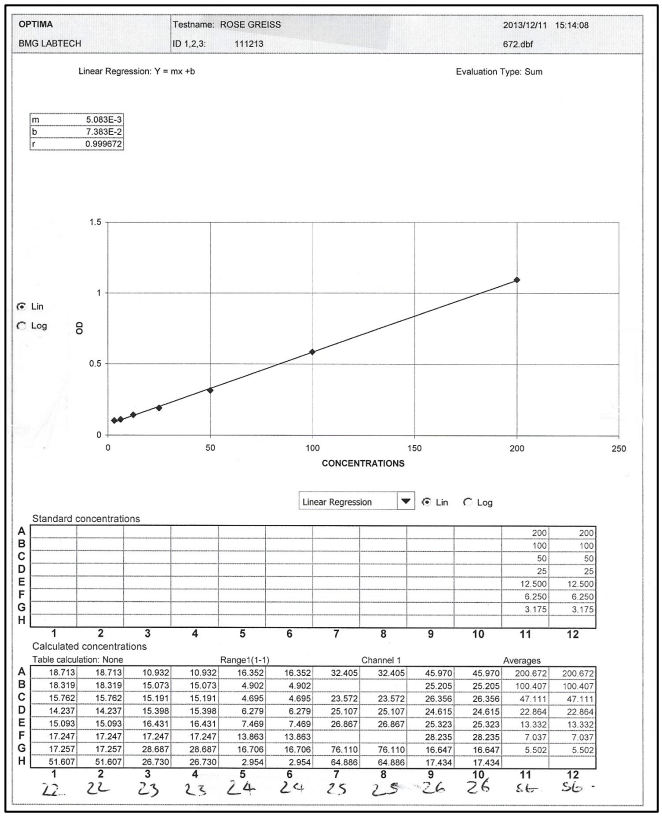


Figure 2.1. An example of Griess reaction results. *This shows the standard curve and the calculation of results that is produced by the plate reader. Standard curve r = 0.99742*

2.1.4. Statistical analysis

Clinical data from the patients was collected around the time of transplantation and during the follow up period of 3 - 6 months from the departmental database (VITALDATA). Statistical analysis was performed using IBM SPSS Statistics

version 20. Data was expressed as a ratio between the pre and post-operative nitrate concentration and handled using non-parametric tests. The nitrite values were not reported, as many of the tests returned un-recordable results; this problem is addressed in more detail in part two of the study (see section 2.4). In all analysis differences with $p < 0.05$ were considered statistically significant.

2.2. Part 2 Methods

2.2.1. Experimental subjects

A total of 35 consecutive DCD kidney graft recipients transplanted at Cardiff Transplant Unit between January 2012 and October 2014 were studied. Patients were excluded only if they were not deemed competent to consent. All patients who were approached agreed to participate in the study, the consent form is shown in appendix 3.

This was a non-interventional study and patient immunosuppression was managed according to the unit's standard protocols and the one of a separate, concurrently running study (197, 240). The departmental protocol for recipients of DCD renal transplants during the study period was to use ATG as induction; as we discussed in chapter 1, ATG causes release of cytokines; particularly TNF-α, IL-1 and IL-6. These in turn contribute to up regulation of iNOS and therefore we would expect ATG induction to have an effect on NO production. However, we should bear in mind that ATG is administered over a period of 4 hours (which commences after the induction of anaesthesia) with a high dose steroid and an antihistamine (in order to reduce the clinical side effects of cytokine release syndrome) and these factors may interfere with this anticipated effect.

During the period of patient recruitment there was a concurrently running national trial that the unit participated in (Campath, calcineurin inhibitor reduction and chronic allograft nephropathy (3C) study (197, 240)). Patients who were also enrolled in this study were randomized to receive either Campath or Simulect induction as opposed to the usual ATG induction.

The immunosuppression regimens were as follows:

1. ATG (1.25 mg/kg day 0-4) and Methyprednisolone (500 mg IV, day 0) with oral maintenance of Tacrolimus (0.025 mg/kg BD), MMF (1 g BD) and Prednisolone (20 mg OD).
2. Simulect (20mg IV, day 0 and day 4) and Methyleprednisolone (500 mg IV) with oral maintenance of Tacrolimus (0.05 mg/kg BD), MMF (1g BD) and Prednisolone (20mg OD).
3. Patient participating in a different study were randomized to receive either, Campath (30mg s/c day 0 and day 1) or Simulect (20 mg IV, day 0 and day 4) and Methyplrednisolone. Followed by either Tacrolimus or Sirolimus, MMF or Myfortic, and either 20mg of Prednisolone or no maintenance steroid.

Information on the immunosuppression regimen was recorded as part of the data collection.

2.2.2. Sample preparation

If a patient consented to participate in our study then 10mls of peripheral whole blood was collected in EDTA bottles at eight time points (shown below) around the time of transplantation. Peripheral blood was selected to analyse (as opposed to urine) as DGF was expected in at least 50% of the subjects and may cause anuria. Taking samples from the renal vein would have allowed us to more accurately reflect changes of NO metabolites directly attributed to the kidney. The ethics committee felt that the risks of renal vein sampling were an unknown quantity in this context and therefore this was not an acceptable intervention. We also anticipated that renal vein sampling may deter the operating surgeon from allowing their patients to participate in the study.

Timing of sample collections:

1. Sample A: Pre-operatively
2. Sample B: 30 minutes post-induction of anaesthesia
3. Sample C: Post skin incision but prior to the application of vascular clamps
4. Sample D: 30 minutes post-reperfusion of the graft
5. Sample E: 2 hours post-reperfusion of the graft
6. Sample F: 8 hours post-reperfusion of the graft
7. Sample G: 24 hours post-operatively
8. Sample H: 72 hours post-operatively

Plasma samples were prepared by centrifuging the whole blood at 1000 G for 15 minutes, collecting the supernatant and splitting into 500 µl aliquots, which were stored at -80 °C. The precipitate was discarded.

Data was collected on donor and recipient characteristics operative details, immunosuppressive regimen, post-operative course and graft function for 6 months after transplantation.

2.2.3. Identification and coding

Each patient was assigned a sequential number (1-35); all samples were labelled according to this number and the timing of the sample (A-H). The labelled samples were stored at -80 °C in the Institute of Nephrology, University Hospital of Wales (UHW), Cardiff. The patient details were kept only by the lead researcher and were stored in a locked office at UHW.

2.3. Plasma Analysis

The following analysis was undertaken on the samples:

1. Nitrate and nitrite analysis using the Griess reaction
2. Nitrate and nitrite analysis using ozone chemiluminescence
3. Cytokine analysis using Luminex
4. IFN-γ analysis using an enzyme linked immunosorbent assay (ELISA)

All samples were run in duplicate for each method.

2.3.1. Griess reaction

Nitrate and nitrite levels were determined using the *Parameter, Total NO/Nitrite/Nitrate* kit from R&D Systems as described in 2.1.3.2. We determined the nitrite and nitrate levels using the Griess reaction in all samples taken from the first 11 recruited patients (88 samples in total).

It became apparent when analysing the initial results and comparing them with those obtained from using the ozone chemiluminescence method (described below) that the level of nitrite in our samples was so small that it was below the levels of sensitivity for this assay; the minimum detectable amount quoted by the manufactures of the kit is 0.78 μmol/l. Due to the limited sensitively of the Griess reaction we only used ozone chemiluminescence to determine nitrite and nitrate concentration in the remaining samples. All testing was performed in duplicate and the mean of the two values used for analysis.

2.3.2. Ozone chemiluminescence

We measured NO metabolites, nitrite and nitrate using ozone-based chemiluminescence (*NO analyser, NOA280i, Sievers*) in all of our collected samples.

2.3.2.1. Scientific principle

The principle of the technique is as follows: a cleavage agent specific to the metabolite of interest is used to cleave NO. This NO is then carried through a sodium hydroxide (NaOH) trap by nitrogen gas. The purpose of this "trap" is to prevent contamination of the sample with any extra NO, which may be generated from other constituents of the plasma and to protect the NO analyser (NOA) from acidic vapours. After bubbling through the NaOH the NO is carried to the NOA, here it is forced to react with ozone (O_3), forming unstable nitrogen dioxide (NO_2). In order to stabilize itself NO_2 very quickly gives up a photon (*hv*), and the electric discharge from this is measured in milliVolts (mV) by the NOA photomultiplier tube.

$$\mathbf{NO + O_3 \rightarrow NO_2 + O_2 \rightarrow NO_2^- + O_2 + \boldsymbol{hv}}$$

Sievers NOA Analysis™ Liquid Version 3.2.1 acquires the data as a series of peaks and is capable of calculating the area under the curve to give a value in mV (millivolts), this is then read from a standard curve constructed of known concentrations and is measured in nanomols per litre (nmol/L). This standard curve must be constructed on each day of experiments as there may be variation in the rate of NO detection by the NOA.

2.3.2.2. Chemiluminescence methods

The testing took place in the laboratory at the Institute of Cardiology, Cardiff under the guidance of Dr. Phillip James. Good laboratory practice was followed during these experiments.

Sample preparation

The reagents that were used were dependent upon whether we were doing nitrite or nitrate analysis and will be described in more detail subsequently, however sample preparation was uniform to both reactions. The frozen plasma samples were stored at -80 ^{o}C until needed then thawed in a water bath at 37 ^{o}C for three minutes and used (undiluted) immediately.

Method – Plasma nitrite

Tri-iodide is the cleaving agent used to measure nitrite; it consists of glacial acetic acid, potassium iodide and iodine. The acid converts nitrite into nitric acid then the following reaction occurs to release NO

$$\mathbf{HNO_2 + 2I^- + 2H^+ \rightarrow 2NO + I_2 + 2H_20}$$

Reagent preparation

Tri-iodide was made up daily by combining 1g of potassium iodide dissolved in 20 millilitres (ml) of purified water with 650 mg of iodine crystals dissolved in 70ml of glacial acetic acid. The mixture was stirred for 30 minutes prior to use. Standards were prepared using serial dilutions of a known concentration and 1 molar (M) NaOH was taken from stock, see figures 2.2, 2.3 and 2.4.

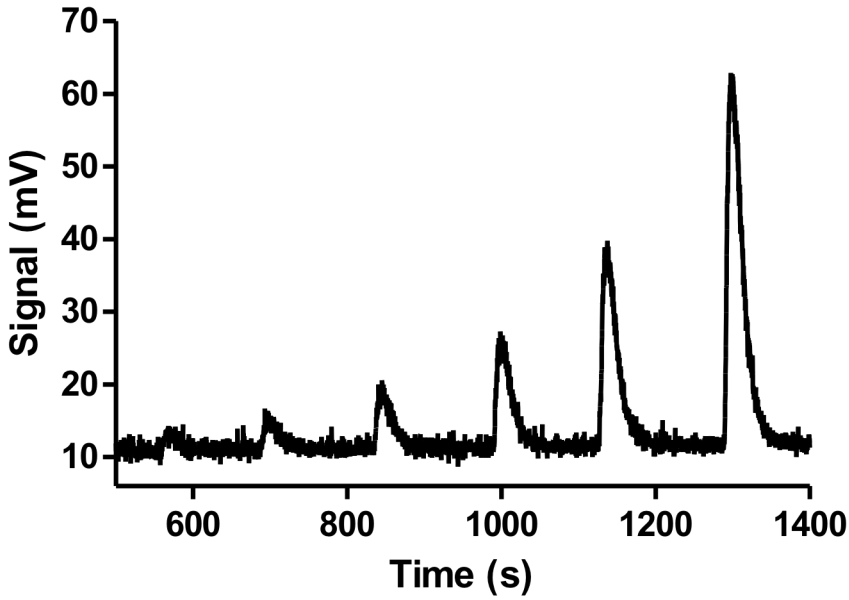


Figure 2.2: Standard curve of nitrite. *An example of the peaks used to construct a daily standard curve, concentrations used are: Water, 62.5 nanomolar (nM), 125 nM, 250 nM, 500 nM and 1000 nM sodium nitrite.*

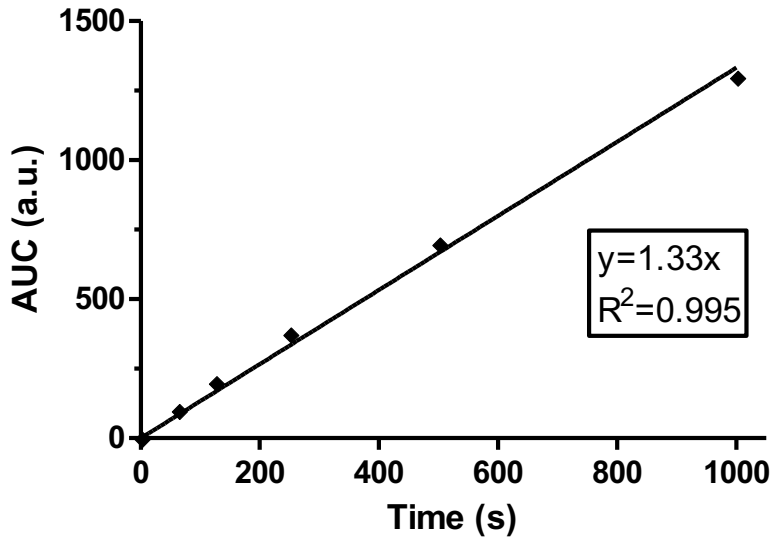


Figure 2.3: Area under the curve nitrite. *The area under the curve (AUC) of each peak was used to calculate the coefficient, the figures were corrected by the AUC of water to ensure the curve went through 0.*

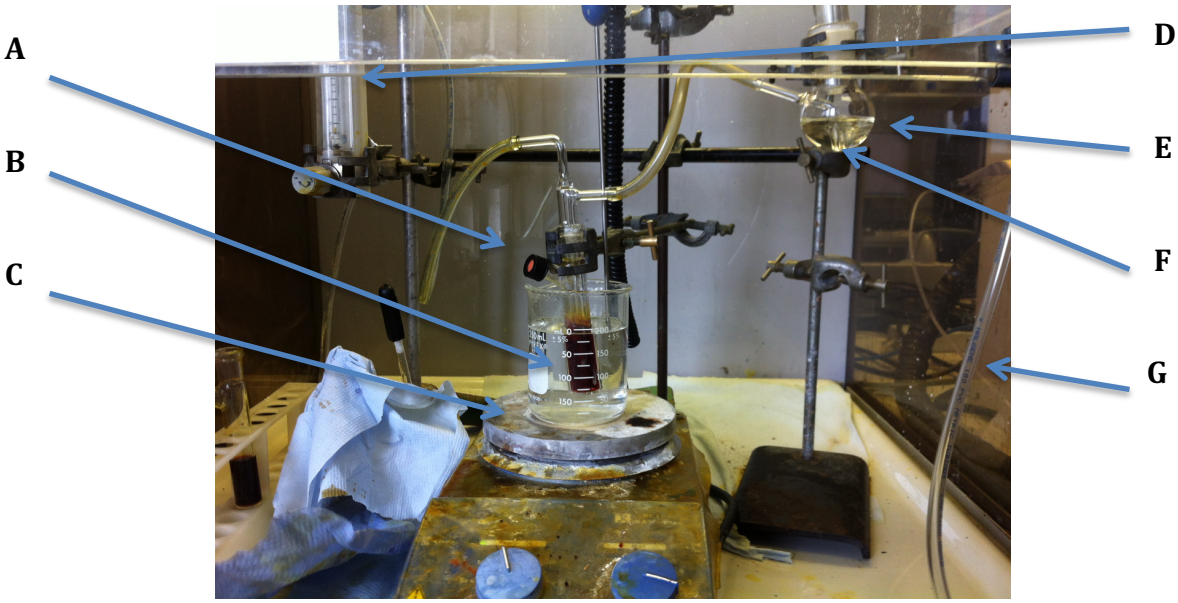


Figure 2.4: Nitrite analysis.

A - 100 µl sample is injected through the rubber septum

B - Custom made vessel containing 5mls of tri-iodide

C - Temperature is maintained at 50 °C by a water bath and hot plate

D - NO is carried by N_2 gas stream controlled by a flowmeter

E - Released NO is carried into a round-bottom flask

F – Containing 1 M NaOH

G – NO vapor is directed to the NO analyser

Laboratory procedure

1. 5mls of tri-iodide were pipetted into a custom made vessel (a drop of Antifoam™ was added into the vessel).
2. The temperature was maintained at 50 °C by a beaker of water kept on a hot plate.
3. 15 mls of NaOH was added to the round-bottomed flask.
4. The tubing was connected to the nitrogen gas and the NO analyser.
5. 100µl of prepared sample was injected in to the rubber bung and the data collected.
6. After 5 injections of plasma the tri-iodide was discarded and replaced.

Values were recorded as AUC in mV for each sample (run in duplicate) as calculated by *Sievers NOA Analysis™ Liquid Version 3.2.1*. Results were calculated on a daily basis using the standard curve constructed that morning and a mean value in nmol/L taken.

Method – Plasma nitrate

Plasma nitrate levels were measured by using vanadium chloride. The following reaction occurs to reduce nitrate in to NO.

$$\mathbf{2VCL_3 + 4HCL + NO_3^- \rightarrow 2VCL_5 + 2H_2O + 2NO}$$

Cleaving of NO will only occur at high temperatures so the reaction chamber is submerged in a water bath at 85 °C.

Reagent preparation

Vanadium chloride solution is prepared by adding 80 mls 1M HCL to 20ml of purified water then dissolving 785 mg of vanadium chloride in the diluted acid. The mixture is stirred for 15 minutes before being filtered using a 22 µm filter. Once filtered the solution is a bright clear turquoise colour (as shown in the flask in figure 2.7).

Standards were prepared using serial dilutions of known concentrations of sodium nitrate, see figures 2.5 and 2.6.

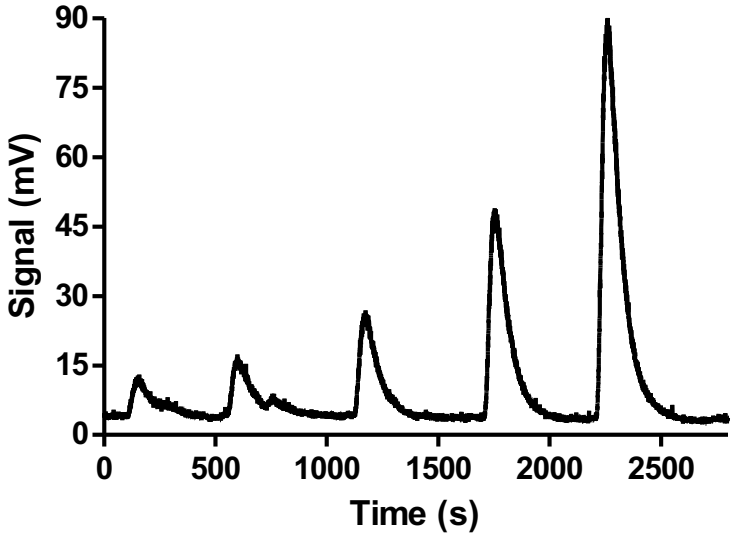


Figure 2.5: Standard curve of nitrate. *An example of the peaks used to construct a daily standard curve, concentrations used are: Water, 6.25 µM, 12.5 µM, 25 µM, 50 µM and 100 µM sodium nitrate*

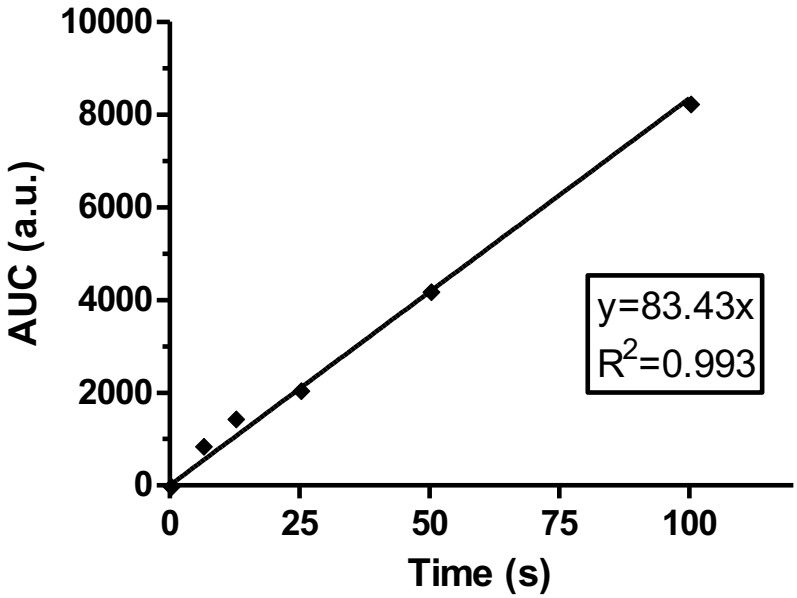


Figure 2.6: Area under the curve nitrate*. The AUC of each peak was used to calculate the coefficient, the figures were corrected by the AUC of water to ensure the curve went through 0.*

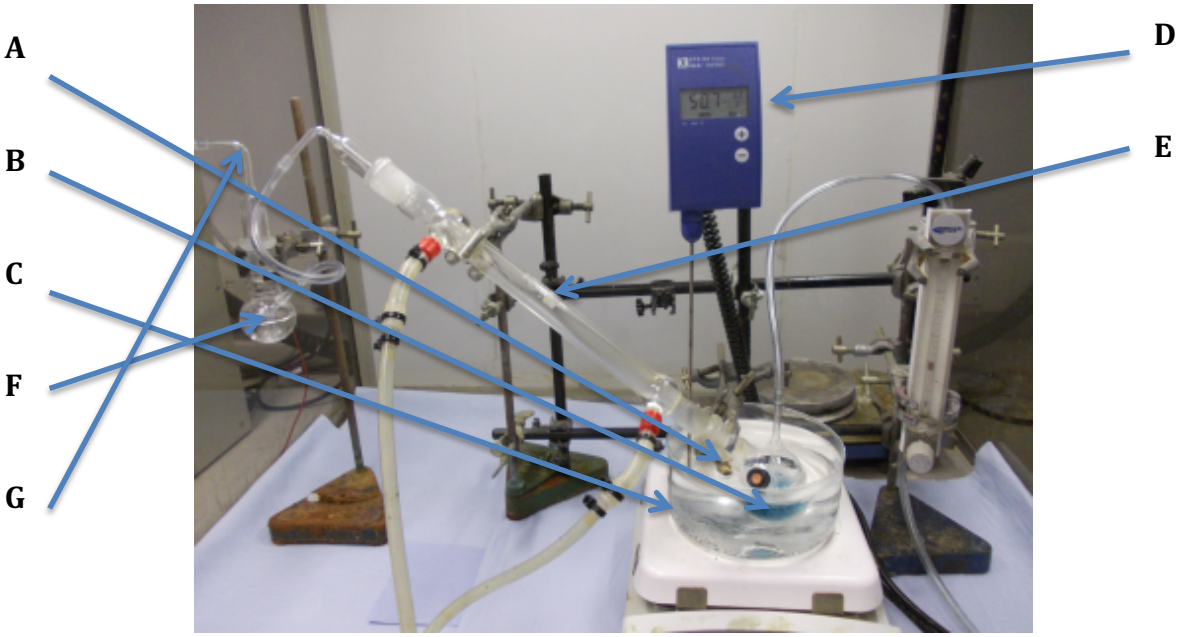


Figure 2.7: Nitrate analysis.

A - 20 µl sample is injected through the rubber septum

B - 2-neck round bottom flask containing 30 ml vanadium chloride reagent.

C – Temperature is maintained at 85 °C by a water bath

D – Water bath linked to IKATRON® ETS-D4 fuzzy

E – NO is carried in N_2 gas stream controlled by a flowmeter through a condenser

F – Released NO is carried to a round-bottom flask containing 1 M NaOH

G – NO vapor is directed to the NO Analyser

Laboritory procedure

1. 30 mls of vanadium chloride is added to the purge vessel as shown.
2. The temperature of the water bath is maintained at 85 ^{o}C.
3. Evaporated vanadium runs back into the reaction chamber when it makes contact with the custom designed water-cooled funnel.
4. The tubing is connected to nitrogen gas and the NO analyser.

5. 15 µl of prepared sample is injected in to the rubber bung and the data collected.
6. After 20 injections of plasma the vanadium chloride is discarded and replaced.

Analysis

Standard curves were generated daily for both nitrite and nitrate analysis. This is essential to the chemiluminescence technique as daily variation in detection is known to occur. This is thought to be due to sensitivity of the photomultiplier tube to changes in room temperature or oxygen pressure. Standard curves were generated by plotting area under the curve on the y axis against serial dilutions of known nitrite or nitrate on the x axis. All samples were run in duplicate and average values in µmol/l calculated.

2.4. A Comparison of Methods for NO Analysis

2.4.1. Introduction

Attenuating the effects of IRI is undoubtedly an essential part of the future of renal transplantation. In chapter I we reviewed the complexities of IRI and explored the rationale behind the objectives of this study; namely to look at metabolites of nitric oxide and the inflammatory cytokines that stimulate their production in patients receiving deceased renal transplants, with a view to correlating this with the clinical outcome of DGF. We have seen how current evidence suggests conflicting roles for NO metabolites; how NO_2^-, a metabolite itself, may act as a source of NO during times of hypoxia (241), how endothelial dysfunction (a fundamental step in IRI) results in uncoupling of eNOS and therefore reduced NO production (60) and how this has lead to research into the possibility of a therapeutic role for supplementation of NO metabolites around the time of transplantation (77). With regards to renal transplantation it is important to remember that NO may be produced by both eNOS and iNOS and that the ROS produced as a consequence of NO metabolization have physiological effects that also appear to be dose dependent (146).

From our review of the literature surrounding the topics of IRI, NO and renal transplantation, it became clear that concentration of NO is likely to be a significant factor influencing the effects of NO and its metabolites. It was therefore of paramount importance to be able to measure these metabolites i.e. NO_2^- and NO_3^- accurately with precision and low intravariability. From our preliminary research into the topic it seemed that we were expecting to find only nanomolar concentrations of NO_2^- in plasma samples. Studies conducted previously in this unit and by others on similar topics had been performed using the Griess reaction for the measurement of NO metabolites. This is an effective method for looking at NO_3^- however we were skeptical about the ability of this method to accurately quantify NO_2^- in our plasma samples. To investigate this further we used two different methods to analyze the same 88 samples and compared the results. The aim of this was to determine which, of two well-established methods of NO analysis, was most appropriate for our study group.

In order to handle the data correctly, we first looked at the distribution of the values obtained. Neither NO_2^- nor NO_3^- were found to reliably have a normal distribution, therefore, non-parametric tests were used for analysis.

2.4.2. Results of Griess/chemiluminescence correlation

We used all samples from the first 11 patients recruited to determine which of the two methods that were available to us was the most appropriate for our study. Eighty-eight plasma samples from 11 patients collected at specific time points around the time of renal transplantation were analysed using the two different methods. Both the reaction and ozone chemiluminescence may be used to quantify NO_2^- and NO_3^- in plasma; this analysis was performed in duplicate on all 88 samples. The correlations between the values obtained with the two different methods were calculated using the Spearman's rank correlation coefficient.

2.4.2.1. Nitrite

When we examined the initial results for NO_2^- the values obtained were outside the range of the Griess assay (3.12-200 µmol/L) although the R&D kit which we used does quote a minimum detectable value of 0.25 µmol/L. Moreover, there was no correlation between the values obtained for each sample using the two tests see table 2.1 below. The physiological range of NO_2^- in fasted human plasma is 56-210 nmol/L (242), which is considerably below the range detectable by the reaction. Even taking into account that we were expecting a rise in concentration this was not an acceptable method for analysis in physiological samples.

Ozone chemiluminescence *(Seivers, NOA 280i)* is capable of detecting nM concentrations in liquid samples with a sensitivity of ≈ 1 picomole. It is therefore over 1000 times more sensitive than the Griess reaction and as such we decided not to perform NO_2^- analysis using the Griess method on the remaining 24 samples. Any reference to NO_2^- values henceforth will therefore refer to those obtained using ozone chemiluminescence. Table 2.1 below demonstrates the lack of correlation found between the values obtained using each method using the Spearman's rank correlation coefficient at each time point.

Sample	Correlation coefficient NO_2^-	Significance p=
A	0.46	NS
B	0.14	NS
C	0.13	NS
D	0.08	NS
E	-0.3	NS
F	-0.2	NS
G	1.53	NS
H	0.41	NS

Table 2.1: The correlation coefficient of nitrite measurement. *The correlation coefficient of NO_2^- between samples measured using ozone chemiluminescence and the Griess reaction.*

2.4.2.2. Nitrate

When we looked at the results for NO_3^- tight correlation was found between the values obtained using the Griess reaction and Ozone Chemiluminescence $r_s(267) = 0.87$, $p = <0.0001$. Table 2.2 below illustrates the correlation coefficient found between the values taken at each sampling time point using the Spearman's rank correlation coefficient.

Sample	Correlation coefficient NO_3^-	Significance p=
A	0.88	<0.0001
B	0.9	<0.0001
C	0.82	<0.0001
D	0.87	<0.0001
E	0.86	<0.0001
F	0.8	<0.0001
G	0.84	<0.0001
H	0.93	<0.0001

Table 2.2: The correlation coefficient of nitrate measurement. *The correlation coefficient of NO_3^- between samples measured using ozone chemiluminescence and the Griess reaction*

This concordance is attributable to the higher levels of NO_3^- found, bringing the analyte (NO_3^-) into the detectable range of the Griess assay.

2.4.3. Discussion

We chose to collect plasma from our subjects as opposed to urine; urine has been used in other studies looking at NO metabolites and the kidney however we did not think that this would provide us with accurate results for two reasons. The first being any renal transplant recipient (unless they have undergone a bilateral native nephrectomy) may still excrete some urine produced by the residual function of their native kidneys. The second consideration is that we were expecting around 50% of our study group to have DGF in the first few days post transplant; some of these patients may not produce any or very little urine at all

during this time. Urine was therefore not a reliable medium in which to measure NO production or cytokines for our study.

One of the challenges when undertaking this study was the measurement of the NO metabolites themselves. Previous work done by this unit (and indeed the majority of the published literature within the field of transplantation) uses the griess reaction for measurement of nitrite and nitrate, although this is not the case in other areas of research (for example in the field of cardiology (243-249)). The benefits of the Griess reaction are that it is an inexpensive and quick method, allowing multiple samples to be quantified in duplicate simultaneously. In contrast, ozone chemiluminescence is an extremely labor and time intensive method but is much more sensitive and accurate. We were therefore hoping to find good correlation between our measurements using the two different assays, thus validating the use of the Griess reaction in future work. This was unfortunately not the case (as is reported above); when measuring NO_2^- in the plasma samples of the renal transplant recipients the levels were too low to allow accurate quantification using the Griess reaction and so we performed our quantification of NO_2^- using ozone chemiluminescence. However, in the NO_3^- measurements we found good correlation between the values obtained using both methods and therefore we believe the Griess reaction could be used in future work for detection of NO_3^- only. For consistency in this study we used the values obtained from ozone chemiluminescence for our analysis of both NO_2^- and NO_3^-.

We would conclude that when measuring NO metabolites in the plasma of renal transplant recipients the griess reaction is an acceptable method if solely measuring NO_3^-. If, however, NO_2^- measurement is required the Griess reaction is not sensitive enough for accurate quantification of the levels found in human plasma and a more sensitive technique such as ozone chemiluminescence should be employed.

2.5. Cytokine Measurement

Cytokines were measured using 2 techniques; Luminex and an ELISA

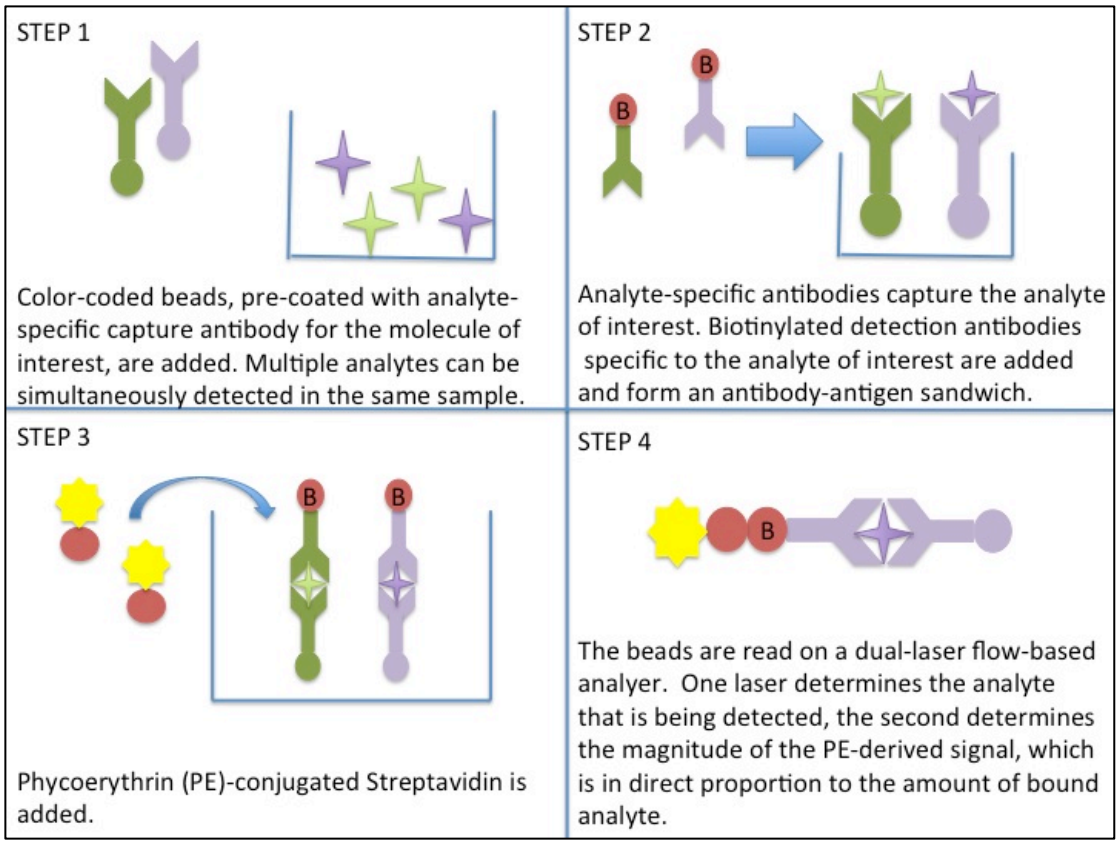
2.5.1. Luminex for cytokine measurement

2.5.1.1. Scientific principle

Luminex is a multiplex immunosorbant assay, it's roots lie in the ELISA however luminex assays have the advantage of being able to detect multiple analytes on one plate (unlike ELISAs which can only detect a single analyte at a time). Their common feature is that they are both antibody sandwich assays. The principle of the luminex assay is as follows:

1. Colour coded beads (or microparticles) with an analyte specific antibody are mixed with samples or standards.
2. Another analyte specific detection antibody is added which binds to its particular target.
3. Streptavidin-PE is then added which binds to the detection antibody.
4. A dual laser flow cytometer is used to quantify the analytes of interest. One laser is able to distinguish between different coloured beads and therefore identifies the analyte, whilst the other one detects the signal intensity given off by the bound streptavidin-PE there by quantifying the analyte.

Luminex technology has many applications in medicine, including HLA typing and antibody monitoring in organ transplantation, see figure 2.8.



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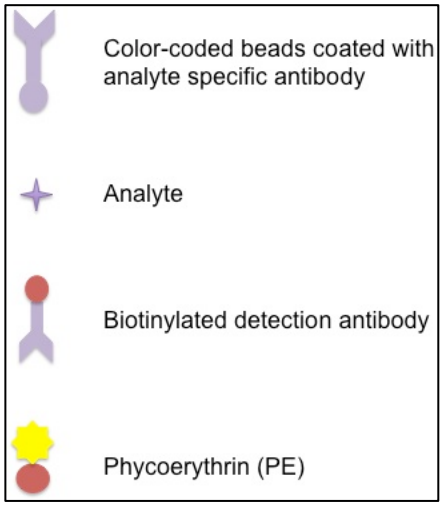


Figure 2.8: Steps of the Luminex assay

Kits are available to detect a variety of biomarkers; we used the *Luminex Performance Assay, Human Cytokine Premixed Kit A* from R&D Systems to investigate levels of IL-2, IL-6, IL-10, IL-17, TNF-α and IFN-γ in our study group. These cytokines were quantified in samples taken around the time of transplantation at the following points; pre-operatively (A), post skin incision but prior to the application of vascular clamps (C), at 30 minutes post reperfusion of the graft (D), at 2 hours post reperfusion of the graft (E) and at 24 hours post operatively (G).

2.5.1.2. Luminex method

The testing took place in the Welsh transplantation and immunology laboratory at the Welsh blood service under the guidance of Dr. Francis Edwards and Dr. Emma Burrows. Good laboratory practice was followed during these experiments the procedure is described in appendix 4.

Analysis of Luminex cytokine results

Results were collected on the Bio-Rad® analyser. A standard curve was generated using a five parameter logistic (5 PL) curve fit. Using a logistic regression model ensures greater accuracy across a broader range of concentrations and is the recommended method for analyzing multiplex sandwich immunoassays. The samples were run in duplicate and the average calculated before being multiplied by the X4 dilution factor.

2.5.2. ELISA - IFN-γ

When we looked at our luminex results, although the standard curves had all given acceptable r^2 values and the assay appeared to be working, we detected less IFN-γ, IL-2 and IL-10 in the samples than was expected. We contacted R&D Systems, who produced the kit, and sent them all of our results to check; they were not able to ascertain why the levels in the sample were so low and were confident that the assay had worked correctly however, as we still remained suspicious, they sent us a specific human IFN-γ ELISA kits to check the results.

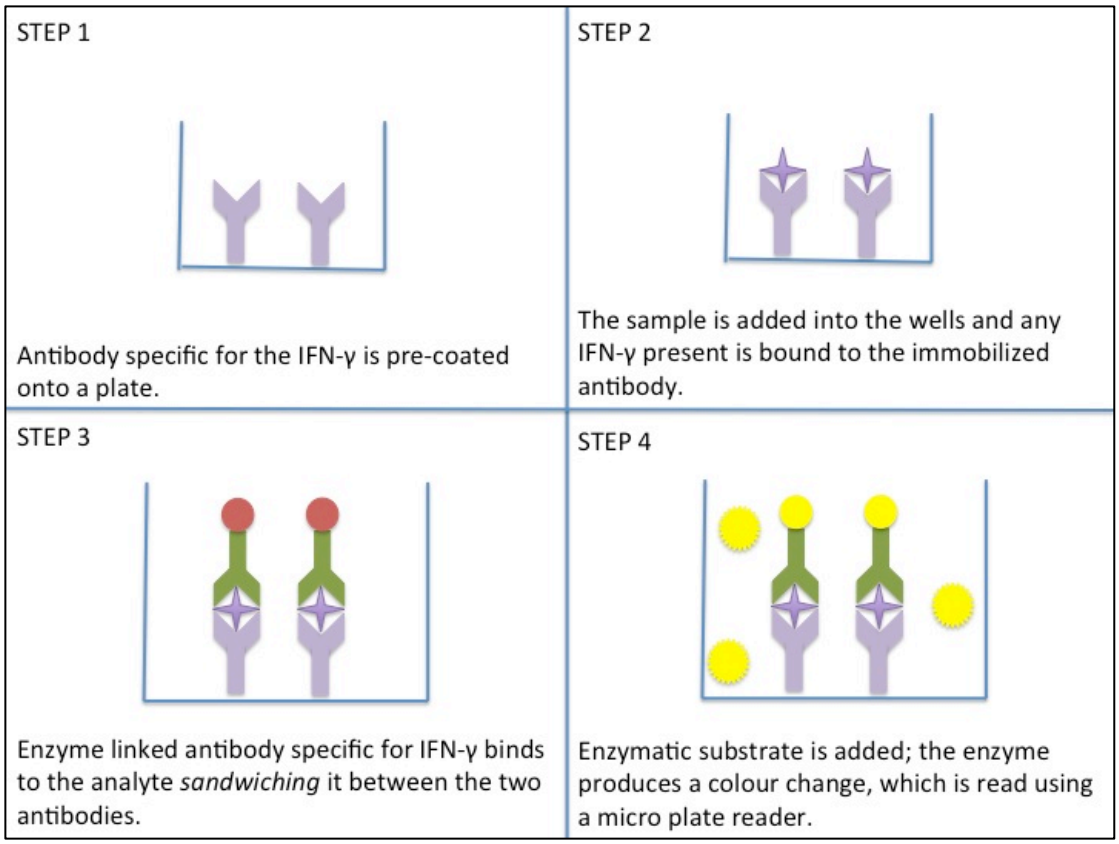
A representative from the company came out to assist us with the initial runs of the assay and to look through the data from the luminex experiments to see if there was any identifiable cause for the unexpected discrepancy. They were unable to identify any technical problems with the data or the equipment in the laboratory. The ELISA revealed much higher levels of IFN-γ as we would have expected in 9/35 patients, however in 26/35 patients no IFN-γ was detected at all in any of the samples using either method.

One possible explanation may be that the amount of cytokines in the plasma samples is below the level of detectability of the assays. An alternative possibility, based on the experience of immunologists who use these assays routinely, is that although the luminex assay was accurately detecting them in the standards provided in the kit by R&D Systems it was not binding to the human forms in patient plasma samples. A future extension to this work may be to look into other techniques for quantifying these cytokines. We did not feel data from 9 patients could provide any meaningful result therefore there is no report of IFN-γ in the results chapter.

The results for the other cytokines IL-6, 10 and TNF-α all returned quantities in line with what we would expect. The standard curves were acceptable and the duplicate readings were concordant; in conclusion we have no reason to suspect that these results are not accurate and reliable.

2.5.2.1. Scientific principles

ELISA stands for enzyme linked immunosorbant assay; it uses a polyclonal antibody specific for the analyte, in this case IFN-γ, which is pre-coated onto a plate. The sample is added into the wells and any IFN-γ present is bound to the immobilized antibody. After washing, a further antibody specific for IFN-γ is added to the wells. This binds to the analyte, *sandwiching* it between the two antibodies. After a final wash to remove any unbound substances an enzymatic substrate is added. As the plate develops the enzyme produces a colour change, which is read using a microplate reader, see figure 2.9.



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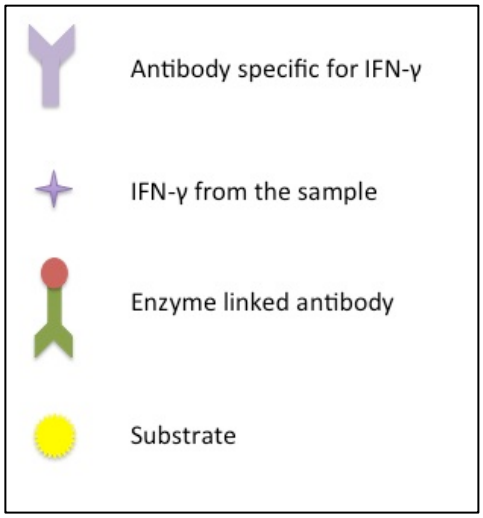


Figure 2.9: Steps of the ELISA assay.

2.5.2.2. ELISA method

The testing took place in the Welsh transplantation and immunology laboratory at the Welsh blood service under the guidance of Dr. Francis Edwards and Dr. Emma Burrows. Good laboratory practice was followed during these experiments, the procedure followed is detailed in appendix 5.

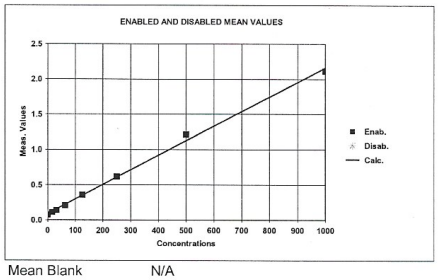
Anaylsis of ELISA

Results were collected on the Bio-Rad® analyser. A standard curve was generated using a four parameter logistic (4 PL) curve fit. Using a logistic regression model ensures greater accuracy across a broader range of concentrations see figure 2.10. The samples were run in duplicate and the mean calculated.

Sheet: Measure1, Assay: Assay1

Name	Meas.	Conc.
Cal_1	2.116	
	2.108	
	2.112	1000.000
Cal_2	1.195	
	1.231	
	1.213	500.000
Cal_3	0.595	
	0.639	
	0.617	250.000
Cal_4	0.344	
	0.371	
	0.358	125.000
Cal_5	0.203	
	0.210	
	0.207	62.500
Cal_6	0.144	
	0.136	
	0.140	31.200
Cal_7	0.113	
	0.110	
	0.112	15.600
Cal_8	0.081	
	0.073	
	0.077	0

	Conc.	Meas.	CalcConc.	Residual
Cal_1	1000.000	2.112	977.938	22.062
Cal_2	500.000	1.213	542.715	-42.715
Cal_3	250.000	0.617	254.180	-4.180
Cal_4	125.000	0.358	128.551	-3.551
Cal_5	62.500	0.207	55.449	7.051
Cal_6	31.200	0.140	23.255	7.945
Cal_7	15.600	0.112	9.457	6.143
Cal_8	0	0.077	-7.245	7.245



Mean Blank	N/A		
Status:			
Remark:	Bold values are extrapolated		
Fit type:	Linear regression (SVD): y = a+b*x		
Meas. transformation:	Linear		
Conc. transformation:	Linear		
Parameters:	a	b	
	0.092	0.002	
Corr. coeff. R2:	0.994		

Figure 2.10. An example of the 4 PL curves used for interpretation of the ELISA.

2.6. Statistical Analysis and Data Reporting

Clinical data from the patients was collected around the time of transplantation and during the follow up period of 6 months from the departmental database (VITALDATA). Statistical analysis was performed using IBM SPSS Statistics version 20. Data was expressed using median, range and interquartile range as it was not found to be normally distributed. Mann-Whitney U, Friedman and Kruskal-Wallis H tests (with post-hoc Bonferroni correction) were used to look at differences between groups. Multiple regression analysis and Spearman's rank correlation coefficient were used to look for associations. In all analyses differences with $p < 0.050$ were considered statistically significant.

NO metabolites were measured in nmol/L using ozone chemiluminescence, the results were converted to μmol/L and given to one decimal place for ease of reporting in the results section. Test were performed looking at the change in concentration of NO metabolites this was calculated to address the variation in baseline production within the population. In order to look at the relationship between NO_3^- and NO_2^- we created a ratio and used this to look for associations with our variables. When describing the graphs, median values were quoted if reporting a significant test; illustrations that did not show significant differences do not have individual values quoted.

The analysis performed was aimed at addressing three areas:

1. Ozone chemiluminescence and the Griess reaction were compared for measuring metabolites of nitric oxide.
2. Risk for IRI in renal transplantation were correlated with nitric oxide levels using regression models.
3. Nitric oxide levels were correlated with production of inflammatory cytokines

CHAPTER 3. RESULTS – Demographics of the Study Group and NO Methods Analysis

3.1. Introduction

The following chapter will describe the demographics of the study group. This study consisted of two parts the results for each study follow later in the chapter.

3.2. Demographics of Part 1 Study Group

As part of previous work at the Cardiff Transplant Unit that had investigated the levels of NO metabolites in acute rejection (239), plasma samples had been collected pre and post reperfusion from patients undergoing both living and DBD renal transplantation and DCD transplants to investigate our theory that factors influential in renal IRI may lead to changes in NO levels. This cohort's demographics are presented in Table 3.1.

Total		69
Donor type	LD	9
	DBD	31
	DCD	29
Median cold ischemic time in hours in DCDs (range)		11 (4 - 21)
Donor median age in years (range)		50 (5 - 74)
Donor gender	Male	40
	Female	29
Recipient median age in years (range)		49 (23 - 73)
Recipient gender	Male	54
	Female	15
Median baseline NO metabolites µmol/L (range)		39 (7.1 - 481)
Median post perfusion NO metabolites µmol/L (range)		34 (2.9 - 431)

Table 3.1: Patient characteristics of part 1 study group.

The population included 9 LD, 31 DBD and 29 DCD renal transplant recipients. The samples we used for analysis were collected pre-operatively and 2 hours

post reperfusion of the organ. To accommodate the variability in baseline measurements that occurs naturally within the population, we calculated a reperfusion ratio (RRT) i.e. the ratio between the pre and post operative nitrate concentration and looked initially at the differences between donor types, see 3.4 Part 1 results.

3.3. Demographics of Part 2 Study Group

Due to the study design the patient cohort were not selected in anyway other than that they underwent DCD renal transplant at Cardiff transplant unit within the study period and consented to take part in the study. The rate of consent for those who were approached to participate was 100%, this reflects the minimal intervention that was required for the patients to take part and that they were all approached by two experienced registrars.

Thirty-five patients were recruited between March 2012 and April 2014. During this time, a total of 64 DCD renal transplants were done that were not recruited to the study due to unavailability of the researcher (in clinical practice in London October 2012 – October 2013). A total of 280 plasma samples from 35 patients were collected. The samples were stored at -80 degrees Celsius and investigated for metabolites of nitric oxide and pro-inflammatory cytokines as described in the previous methods section. The demographics are summarized in the Table 3.2.

Total		35
Donor median age in years (range)		63 (26-76)
Donor Gender	Male	16
	Female	19
Median cold ischemic time in hours (range)		14 (7-36)
Median primary warm ischemic time in minutes (range)		14 (8-34)
Median secondary warm ischemic time in minutes (range)		45 (25-58)
Storage method	Cold static	18
	Machine perfusion	17
Recipient median age in years (range)		58 (29-75)
Recipient gender	Male	27
	Female	8
Mode of dialysis	Pre-dialysis	2
	PD	16
	HD	17
Induction immunosuppression	ATG	13
	Campath	10
	Simulect	12
Median baseline NO_2^- µmol/L (range)		0.3 (0.1-0.9)
Median baseline NO_3^- µmol/L (range)		42.2 (8.3-103.6)

Table 3.2: Patient characteristics of part 2 study group.

3.3.1. Sex

Of the 35 participants in the study 8 (22.9%) were female and 27 (77.1%) were male. Of the 35 donor kidneys used 19 (54.3) were from female donors and 16 (45.7%) were from male donors.

3.3.2. Age

The recipient ages ranged from 29 to 75 years with a mean age of 55.8 years and a median age of 58 years. The donor ages ranged from 26 to 76 years with a mean age of 56.1 and a median age of 63 years. Data from a recent paper using statistics from the UK transplant registry reported national figures; median recipient age was 53 years (interquartile range (IQR) 43–62) and median donor age was 49 years (IQR 37–59) (32). In this study cohort both donor and recipient ages are found to be higher than the national averages this may represent the use, by our unit, of ECD DCD kidneys which some centres nationally are less likely to accept for transplantation.

3.3.3. Immunosuppression regimens

3.3.3.1. Induction immunosuppression

The induction immunosuppression received by the participants in our study was of particular importance since this was one of the research questions; namely whether the induction immunosuppression played a role in the levels of nitric oxide following reperfusion. As described in the methods chapter, subjects received one of three induction immunosuppression medications; 13/35 (37%) received ATG; 10/35 (29%) received Campath and 12/35 (34%) received Simulect.

3.3.3.2. Maintenance immunosuppression

Calcineurin inhibitors

All patients were commenced on Tacrolimus on the first postoperative day.

Inhibitors of nucleotide synthesis

MMF was commenced on the first post-operative day in 17/35 (49%) of the patients; Myfortic in 14/35 (40%) and 4/35 (11%) were not prescribed either medication.

Corticosteroids

Twenty-three out of 35 (65.7%) patients were commenced on 20 mg of prednisolone on the first postoperative day. Twelve out of 35 (34.3%) patients did not receive an oral corticosteroid.

3.3.4. Mode of dialysis

Two (6%) of the 35 patients included in the study were pre-dialysis, 17 (48%) used haemodialysis and 16 (46%) were peritoneal dialysis patients.

3.3.5. Previous transplantation

For 32/35 (91%) of the patients this was their first renal transplant, in 2/35 (6%) cases it was their second and one (3%) patient received their third kidney.

3.3.6. Ischemic times

Cold ischemic times were recorded in hours; the median time was 14 hours and the mean 13 hours 30 minutes with a range of 7 hours 36 minutes to 25 hours 12 minutes. Nationally, the median cold ischemic time as reported by NHSBT in their *Annual Report On Kidney Transplantation* was 13 hours for the recruitment period.

The median and mean 1st warm ischemic time (defined as time of asystole until the time cold perfusion was instigated) were both 14 minutes with a range of 8-34 minutes. The median second warm ischemic time (out of ice until reperfusion of the organ) was 45 minutes and mean 47 minutes with a range of 25 to 58 minutes, shown in Figure 3.1.

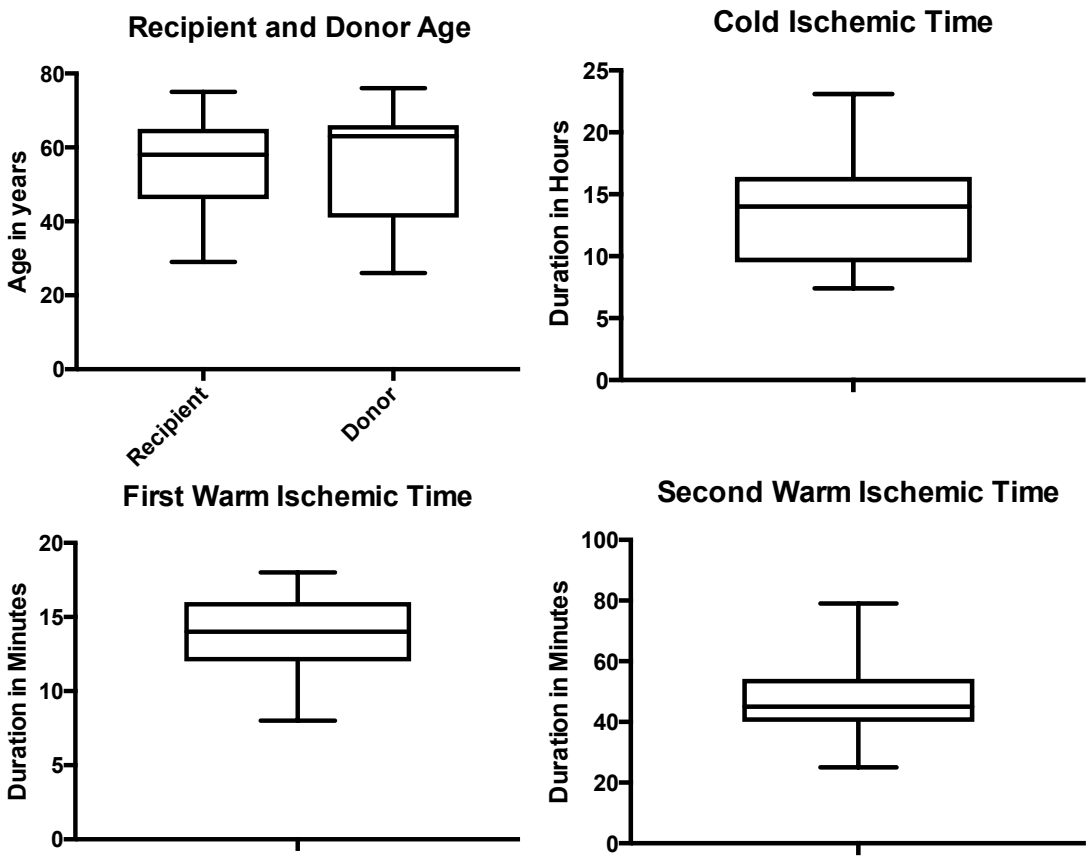


Figure 3.1: The distribution of important risk factors of part 2 of the study. *Box and whisker plots showing the median, interquartile and central ranges of the donor and recipient age, the CIT in hours and the 1st and 2nd WIT in minutes. Median (range): donor age in years = 63 (26-76), recipient age in years = 58 (29 – 55), CIT in hours 14 (7 – 36), 1st WIT in minutes = 14 (8 – 34) and 2nd WIT in minutes = 45 (25 – 58).*

3.3.7. DGF

As previously discussed, DGF is commonly defined in the scientific literature in multiple ways, two of the most common being;

1) Where creatinine does not reduce by 10% in the first 48 hours post-operatively.

2) Requirement for dialysis in the first 7 days post operatively.

The first of these definitions is a more objective measure (as the use of dialysis may be subject to differing local criteria) and is therefore the one we shall apply to our results.

Using this definition, DGF in this cohort occurred in 25/35 (71%) of patients. There was immediate function in 9/35 (26%) grafts and there was one early graft loss at 16 hours due to venous thrombosis. Of those 25 patients with DGF, only 23 required dialysis. 1/23 (4%) was only dialysed once in the first 24 hours, 14/23 (16%) were dialysed for up to 7 days and 8/23 (35%) required dialysis for more than 7 days.

The incidence of 71% DGF is on the higher side of the range reported in the literature, probably due to the higher than average use of older donor organs.

3.3.8. Rejection episodes

Seven of the 34 patients with functioning grafts developed acute rejection within the first six months after transplantation. The median time from transplantation to acute rejection was 90 days and none occurred within the sample collection period. Acute rejection is outside the scope of this project and will not be discussed further.

3.3.9. Storage method

After organ retrieval all kidneys are transported to the recipient centre in cold static storage. Once the organs arrived at Cardiff Transplant Unit they were prepared for implantation and then a further storage period followed whilst the patient was prepared for implantation, during this time 17/35 (48.6%) donor kidneys underwent hypothermic machine perfusion, 18/35 (51.4%) underwent just cold static storage. Selection of storage method was based on the anticipated duration of CIT; at the time the vast majority of DCD organs were

received as a pair therefore the second organ to be transplanted underwent hypothermic machine perfusion whilst the first was being transplanted. The first organ underwent cold static storage.

3.3.10. Baseline nitrite and nitrate concentration

Within the population there is variation in the circulating concentration of NO; the baseline values of NO_2^- and NO_3^- was measured (time point A) for all participants in the study. The baseline median (range) concentration of NO_2^- in μmol/L was 0.3 (0.1-0.9) and the baseline NO_3^- in μmol/L was 42.2 (8.3-103.6).

3.4. Part 1 Results

The data included Nitrate levels measured (as previously described) in the plasma samples of 9 LD, 31 DBD and 29 DCD renal transplant recipients using the Griess method. The samples we used for analysis were collected pre operatively and 2 hours post reperfusion of the organ. We calculated a reperfusion ratio (RRT) i.e. the ratio between the pre and post-operative nitrate concentration and looked initially at the differences between types of transplant, shown in Figure 3.2.

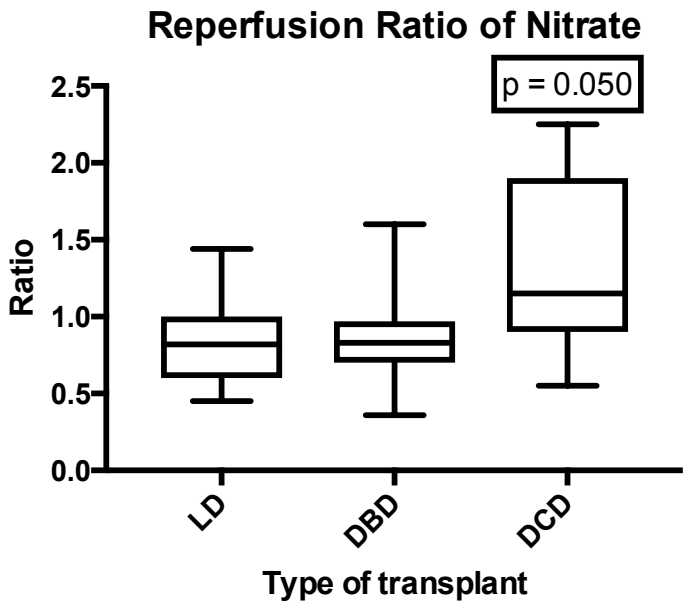


Figure 3.2: Reperfusion ratio of nitrate in LD, DBD and DCD kidneys. *Reperfusion ratio (post-operative:pre-operative) of the levels of the NO metabolite nitrate in a series of patients undergoing renal transplantation from live donors (LD), brain death donors (DBD) and circulatory death donors (DCD). The values are median, interquartile and central range. The reperfusion ratio was significantly higher in patients receiving a kidney transplant from a DCD donor compared to both LD and DBD kidneys using a Mann Whitney U test (n = 69, p=0.050).*

We found that as we move from LD to DBD and then to DCD transplants the mean RRT of nitrate increases, that is to say that the difference between the pre-

operative and the post-reperfusion nitrate level is greater. Significant difference was found between DCD transplants and the two other types of transplantation ($p = 0.050$). We hypothesized that this may reflect the increasing IRI between these modes of donation. This finding prompted us to look in more detail at the DCD group with a particular interest into the risk factors for IRI.

Our research question was; is there an association between risk factors for increased IRI in renal transplantation (warm and cold ischemic times, donor and recipient age, donor sex) and changes in NO metabolites in DCD kidney transplants?

In DCD transplants, we found statistically significant increases in RRT when cold ischemic time was greater than 12 hours ($p=0.03$) and where the donor age was greater than 55 years ($p=0.04$). Although, when considering donor age, one can see that there is a significant overlapping of the values involved, as shown in Figure 3.3 and 3.4.

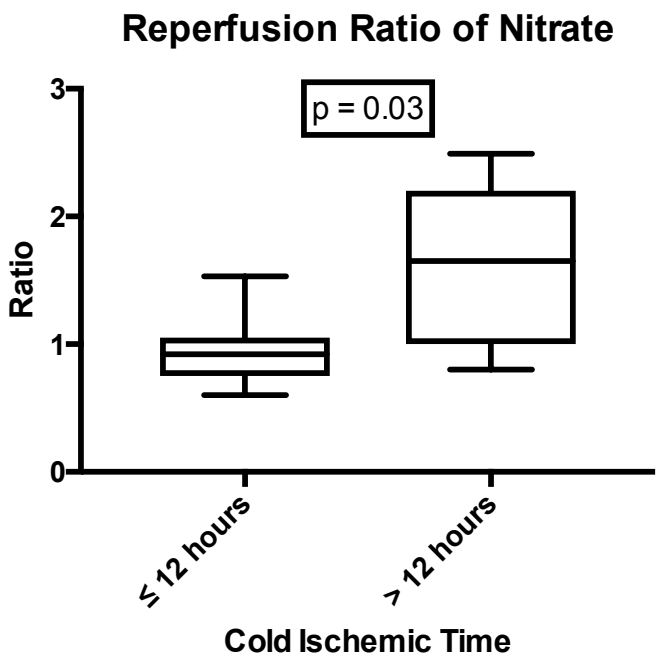


Figure 3.3: Difference in RRT between DCD kidney transplants with CIT of less than or equal to 12 hours and greater than 12 hours. *RRT of the levels of the NO metabolite nitrate in a series of patients undergoing renal transplantation from DCD donors. The values are median, interquartile and central range. RRT was significantly higher in patients who's CIT is greater than 12 hours using a Mann Whitney U test (n= 29, p=0.03).*

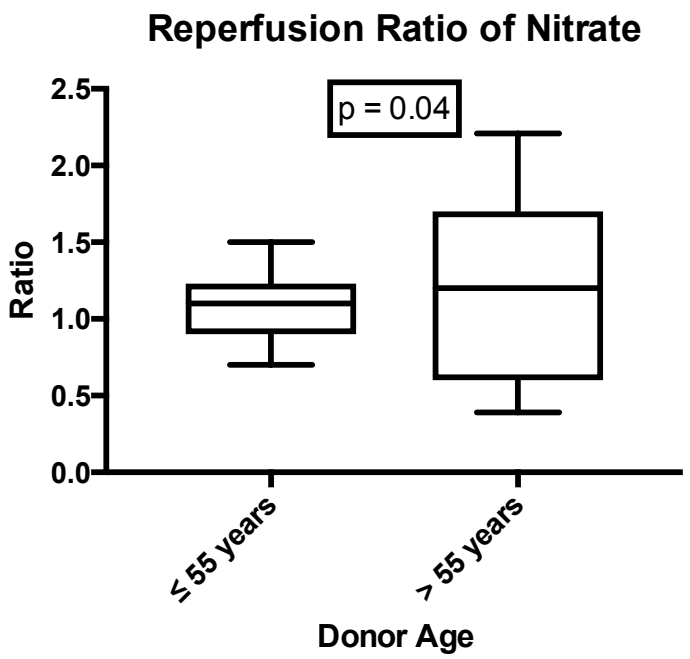


Figure 3.4: Difference in RRT between kidney transplants whose donor age is less than or equal to 55 years and greater than 55 years. *RRT of the levels of the NO metabolite nitrate in a series of patients undergoing renal transplantation from DCD donors. The values are median, interquartile and central range. RRT was significantly higher in patients who's donor age was greater than 55 years using a Mann Whitney U test (n= 29, p=0.04).*

Both CIT and donor age are risk factors for IRI, as discussed previously. No changes in RRT were seen when looking at other risk factors for DGF, such as WIT or recipient age. These results correlate with data from a recent UK study looking at outcomes in deceased renal transplants; the paper reported decreased graft survival in DCD transplants whose CIT was greater than 12 hours (interestingly this effect was not seen in DBD transplants) and decreased graft survival in kidneys from older donors (regardless of mode of donation) (14).

3.4.1. Summary of findings

This pilot investigation has shown that changes in nitrate vary significantly depending on donor type and that the greatest change is associated with DCD donors. Within the DCD subgroup the data suggests that CIT is the predominant factor, although, without adjustment for confounding variables is inconclusive.

CHAPTER 4: RESULTS – Part 2: Metabolites of NO in Recipients of DCD Kidneys in the Peri-operative Period and Associations with Risk Factors for DGF.

4.1. Introduction

In the previous chapter we have seen how changes in NO metabolites are more pronounced in DCD renal transplants. In the methodology section 2.4 (page 68) we concluded that in order to accurately quantify both nitrite and nitrate, in the context of this study, ozone chemiluminescence should be used. In this chapter we will investigate the levels of NO we found around the time of transplantation using the chemiluminescence method and see what factors may be affecting the production of NO. When considering the data presented it is important to remember that NO (and therefore NO_2^- and NO_3^-) were being produced in tissues throughout the body and also that those extra-renal tissues were subject to the systemic insult of IRI. To accommodate this and baseline variability within the population, our analysis will be focused on changes in NO_2^- and NO_3^- as opposed to the absolute values measured.

For each of the NO metabolites analysed, we will consider variables known to be risk factors for the extent of IRI namely WIT, CIT, age of the donor and recipient. We will also look to see if NO is affected by the method of storage of the organ before transplantation. i.e. machine perfusion as opposed to cold static storage.

In a separate section we will explore the temporal pattern of change of pro-inflammatory cytokines post reperfusion and their relation with NO metabolites. We will conclude by investigating whether changes in NO metabolites are affected by differing induction regimes, as they are known to affect the release of pro-inflammatory cytokines.

4.2. Nitrite

4.2.1. Nitrite concentration

All 280 samples collected from the 35 patients were analysed in duplicate using tri-iodide to cleave NO from NO_2^-, which was subsequently quantified by the NOA photomultiplier (ozone chemiluminescence). The graph below, Figure 4.1 depicts what happens to the NO_2^- levels in the 8 hours after reperfusion, shown with the pre and post op values for comparison.

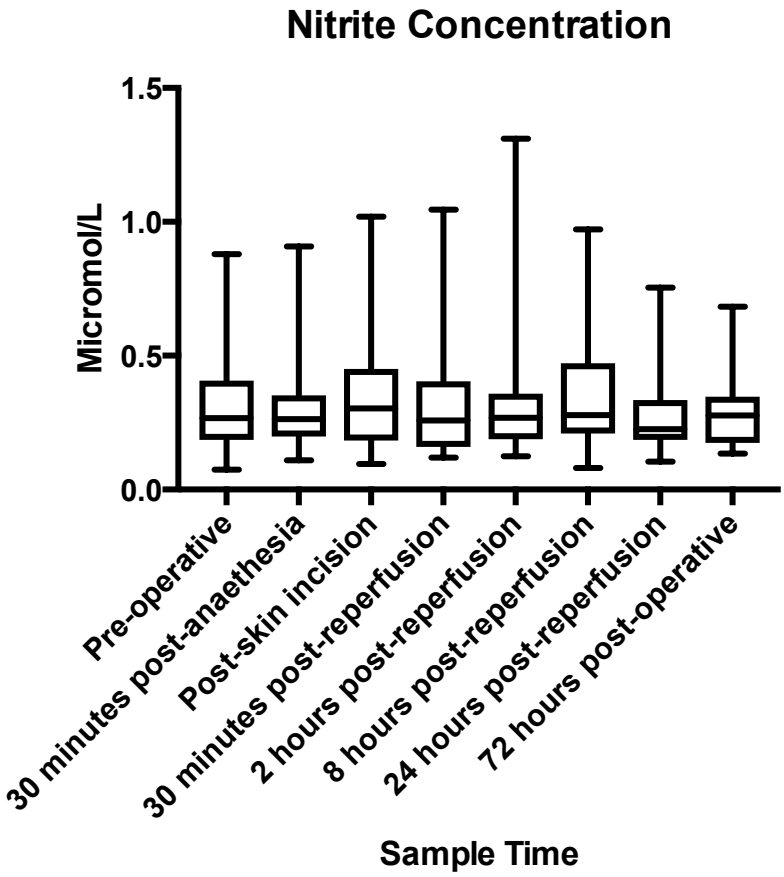


Figure 4.1: Box and whisker plots illustrating the nitrite distribution at each sample time point. *Box and whisker plots showing the median, interquartile and central range of nitrite at each sample point. There are no significant differences between the samples in the peri-operative period (n= 35, $\chi^2(6) = 4.779$, $p = 0.572$: Friedman test).*

There were no significant changes in NO_2- over time using a Friedman test ($\chi^2(6) = 4.779$, $p = 0.572$). We were interested to see if there was an inherent differing ability to produce NO amongst the recipients, which would explain why overall changes were not significant; therefore we looked to see if there was any relationship between the age of the recipient and the change in NO_2^-. There was no correlation between the baseline NO_2^- and the age of the recipient however there was a strong negative correlation between the age of the recipient and the change in NO_2^-at 8 hours post reperfusion r_s (33) = -0.435, p = 0.01 see Figure 4.2 below.

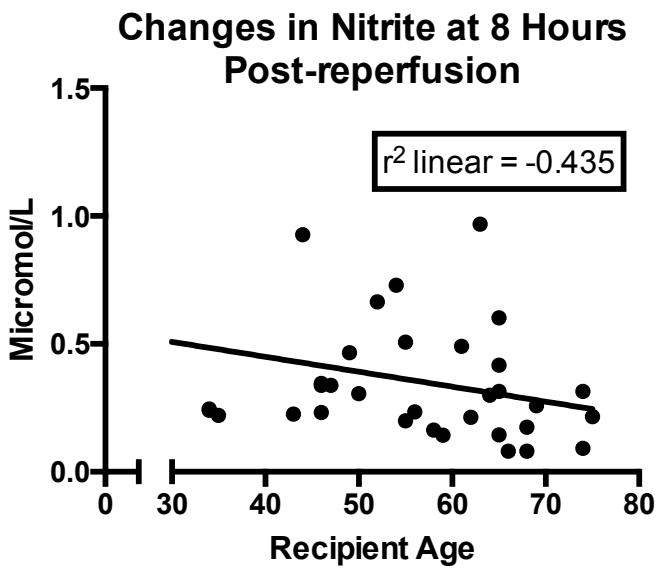


Figure 4.2: Changes in nitrite 8 hours post-reperfusion. *Scatterplot showing the relationship between changes of NO_2^- at 8 hours post-reperfusion and recipient age, shown with line of best fit (n=35, r_s (33) = -0.435, p = 0.01: Spearman's rank order correlation).*

4.2.1.1. Conclusion

We have found that increasing age is associated with an impaired ability of the body to mount a NO_2^- response to IRI in the context of renal transplantation. This finding is in keeping with the known changes that occur as the vasculature ages. In both humans and animals, studies have found that increased production of reactive oxygen species lead to endothelial dysfunction and that this process

becomes more prevalent with age (250-252). High levels of superoxide cause functional inactivation of NO (253) and production of peroxynitrite (251), thus compromising endothelial function. This is aggravated by an age related decline in eNOS expression (254, 255) as well as eNOS uncoupling (causing further peroxynitrite release) as a result of mitochondrial ROS formation and mitochondrial DNA lesions (256, 257). This study confirms that this is a biochemically detectable phenomenon in the renal transplant recipient.

As a result of the differing inherent ability of individuals to mount a nitrite response depending on their age, it became apparent that subsequent analysis to look for other factors associated with IRI that may contribute to changes in NO_2^- should use regression in order to account for the mal-distribution of the age factor.

4.2.2. IRI and changes in nitrite

IRI is a multifactorial and complex phenomenon. We do know that the age of the recipient, the donor and the duration of the warm and cold ischemic times are all contributing factors to DGF in a transplanted organ (198, 258) and that DGF may reflect a more severe IRI. We were therefore interested to see if there was any relationship between these variables and changes of NO_2^- found in plasma around the time of transplantation.

In order to correct for the natural variation in the baseline concentration we calculated the change of NO_2^- and used this as the dependent variable as opposed to absolute values measured. We looked at how the concentration changed from the baseline value to ensure that other factors, such as induction of anaesthesia and surgical stimulus, were not influencing our results. When looking at the period post-reperfusion of the transplanted kidney, we calculated the change between the pre and post-reperfusion value as this was felt to most accurately isolate any effects attributable to reperfusion. We used regression analysis to see if there were any associations between the age of the donor or recipient, the

CIT and the 1st and 2nd WITs and changes in NO_2^- that occur after reperfusion of the transplanted kidney. There was no relationship between the factors listed above and the change of NO_2^- in our earliest post reperfusion sample (30 min. post perfusion of the transplanted kidney). However at 2 and 8 hours post perfusion we found some of these factors to be significantly influencing NO_2^- changes.

At 2 hours post-reperfusion, regression analysis revealed that the change of NO_2^- was negatively related to the age of the recipient ($p=0.050$) and the model that included these factors was predictive with an $R^2= 0.159$. The regression coefficient and standard errors can be seen in the table below *Table 4.2.*

At 8 hours post-reperfusion regression analysis revealed that the change of NO_2^- positively related to the primary WIT ($p < 0.0005$) and negatively related to the age of the recipient ($p=0.001$). The model for NO_2^- at 8 hours that included these factors was predictive with an $R^2= 0.546$ ($p < 0.0005$). Regression coefficient and standard errors can be seen in the table below *Table 4.2.*

2 Hours Post reperfusion	SE	Beta	p
Age of Recipient	0.003	-0.443	**0.050**
Age of Donor	0.002	0.326	0.149
CIT	0.006	-0.032	0.858
1st WIT	0.006	0.173	0.362
2nd WIT	0.002	-0.218	0.247

8 Hours Post reperfusion	SE	Beta	p
Age of Recipient	0.003	-0.666	**0.001**
Age of Donor	0.003	-0.272	0.129
CIT	0.007	-0.139	0.302
1st WIT	0.008	0.567	**<0.0005**
2nd WIT	0.003	-0.112	0.421

Table 4.1: Change of nitrite at 2 and 8 hours post-reperfusion

Summary of multiple regression analysis, change of NO_2^- in µmol/L at 2 and 8 hours post reperfusion. SE = Standard error of the coefficient; Beta = standardized coefficient.

Twenty four and 72 hours post operatively there were no relationships found between our factors and changes in NO_2^-.

4.2.2.1. Conclusion

In this section we have found that changes in NO_2^- are related to certain risk factors for IRI and that this change appears to become biochemically detectable between 2 and 8 hours post reperfusion. Both primary WIT and recipient age are recognised risk factors for DGF and from our analysis we can see that they both are influential in NO_2^- change at 8 hours post-reperfusion. Changes at 2 hours are affected only by the age of the recipient. This reinforces our findings in 4.2.1 that aging decreases the body's ability to produce NO in the context of transplantation. Decreased production of NO may therefore be a contributing mechanism to the greater incidence of DGF (and therefore IRI) found in older recipients. (14). Prolonged WIT is a risk factor for IRI; we did not find any

previous studies linking it specifically to changes in NO however, in this analysis, it was found to be influential. We hypothesised that this may be due to changes occurring in the endothelium of the transplanted organ as a result of reperfusion injury.

Other factors that may play a role in nitrite changes:

4.2.3. The effect of machine perfusion on nitrite concentration post reperfusion

To examine if the use of machine perfusion had an effect on change in NO_2^- concentration a, Mann Whitney U test was performed to compare the two groups. There were no statistically different changes in NO_2^- concentration between the group that underwent machine perfusion and the group that underwent cold static storage at any point post-reperfusion see Figure 4.3 below.

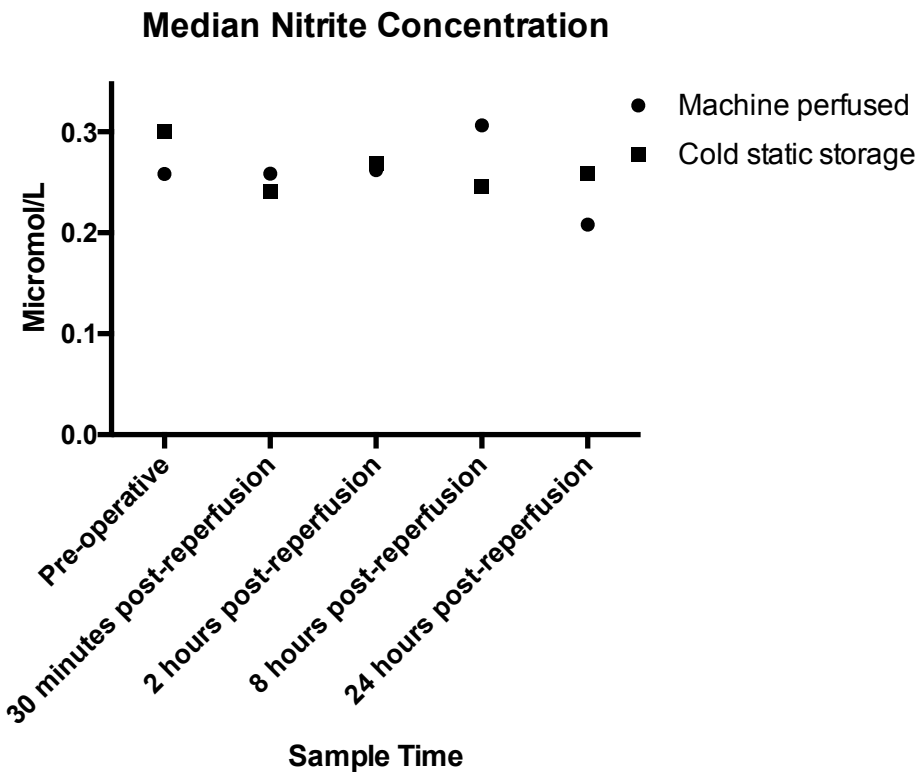


Figure 4.3: Nitrite concentration according to storage method. *Data showing the median nitrite concentration in µmol/L during the period post-reperfusion, according to storage method. No significant differences were found between groups (n=35, pre-operative p=0.483, 30 minutes post-reperfusion p=0.878, 2 hours post-reperfusion p=0.832, 8 hours post-reperfusion p=0.484, 24 hours post-operative p=0.102: Mann Whitney U test).*

4.2.4. Effect of recipient and donor sex on changes in nitrite post reperfusion

To see if sex of the recipient or donor had an effect on change in NO_2^- concentration, a Mann Whitney U test was performed to compare the two groups. There were no statistically different changes in NO_2^- concentration between male and female recipients at any point. Similarly there was no difference in NO_2^- concentrations found in recipients receiving kidneys from male or female donors. See Figures 4.4 and 4.5 below. The numerical difference seen in the post -reperfusion samples was pre-existing in the base line ones.

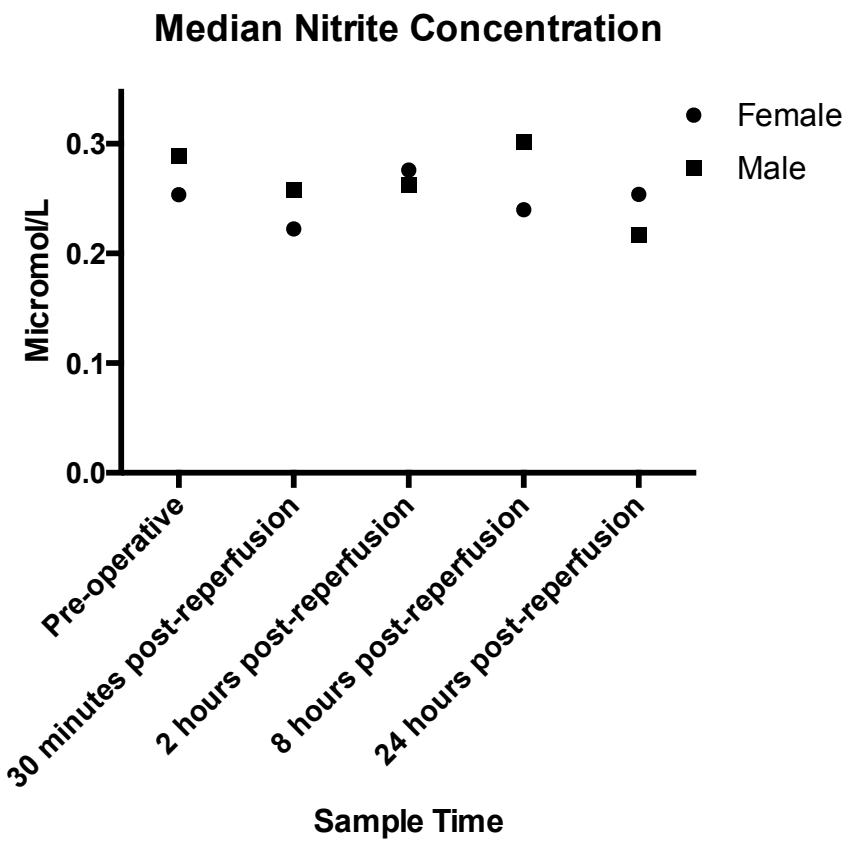


Figure 4.4: Nitrite concentration according to recipient sex. *Data showing the median nitrite concentration in µmol/L during the period post-reperfusion according to sex of the recipient. No significant differences were found between groups (n=35, pre-operative p=0.451, 30 minutes post-reperfusion p=0.390, 2 hours post-reperfusion p=0.954, 8 hours post-reperfusion p=0.952, 24 hours post-operative p=0.424: Mann Whitney U test).*

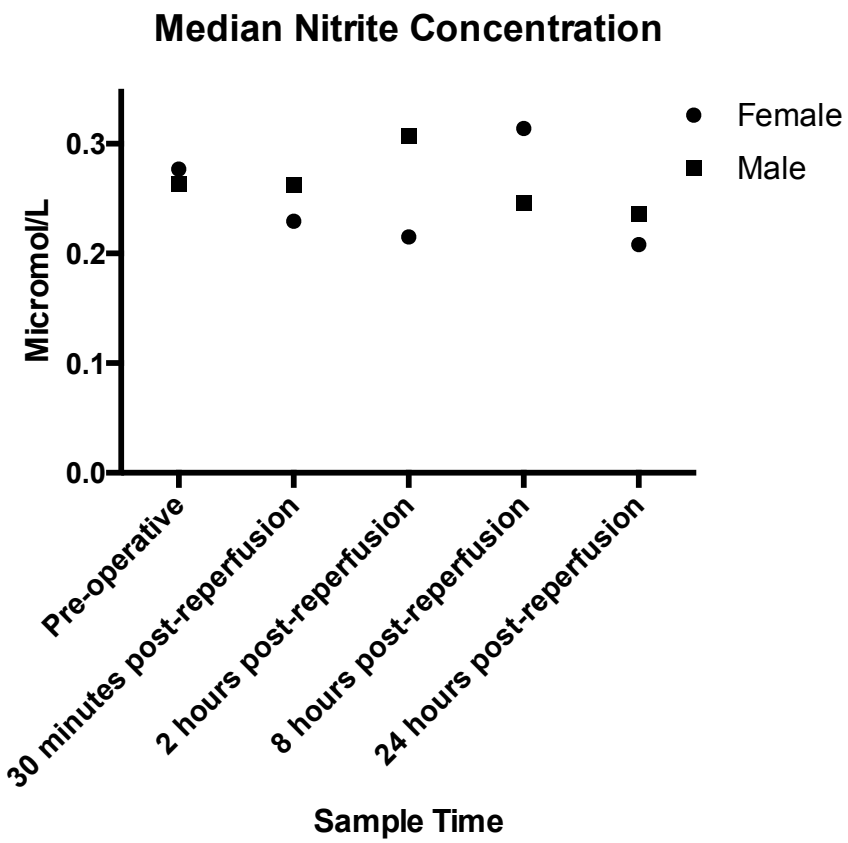


Figure 4.5: Nitrite concentration according to donor sex. *Data showing the median nitrite concentration in µmol/L during the period post-reperfusion according to sex of the donor. No significant differences were found between groups (n=35, pre-operative p=0.523, 30 minutes post-reperfusion p=0.506, 2 hours post-reperfusion p=0.707, 8 hours post-reperfusion p=0.471, 24 hours post-operative p=0.581: Mann Whitney U test).*

4.2.5. Summary of findings

In this section we have seen that aging appears to impair the body's ability to produce NO_2^- and that the 1st WIT, that is associated with IRI, is also influential in the change of NO_2^- levels during the process of renal transplantation.

We have also seen that these nitrite changes are not influenced by the sex of the donor or the recipient, and not statistically by the organ storage method. Next we will look to analyse NO_3^- to see if it is influenced by the same factors.

4.3. Nitrate

4.3.1. Nitrate concentration

We looked to see if changes in NO_3^- followed the same pattern as nitrite and if its changes were influenced by the same risk factors for IRI. Figure 4.6 shows how the nitrate concentration changed over the sampling period.

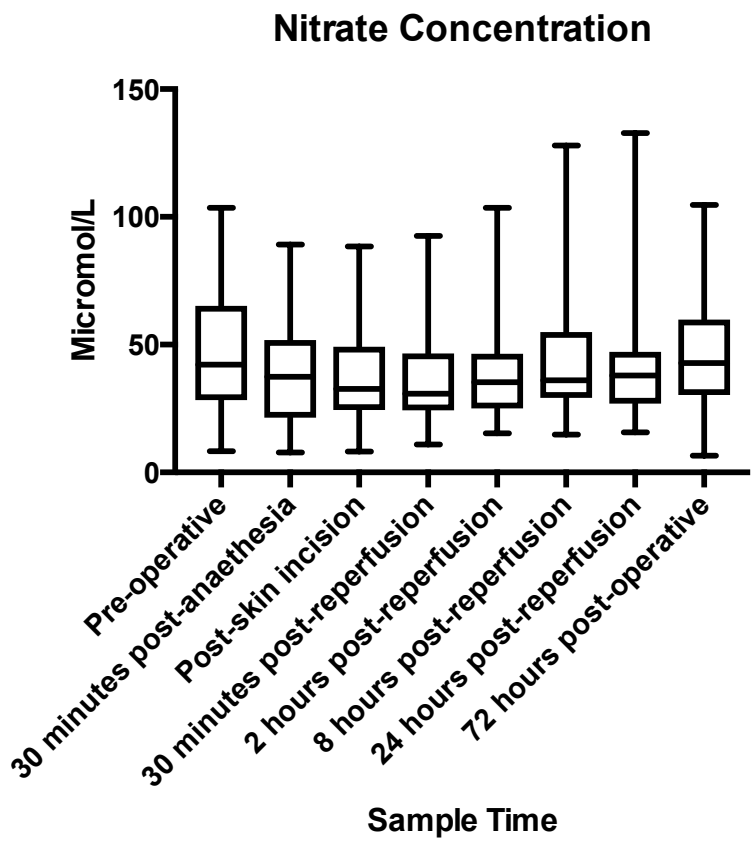


Figure 4.6: Box and whisker plots illustrating the nitrate distribution at each sample time point. *Box and whisker plots showing the median, interquartile and central range of nitrite at each sample point. Changes in NO_3- over time were statistically significant, ($n=35$, $\chi^2(3) = 12.097$, $p = 0.003$: Friedman test). Pairwise comparisons with a Bonferroni correction for multiple comparisons showed NO_3^- concentration increased significantly between samples 30 minutes and 8 hours post-reperfusion ($p = 0.005$).*

Unlike NO_2^- we found that NO_3- levels changed significantly over that sampling period. If we look at the period around *organ reperfusion* we see that the concentration rises steadily. A Friedman test was run to determine if there were statistical differences in the change of NO_3^- levels during the sample collection. Changes in NO_3- over time were statistically significant, $\chi^2(3) = 12.097$, $p = 0.003$. Pairwise comparisons were performed with a Bonferroni correction for multiple comparisons; NO_3^- concentration increased significantly between samples taken at 30 minutes and 8 hours post-reperfusion ($p = 0.005$).

To see if changes in NO_3^- were influenced by the age of the recipient (as we had found with NO_2^-) we performed correlation analysis using Speaman's rank correlation. Once again there was a strong negative correlation between the age of the recipient and the change of NO_3^- at 8 hours post reperfusion r_s (32) = -0.508, p = 0.002, see Figure 4.7. There was also negative correlation between recipient age and the change in NO_3^- at 30 minutes post-reperfusion r_s (32) = -0.358, p = 0.038, Figure 4.8.

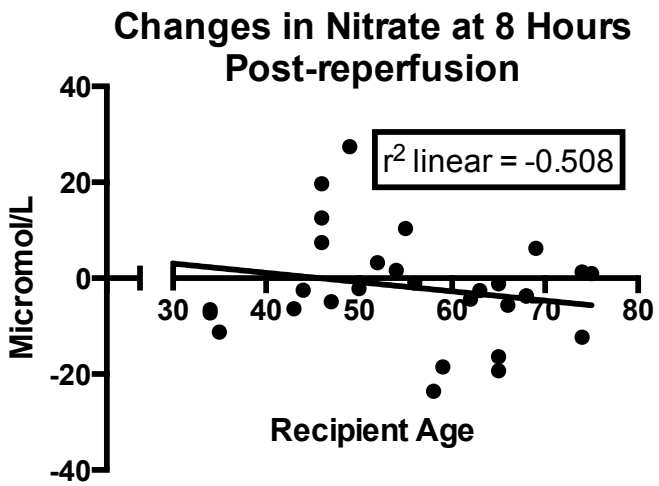


Figure 4.7: Changes in nitrate at 8 hours post-reperfusion. *Scatterplot showing the relationship between changes of NO_3^- at 8 hours post-reperfusion and recipient age, shown with line of best fit. (n=35, r_s (32) = -0.508, p = 0.002: Spearman's rank order correlation).*

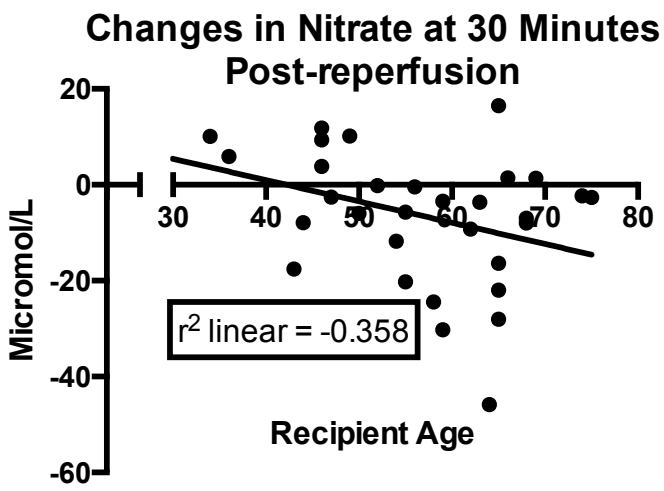


Figure 4.8: Changes in nitrate at 30 minutes post-reperfusion. *Scatterplot showing the relationship between changes of NO_3^- at 30 minutes post reperfusion and recipient age, shown with line of best fit. (n=35, r_s (32) = -0.358, p = 0.038: Spearman's rank order correlation).*

4.3.2. IRI and change in nitrate

Regression analysis was performed using the same variables as we had used during the nitrite analysis to see if these factors were also effecting nitrate changes. The variables we included were WIT, CIT and donor and recipient age. At no point was any relationship found between risk factors for IRI and changes in NO_3^- as shown in Table 4.4. It is surprising that recipient age is not significant as there was good correlation found in the previous analysis it is possibly a reflection that the study is underpowered.

2 Hours Post-reperfusion	SE	Beta	p
Age of Recipient	0.180	-0.194	0.406
Age of Donor	0.158	0.209	0.373
CIT	0.398	-0.010	0.959
1st WIT	0.430	0.046	0.817
2nd WIT	0.149	-0.229	0.248

8 Hours Post-reperfusion	SE	Beta	p
Age of Recipient	0.226	-0.309	0.182
Age of Donor	0.208	-0.028	0.904
CIT	0.475	-0.192	0.277
1st WIT	0.524	0.098	0.605
2nd WIT	0.177	-0.210	0.254

Table 4.2: Change of nitrate at 2 and 8 hours post-reperfusion. *Summary of multiple regression analysis, change of NO_3^- in µmol/L at 2 and 8 hours post-reperfusion. SE = Standard error of the coefficient; Beta = standardized coefficient.*

Other factors that may play a role in nitrate changes:

4.3.3. The effect of machine perfusion on nitrate concentration

To see if use of machine perfusion had an effect on change in NO_3^- concentration the Mann Whitney U test was performed to compare the two groups. There were no statistically different changes in NO_3^- concentration between the group that underwent machine perfusion and the group that underwent cold static storage at any point post reperfusion, see Figure 4.9 below.

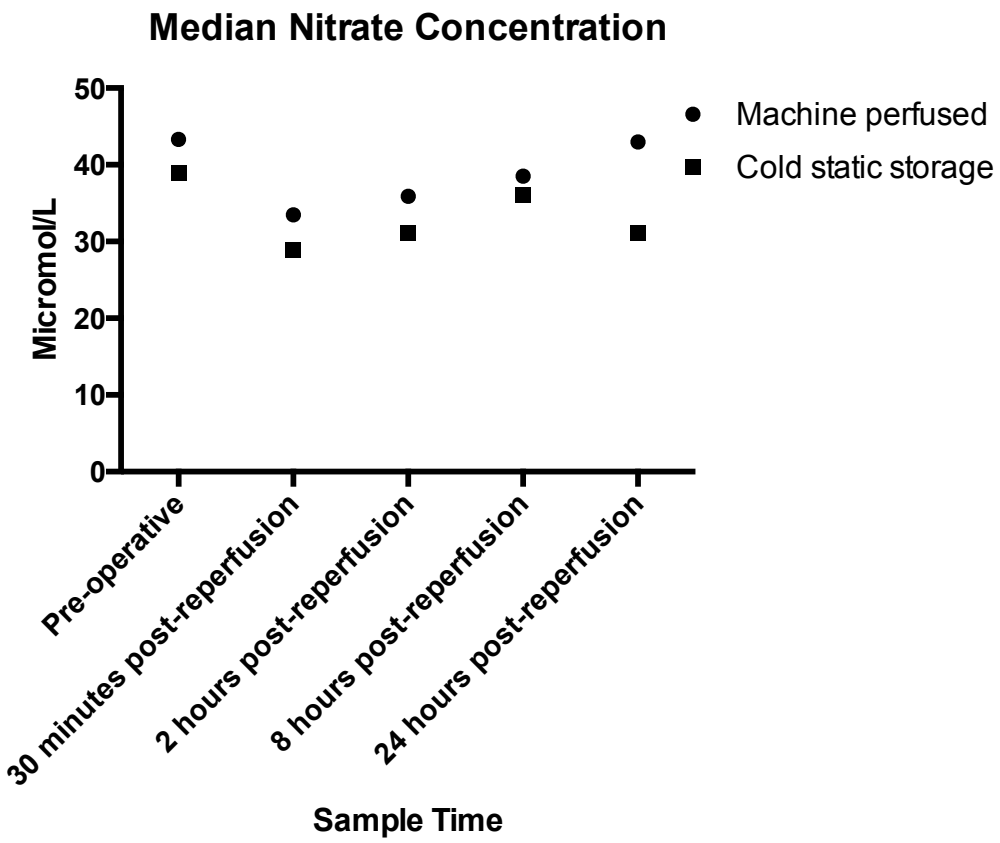


Figure 4.9: Nitrate concentration according to storage method. *Data showing the median nitrate concentration in μmol/L during the period post-reperfusion, according to storage method. No significant differences were found between groups (n=35, pre-operative p=0.258, 30 minutes post-reperfusion p=0.422, 2 hours post-reperfusion p=0.483, 8 hours post-reperfusion p=0.932, 24 hours post-operative p=0.577: Mann Whitney U test).*

4.3.4. The effect of recipient and donor sex on changes in nitrate

To see if sex of the recipient or donor had an effect on change in NO_3^- the Mann Whitney U test was performed to compare the two groups. There were no statistically different changes in NO_3^- concentration between male and female recipients at any point during the sampling period. Similarly there was no difference in NO_3^- concentrations found in recipients receiving kidneys from male or female donors. See Figures 4.10 and 4.11 below.

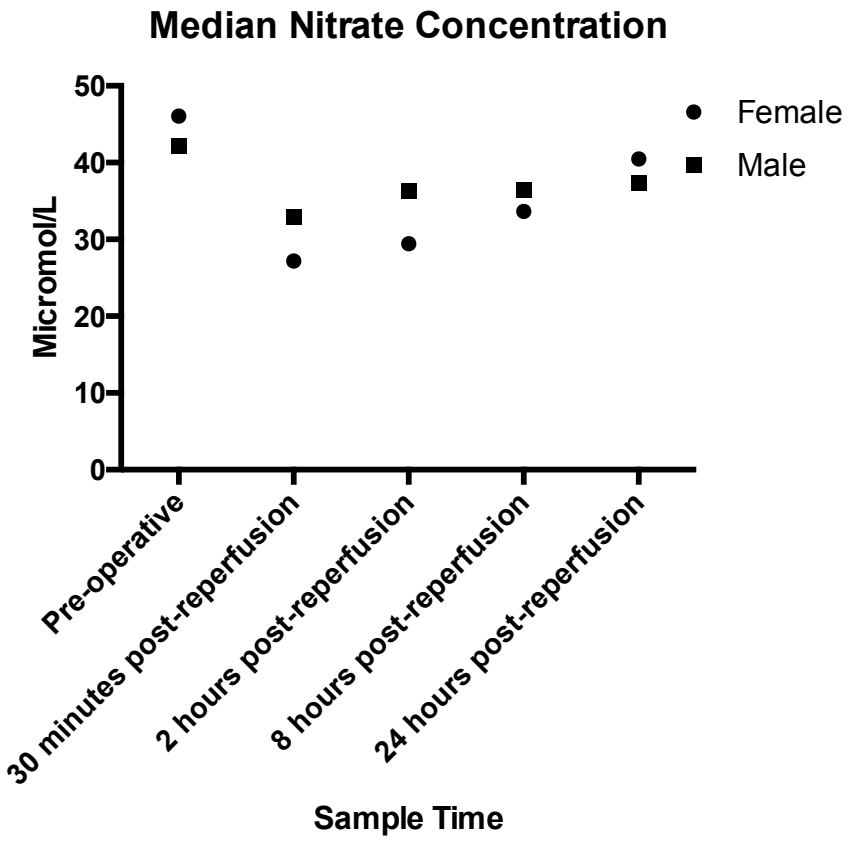


Figure 4.10: Nitrate concentration according to recipient sex. *Data showing the median nitrate concentration in µmol/L during the period post-reperfusion, according to sex of the recipient. No significant differences were found between groups (n=35, pre-operative p=0.832, 30 minutes post-reperfusion p=0.347, 2 hours post-reperfusion p=0.252, 8 hours post-reperfusion p=0.563, 24 hours post-operative p=0.803: Mann Whitney U test).*

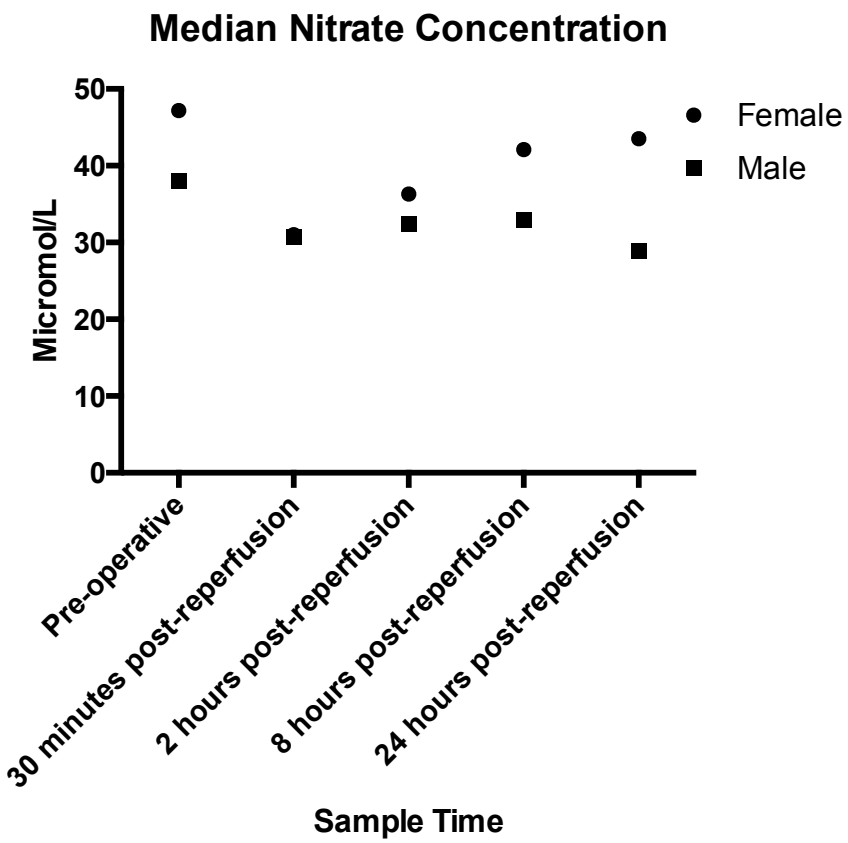


Figure 4.11: Nitrate concentration according to donor sex. *Data showing the median nitrate concentration in µmol/L during the period post-reperfusion, according to sex of the donor. No significant differences were found between groups (n=35, pre-operative p=0.301, 30 minutes post-reperfusion p=0.67, 2 hours post-reperfusion p=0.317, 8 hours post-reperfusion p=0.066, 24 hours post-operative p=0.179: Mann Whitney U test).*

4.3.5. Summary of findings

Nitrate levels rose significantly in the 8 hours after reperfusion of the kidney and this change was found to be negatively associated with the age of the recipient. Unlike NO_2^- we did not find any association between changes in NO_3^- and WIT, (this could represent differing roles for NO_2^- and NO_3^- in the context of IRI), nor did we find that recipient or donor sex, or storage method was influential.

4.4. Nitrate/Nitrite ratio

4.4.1. Introduction

In the previous sections we have found that changes in NO_2^- are linked to factors affecting IRI such as the WIT and the age of the recipient. We have also seen that NO_3^- seems not to be affected by the warm ischemia time.

From our knowledge of the *Nitrate – Nitrite – Nitric oxide axis* we know that:

- NO may be oxidized to NO_2^- in the presence of ceruloplasmin
- NO may be oxidized to NO_3^- in the presence of oxyhaemoglobin
- NO_2^- can be reduced to NO in hypoxic conditions
- NO_3^- can be reduced to NO_2^- by bacteria in the gut
- NO_2^- and NO_3^- may be taken in the diet or formed by oxidation of NO (77)

During the process of retrieval, implantation and reperfusion of the kidney the of the *Nitrate – Nitrite – Nitric oxide axis* will fluctuate depending upon the physiological conditions at the time. In order to analyse the axis in the context of this study we looked at the ratio between NO_3^- and NO_2^- see Figure 4.14 below.

4.4.2. NO_3^- / NO_2^- changes over the sampling period

Figure 4.12 below shows how the ratio of NO_3^- / NO_2^- behaved over the sampling period.

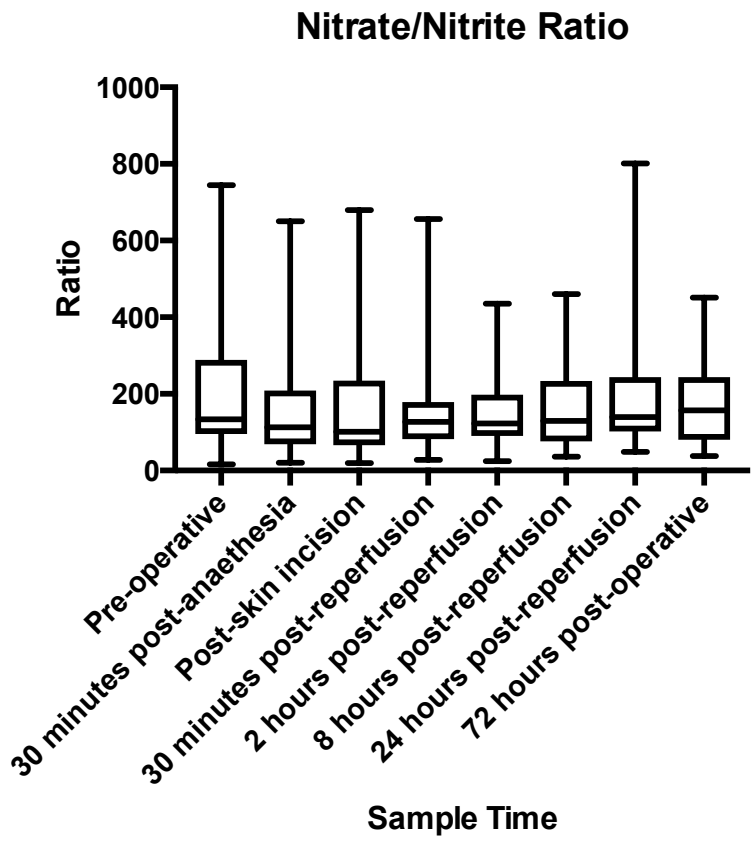


Figure 4.12: Box and whisker plots illustrating the nitrate/nitrite ratio at each sample time point. *Box and whisker plots showing the median, interquartile and central range of nitrate/nitrite ratio at each sample point. There are no significant differences between the samples in the peri-operative period (n=35, $\chi^2(4) = 4.682$, p = 0.322: Friedman test).*

Changes in NO_3-/NO_2^- over time were not found to be statistically significant, $\chi^2(4) = 4.682$, p = 0.322 using a Friedman test.

4.4.3. IRI and changes in the ratio

Having identified recipient age and 1st WIT as important factors for NO_2^- we used these (along with CIT) as variables for regression analysis of the NO_3^- / NO_2^-, along with the pre-operative value (to ensure the model looked at change), see Table 4.6.

2 Hours	SE	Beta	p
Age of Recipient	1.738	0.318	0.141
Age of Donor	1.52	-0.202	0.348
1st WIT	4.162	0.139	0.446
2nd WIT	1.467	0.139	0.477
CIT	4.059	-0.067	0.698

8 Hours	SE	Beta	p
Age of Recipient	1.923	0.417	0.063
Age of Donor	1.766	-0.215	0.331
1st WIT	4.468	-0.243	0.183
2nd WIT	1.536	0.038	0.828
CIT	4.059	0.148	0.378

Table 4.3: Nitrate/nitrite ratio at 2 and 8 hours post-reperfusion. *Summary of Multiple Regression Analysis Change of NO_3^- / NO_2^- at 2 and 8 hours post reperfusion B = unstandardized regression coefficient; SE = Standard error of the coefficient; Beta = standardized coefficient.*

4.4.4. Summary of findings

The ratio between nitrate and nitrite in our samples was not influenced by factors that effect IRI. We could therefore conclude that the ratio between nitrate and nitrite, in spite of their known biochemical relationship, is less useful than the changes in concentration of the individual metabolites.

4.5. Discussion

This chapter has demonstrated that:

1. At 2 and 8 hours post reperfusion of the transplanted kidney, changes in NO_2^- are influenced by WIT and the recipient age.
2. Increasing age is associated with a reduced ability to produce NO_2^- and NO_3^-.
3. NO_3^- concentration increased significantly up to 8 hours post reperfusion.

The WIT and the age of the recipient significantly influenced changes in NO_2^- at 2 and 8 hours after reperfusion regardless of the effect of induction immunosuppression. Both WIT and recipient age are known contributing factors to IRI (198, 258). This study found as WIT increased the so did the change seen in NO_2^-, indicating that the greater the extent of IRI the greater the concentration of NO_2^- at 8 hours post-reperfusion of the kidney. Whether the NO_2^- is produced by the endothelium of transplanted organ or systemically by the recipient is beyond the scope of this study to prove conclusively, however the relationship seen with recipient age implies that this is a systemic response. This reaction is thought to occur as a consequence of cytokine up-regulation (and therefore activation of NOS) produced by the inflammatory mediators released in response to IRI (259). The next chapter will investigate the patterns of cytokine release in our patient cohort and look to see if there are any associations with NO release.

CHAPTER 5: RESULTS – Concentrations of Cytokines in Recipients of DCD Kidneys in the Peri-operative Period and the Effect of Induction Immunosuppression

5.1. Introduction

In the previous chapter we looked at NO changes in the plasma of patients undergoing DCD renal transplant. We found that NO metabolite concentrations were influenced by factors contributing to IRI. Changes in NO may be due to pro-inflammatory cytokines, which stimulate iNOS up-regulation. To investigate this further we measured IL-6, IL-10, and TNF-α in the available plasma samples using a *Luminex Performance Assay.* The samples selected for analysis were times A (pre-operative), C (post-skin incision, prior to clamping of the blood vessels), D (30 minutes post-reperfusion), E (2 hours post-reperfusion) and G (24 hours post-operative) and they were analysed in duplicate for each patient. We selected timings around reperfusion that we thought maybe relevant as well as the baseline and 24 hours post operatively for comparison.

5.2. Cytokine Response to Reperfusion

When the distributions of the cytokines in the baseline samples was initially analysed, the concentrations were not normally distributed and therefore the data was further analysed using non-parametric tests.

5.2.1. IL-6

IL-6 levels rose after reperfusion of the kidney, with the peak found 2 hours after reperfusion. We used a Friedman test to see if these changes were statistically significant; pairwise comparisons were performed with a Bonferroni correction for multiple comparisons. Changes in IL-6 were found to increase significantly $\chi^2(4) = 92.99$, $p < 0.0005$. Post hoc analysis revealed statistically significant differences between the concentrations measured post skin incision (C) (as well as the pre-operative sample (A)) and those found at 30 minutes and 2 hours post-reperfusion (D and E). Figure 5.1 below illustrates the changes found and the box plot shows how the response to reperfusion varied within the sample population. We can see that post reperfusion (D + E), there is a much greater

range in the concentration of IL-6 (see Figure 5.1), this may be attributable to differing propensity to produce cytokines in the individual subjects or may be as a result of the induction immunosuppression. Later on in this chapter we will look to determine further what factors may be influencing this variation.

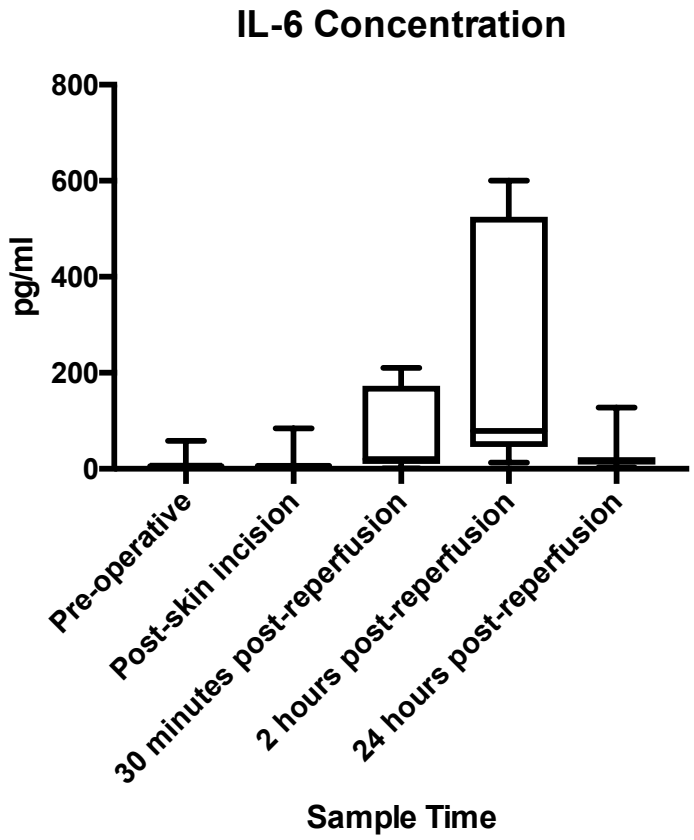


Figure 5.1: IL-6 distribution. *Box and whisker plot showing the median, interquartile and central range of IL-6 in pg/ml around the time of reperfusion and at 24 hours post-operatively. Changes in IL-6 vary significantly (n=35, $\chi^2(4) = 92.99$, $p < 0.0005$: Friedman test). Median (range) in pg/ml: Pre operatively = 5 (0 – 58), post skin incision = 6 (0 – 535), 30 minutes post-reperfusion = 20 (1 – 16200), 2 hours post-reperfusion = 79 (13 – 13135) and 24 hours post operatively = 16 (3 – 128).*

5.2.2. IL-10

The levels of IL-10 also rose after reperfusion with a peak at 2 hours (sample E). A Friedman test found statistically significant changes $\chi^2(4) = 91.604$, $p < 0.0005$ in IL-10 concentration. Post hoc analysis revealed statistically significant differences between the concentrations measured post skin incision (C), (as well as the pre-operative sample (A)) and those found at 30 minutes and 2 hours post-reperfusion (D and E). Once again the range in samples D and E varied greatly as is illustrated in the box and whisker plot below, Figure 5.2.

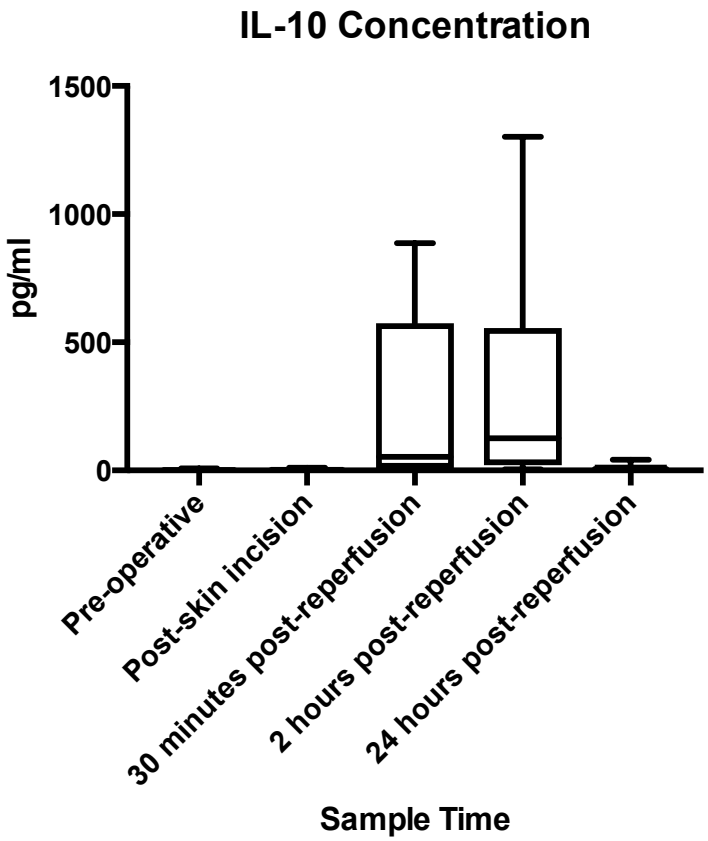


Figure 5.2: IL-10 distribution. *Box and whisker plot showing the median, interquartile and central range of IL-10 in pg/ml around the time of reperfusion and at 24 hours post-operatively. Changes in IL-10 vary significantly (n=35, $\chi^2(4) = 91.604$, $p < 0.0005$: Friedman test). Median (range) in pg/ml: Pre operatively = 1 (0 – 8), post skin incision = 2 (0 – 1306), 30 minutes post perfusion = 53 (1 – 7474), 2 hours post perfusion = 125 (5 – 3925) and 24 hours post operatively = 5 (1 – 151).*

5.2.3. TNF-α

Changes in TNF-α were not found to be significantly different $\chi^2(4) = 2.192$, $p = 0.701$ using a Friedman test. Again the largest ranges were seen in the samples taken after reperfusion of the kidney, see Figure 5.3.

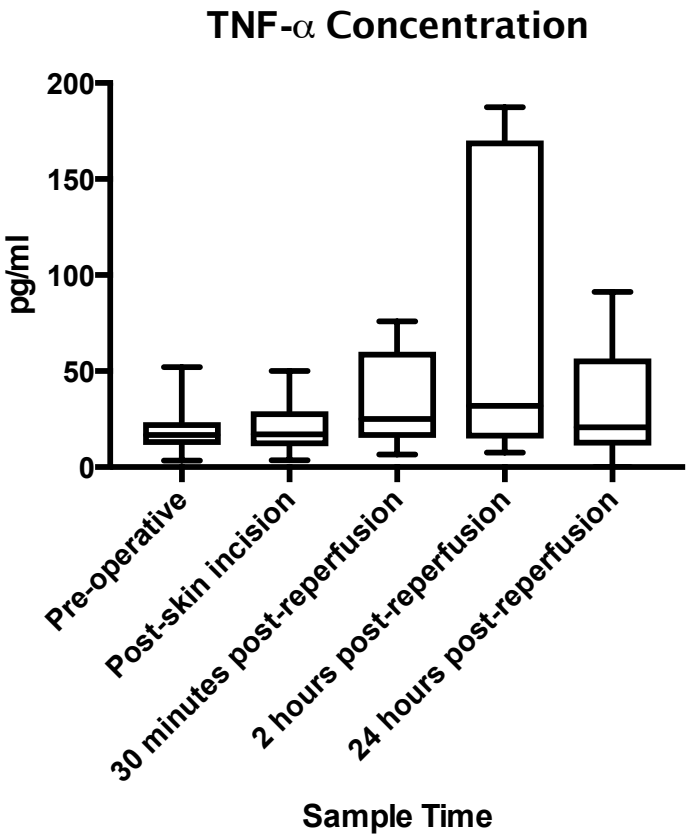


Figure 5.3: TNF-α distribution. *Box and whisker plot showing the median, interquartile and central range of TNF-α in pg/ml around the time of reperfusion and at 24 hours post-operatively. Changes in TNF-α do not vary significantly (n=35, $\chi^2(4) = 2.192$, p = 0.701: Friedman test). Median (range) in pg/ml: Pre operatively = 17 (3 – 52), post skin incision = 17 (4 – 1306), 30 minutes post perfusion = 25 (7 – 2232), 2 hours post perfusion = 32 (8 – 4400) and 24 hours post operatively = 21 (0 – 159).*

5.2.4. Summary

All of these cytokines increased after reperfusion of the kidney; this study found significant rises 30 minutes and 2 hours post-reperfusion in IL-6 and IL-10. The aetiology of this is likely to be multifactorial and include the process of organ reperfusion as well as the recipients' response to the surgical stimulus. However, induction immunosuppression might have exerted a significant influence and the next section of analysis will look at how this differed between groups.

5.3. Cytokine Response to Induction Immunosuppression

This section will look at cytokine changes between groups who received different induction immunosuppression. It is important to be mindful of the way these medications are administered; Simulect was administered approximately one hour pre operatively; Campath during the period of this study was administered subcutaneously during the anaesthetic induction; and finally ATG was administered as an infusion over 4-6 hours during the operative procedure. Both Campath and Simulect infusions are complete by the time the third sample (post induction of anaethesia and prior to skin incision) is taken, however, the ATG infusion is on going whilst samples the third sample is taken, as well as whilst the samples are taken at 30 minutes and 2 hours post perfusion.

5.3.1. IL-6

As shown in the previous section (5.2.1.) we found a significant rise in IL-6 levels after reperfusion. When we broke the group down to look at the effects of induction immunosuppression we can see that this rise was most profound in the ATG group followed by the Campath group and then Simulect, see Figure 5.4.

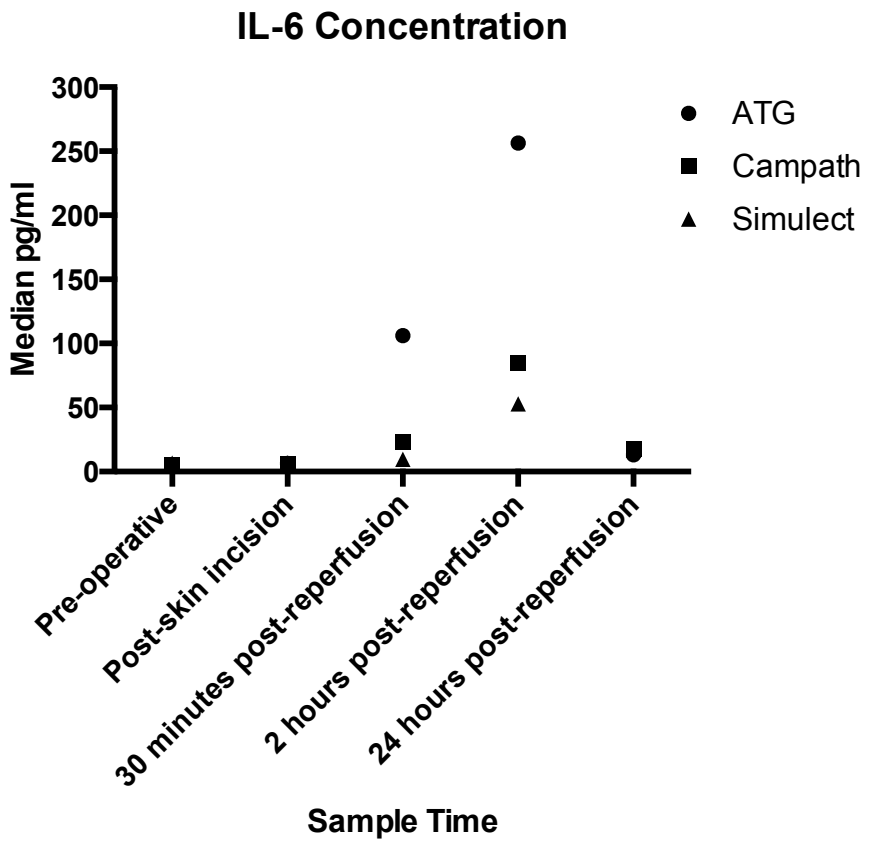


Figure 5.4: IL-6 concentration according to induction immunosuppression group. *Median IL-6 concentrations in pg/ml shown over time according to induction immunosuppression group. Using the Kruskal-Wallis H tests significant differences are found at 30 minutes post-reperfusion (n=35, $\chi^2(2) = 11.92$ $p = 0.003$) and 2 hours post-reperfusion (n=35, $\chi^2(2) = 12.639$ $p = 0.002$) between groups.*

Each immunosuppression group was analyzed looking at the pre-operative and post-reperfusion samples in order to explore further the effects of each induction therapy. Within all of the groups we found statistically significant rises of IL-6 in

the post-reperfusion samples. In the ATG group, a Wilcoxon signed-rank test determined that there was a statistically significant increase in median IL-6 concentration (101 pg/ml) between the pre-operative (5 pg/ml) and the 30 minute post-reperfusion sample (106 pg/ml), z = 3.059, p = 0.002 and also between the pre-operative and the 2 hour post-reperfusion sample (256 pg/ml), z = 3.18, p = 0.001, median increase (252 pg/ml).

In the Campath group there was also a significant difference between the pre-operative and post-reperfusion samples; median increase in IL-6 concentration at 30 minutes was 19 pg/ml, pre-operative median 5 pg/ml and the 30 minute post-reperfusion sample 23 pg/ml, z = 2.083, p = 0.005, at 2 hours the median increase was 80 pg/ml, the 2 hour post-reperfusion median was 86 pg/ml, z = 2.803, p = 0.005.

In the Simulect group there was a statistically significant median increase in IL-6 concentration (3 pg/ml) between the pre-operative (7 pg/ml) and the 30 minute post-reperfusion sample (10 pg/ml), z = 2.66, p = 0.008 and also between the pre-operative and the 2 hour post-reperfusion sample (53 pg/ml), z = 3.059, p = 0.002, median increase (46 pg/ml).

The statistical analysis found significant increases in all of the immunosuppression groups, the graphical representation in Figure 5.4 above shows that this is most profound in the ATG group. As mentioned previously, when considering the immunosuppression groups individually we must remember that the groups are small and therefore we may be seeing the results of a type 1 error. To check to see if one immunosuppression had more of an effect we used a Kruskal-Wallis H tests to compare the groups; at 30 minutes post-reperfusion and 2 hours post-reperfusion there were significant differences between induction groups, $\chi^2(2)$ = 11.92 p= 0.003 and, $\chi^2(2)$ = 12.639 0.002 respectively. Pairwise comparisons were performed using Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons. This post hoc analysis revealed that the statistically significant differences were between the Simulect and ATG groups see Figure 5.5 below.

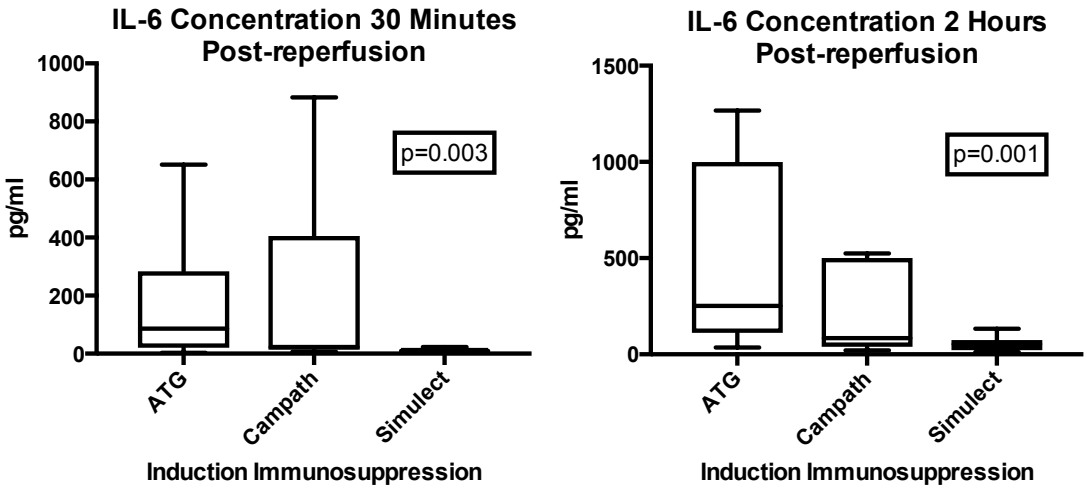


Figure 5.5: Distribution of IL-6 at 30 minutes and 2 hours post-reperfusion according to induction immunosuppression. *Box and whisker plots showing median, interquartile and central range of IL-6 in pg/ml between induction groups at each statistically significant sample. At 30 minutes post reperfusion there is a significant difference in (median) IL-6 concentration in pg/ml between Simulect (10) and ATG (106), n=35, p = 0.003, at 2 hours post reperfusion there is a significant difference in IL-6 concentration between Simulect (53) and ATG (256), n=35, p = 0.001.*

This section has shown that induction immunosuppression has a significant effect on cytokine release in the context of renal transplantation. In the case of IL-6, we found post-reperfusion concentration increases in all of the groups. It is possible that this was caused by organ reperfusion however further analysis found a difference between patients who received ATG and Simulect. IL-6 has been studied after reperfusion in live donor kidneys (166), de Vries et al. found that IL-6 concentration rises after reperfusion in samples taken directly from the renal artery and vein at 30 minutes post-reperfusion and the current study supports that finding. The paper also proposes that it is the allograft itself rather than systemic production by the recipient that causes this rise (it was not specific about the immunosuppression regimen administered to the subjects). Furthermore the same study looked at IL-6 in a mouse model and found it to be protective against renal IRI.

Simulect is a monoclonal antibody specific for the IL-2 receptor (CD25); IL-2 regulates activation and proliferation of cytotoxic T-cells as well as B-cell, NK-cell and regulatory T-cell function (260, 261) thus Simulect suppresses the immune response. Specifically, Simulect binds to the ectodomain of IL-2Rα blocking IL-2 signalling. Other IL-2 receptors, for example IL-2Rβ and γc, are shared with other cytokines (IL-4, IL-7, IL-9 and IL-15) however IL-2Rα is thought to be unique to IL-2 (262), the specificity of the monoclonal antibody target lead us to the conclusion that although IL-6 concentration rose in the Simulect group this may be the effects of reperfusion, where as the rise found in ATG may be due to the drug itself and this could be why we found a difference between the changes in these two groups.

5.3.2. IL-10

In section 5.2.2. we found that IL-10 levels rose significantly after reperfusion of the kidney Figure 5.6 below shows how IL-10 varied according to induction immunosuppression.

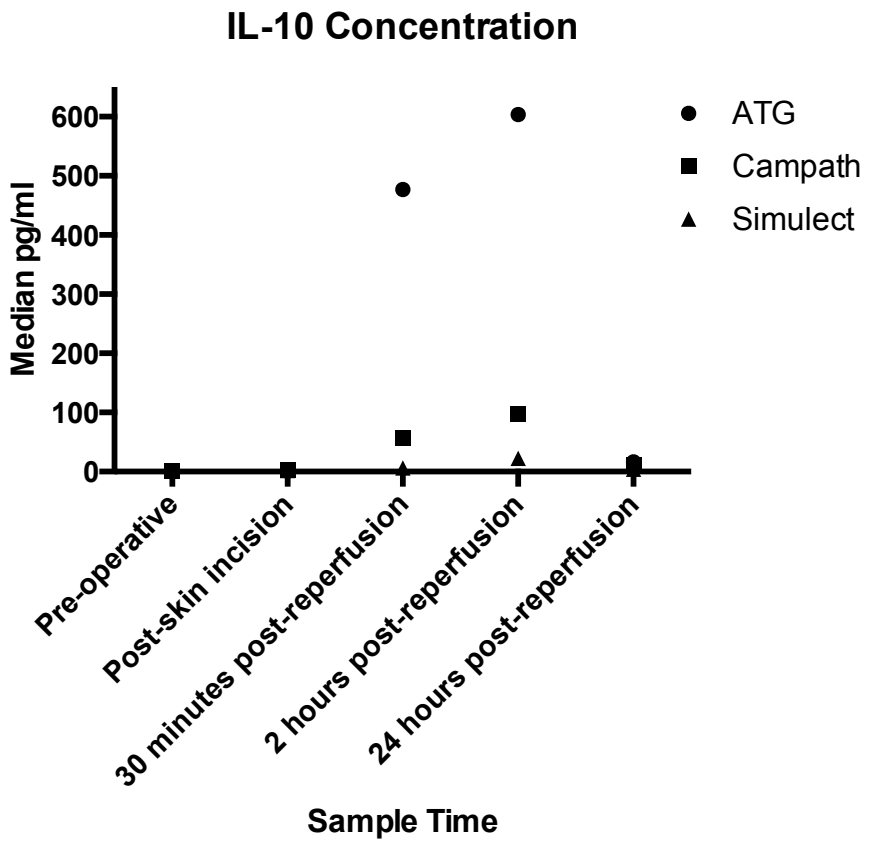


Figure 5.6: IL-10 concentration according to induction immunosuppression group. *Median IL-10 concentrations in pg/ml shown over time according to induction immunosuppression group. Using the Kruskal-Wallis H tests significant differences are found at 30 minutes post-reperfusion (n=35, $\chi^2(2) = 21.124$ $p < 0.0005$) and 2 hours post-reperfusion n=35, ($\chi^2(2) = 19.112$, $p < 0.0005$) between groups.*

As with IL-6, we looked to see if there was a change within the immunosuppression groups post-reperfusion, once again, there were significant

rises in all groups at 30 minutes and 2 hours post perfusion. The results for each group are described and the significant results are illustrated in Figure 5.7.
In the ATG group a Wilcoxon signed-rank test determined that there was a statistically significant increase in median IL-10 concentration (477 pg/ml) between the median pre-operative (1 pg/ml) and the 30 minute post-reperfusion sample (477 pg/ml), $z = 3.18$, $p = 0.001$ and also between the pre-operative and the 2 hour post-reperfusion sample (604 pg/ml), $z = 3.18$, $p = 0.001$, median increase = 603 pg/ml.

In the Campath group again there was a significant difference between the pre-operative and post-reperfusion samples; the median increase in IL-10 concentration at 30 minutes was 56 pg/ml, pre-operative median = 1 pg/ml and the 30 minute post-reperfusion median = 57 pg/ml, $z = 2.803$, $p = 0.005$, at 2 hours the median increase was 96 pg/ml, the 2 hour post-reperfusion median was 97 pg/ml, $z = 2.803$, $p = 0.005$.

In the Simulect group there was a statistically significant median increase in IL-10 concentration (5 pg/ml) between the pre-operative (1 pg/ml) and the 30 minute post-reperfusion sample (6 pg/ml), $z = 2.197$, $p = 0.028$ and also between the pre-operative and the 2 hour post-reperfusion sample (23 pg/ml), $z = 3.059$, $p = 0.002$, median increase = 21 pg/ml.

Although there were increases post-reperfusion in all of the immunosuppression groups, it is clear from the graph and the absolute median values that ATG seems to have had a more profound effect; Kruskal-Wallis H tests were used to see if there were differences between the groups. At 30 minutes post-reperfusion and 2 hours post-reperfusion there were significant differences in IL-10 concentrations between induction immunosuppressants: $\chi^2(2) = 21.124$, $p < 0.0005$ and, $\chi^2(2) = 19.112$, $p < 0.0005$ respectively, see Figure 5.6 above. Pairwise comparisons were performed using Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons. This post hoc analysis revealed that the statistically significant differences at 30 minutes post-reperfusion were between the Simulect and both Campath and ATG groups and at 2 hours post-

reperfusion were found between ATG and both Simulect and Campath; the significance levels of the post-hoc analysis are shown below Figure 5.7.

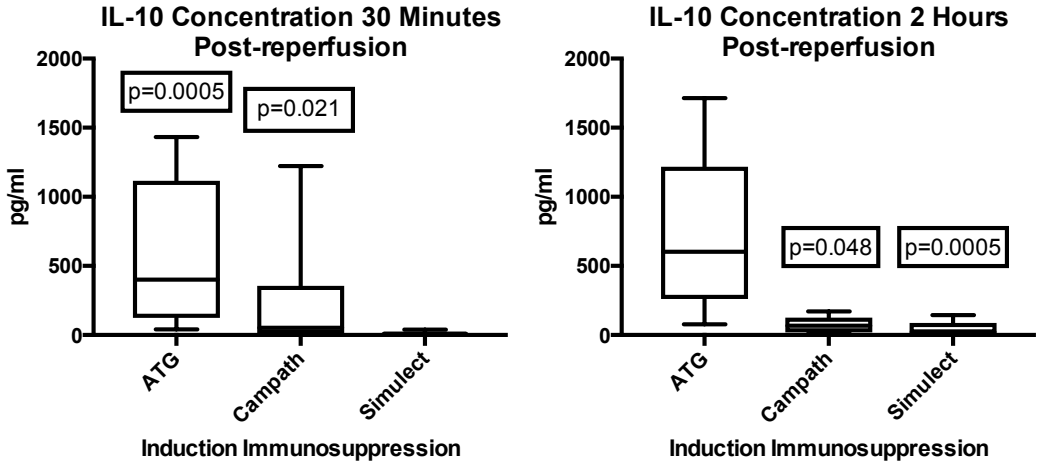


Figure 5.7: Distribution of IL-10 at 30 minutes and 2 hours post-reperfusion according to induction immunosuppression. *Box and whisker plots showing median, interquartile and central range of IL-10 in pg/ml between induction groups at each statistically significant sample. At 30 minutes post reperfusion there is a significant difference in (median) IL-10 concentration in pg/ml between Simulect (6) and both ATG (477), n=35, $p < 0.0005$ and Campath (57) $p = 0.021$, at 2 hours post reperfusion there is a significant difference in IL-10 concentration between ATG (603) and both Campath (97), n=35, $p = 0.048$ and Simulect (23) $p < 0.0005$.*

At 30 minutes post-reperfusion lower levels of IL-10 are seen in the Simulect group, with significantly different IL-10 levels found between the Simulect and both ATG and Campath. At 2 hours post reperfusion our experiments found a significant difference between both the Campath and Simulect group and ATG. After reperfusion of the transplanted kidney, IL-10 production is increased, along with IL-2 and IL-6 (263) our findings are in line with this as shown in section 5.2.2. ATG and Campath appear to be adding to this post-reperfusion of the kidney, with ATG having more of an effect than Campath. It is beyond the scope of this project to determine the mechanism of the changes found however it is clear that the induction immunosuppression administered has a significant effect on the pattern of cytokine release.

5.3.3. TNF-α

In section 5.2.3. we looked for changes in TNF-α concentration over our sampling period and although we saw a general trend of increase post-reperfusion these changes were not significant. Figure 5.8 below illustrates the effect that different immunosuppression had on the cytokine release. It is noticeable from Figure 5.8 that ATG has a dramatic effect on the post-reperfusion samples however in the other immunosuppression groups the post reperfusion peak seen in the interleukins is not found.

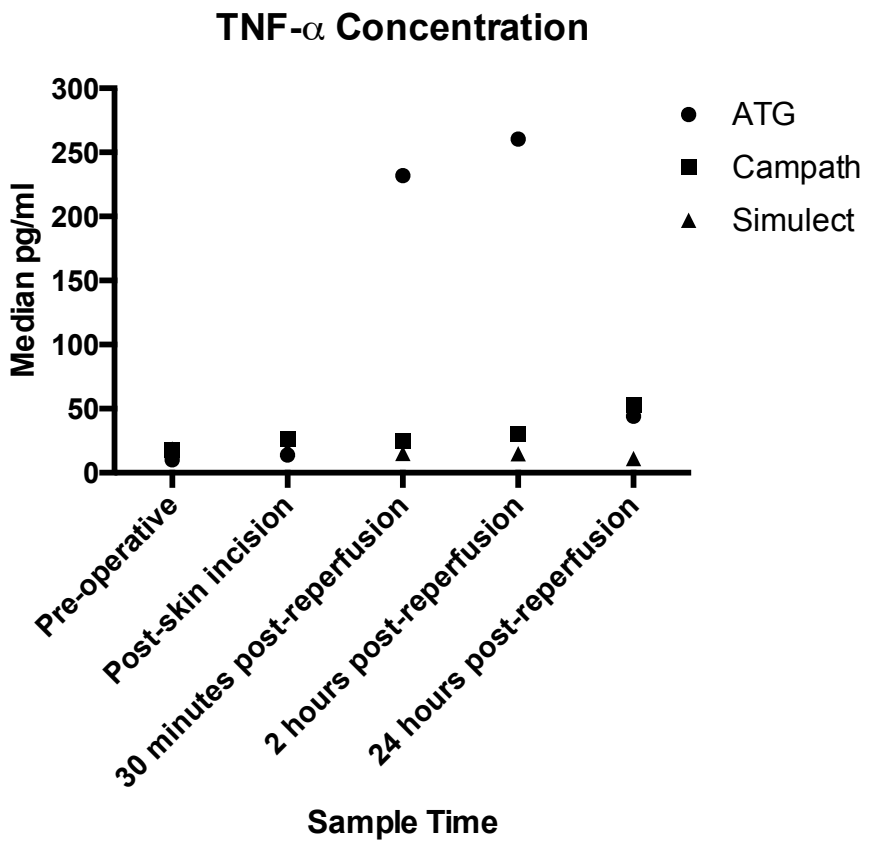


Figure 5.8: TNF-α concentration according to induction immunosuppression group. *Median TNF-α concentrations in pg/ml shown over time according to induction immunosuppression group. Using the Kruskal-Wallis H tests significant differences are found at 30 minutes post-reperfusion (n=35, $\chi^2(2) = 14.235$, $p = 0.001$) and 2 hours post-reperfusion (n=35, $\chi^2(2) = 17.363$, $p < .0005$) between groups.*

As with the other cytokines, we looked to see if there was a change within the immunosuppression groups post perfusion; we found a significant increase in TNF-α in the ATG group and a significant decrease post-reperfusion in the Simulect group. The Campath group was found to rise at 2 hours post-reperfusion.

In the ATG group a Wilcoxon signed-rank test determined that there was a statistically significant increase in median TNF-α concentration (304 pg/ml) between the pre-operative (10 pg/ml) and the 30 minute post-reperfusion sample (314 pg/ml), $z = 2.521$ $p = 0.012$ and also between the pre-operative and the 2 hour post-reperfusion sample (519 pg/ml), $z = 2.521$, $p = 0.012$, median increase = 509 pg/ml.

In the Campath group there were no differences at 30 minutes post-reperfusion, at 2 hours post-reperfusion the median increase was 12 pg/ml, the pre-operative median was 18 pg/ml, the 2 hour post-reperfusion median was 30 pg/ml, $z = 2.073$, $p = 0.038$.

In the Simulect group there was a significant median <u>decrease</u> in TNF-α concentration (4 pg/ml) between the pre-operative (19 pg/ml) and the 30 minute post-reperfusion sample (15 pg/ml), $z = -2.667$, $p = 0.008$ and also between the pre-operative and the 2 hour post-reperfusion sample (15 pg/ml), $z = -2.803$, $p = 0.005$, median decrease (4 pg/ml).

Kruskal-Wallis H tests were run to see if TNF-α concentrations differed between induction groups. At both 30 minutes ($\chi^2(2) = 14.235$, $p = 0.001$) and 2 hours ($\chi^2(2) = 17.363$, $p < .0005$) post-reperfusion significant differences were found between immunosuppression groups. Pairwise comparisons were performed using Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons. This post hoc analysis revealed that the statistically significant differences were between the Simulect and ATG groups at both time points, the significance levels of the post hoc analysis are shown below Figure 5.9.

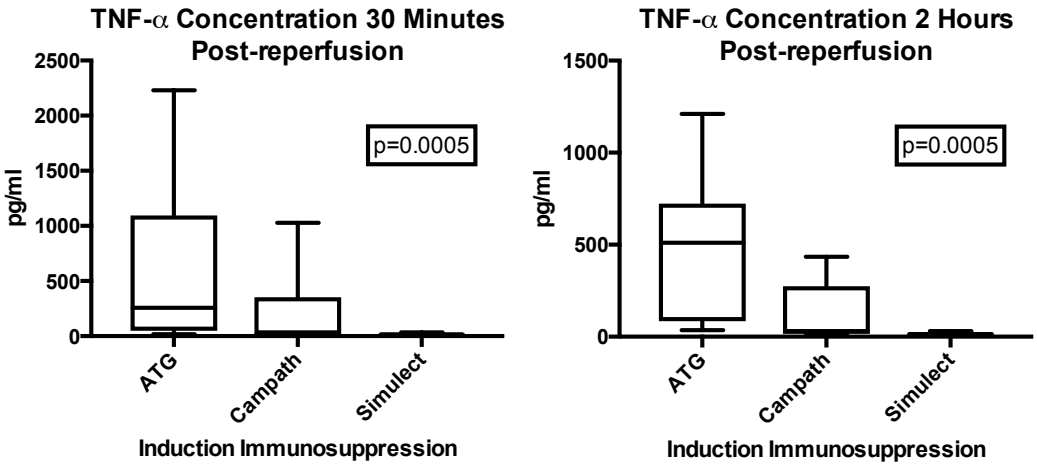


Figure 5.9: Distribution of TNF-α at 30 minutes and 2 hours post-reperfusion according to induction immunosuppression. *Box and whisker plots showing median, interquartile and central range of TNF-α in pg/ml between induction groups. At 30 minutes post-reperfusion there is a significant difference in (median) TNF-α concentration in pg/ml between Simulect (15) and ATG (314), n=35, p < 0.0005, at 2 hours post-reperfusion there is a significant difference in TNF-α concentration between Simulect (15) and ATG (519), n=35, p < 0.0005.*

Post-reperfusion concentration of TNF-α differed between ATG and Simulect; TNF-α plays a significant role in IRI as a pro-inflammatory mediator and anti TNF-α agents have been found to attenuate ischemic injury (264, 265) although not in clinical human trials. In our experiments although we did not find any overall effect of reperfusion on TNF-α concentration, we did see that ATG seems to cause an increase in the levels found and that Simulect may supress its production.

5.3.4. Summary of findings

This section has shown that induction immunosuppression had a significant effect on cytokine release post reperfusion, despite the different timings of administration. All of the cytokines we measured were increased by ATG. Simulect seemed to cause an increase in IL-6 and possibly in IL-10 although the change was small and may be due to type 1 error; Campath was associated with

an increase in IL-6 and IL-10 and also TNF -α (at 2 hours post-reperfusion only). We were limited in the number of the samples we were able to analyse and so further work may be to look at sample F (taken 8 hours post-reperfusion) to complete the picture; alternative methods should also be used for quantifying IL-2, IL-17 and IFN-γ as the Luminex results returned too many undetectable values for useful analysis. The next question this work will address is the relationship, if any, between cytokine concentration and changes in NO metabolites.

5.4. Cytokine Correlation with NO and IRI.

Using Spearman's rank correlation, we looked to see if the rises in cytokines were associated with changes in NO metabolites; in chapter 5.2 we saw that the peak measurement of all cytokines was at 2 hours post-reperfusion and in 4.2 and 4.3 we found that changes of NO_2^- and NO_3^- were associated with risk factors for IRI at 2 and 8 hours post-reperfusion, therefore these were the samples we used. We did not find any significant relationships in our analysis. We also looked to see if the factors affecting NO_2^- and NO_3^-, which we had identified in chapter 4, had any influence over the concentration of cytokines in our samples using regression analysis. We did not identify any significant factors however we can see that the cytokines were significantly affected by induction immunosuppression. The final results chapter will try to investigate the role of induction immunosuppression in more detail.

CHAPTER 6: RESULTS – The Effect of Induction Immunosuppression on Metabolites of NO in the Peri-operative period of a DCD Renal Transplant

6.1. Introduction

All of the patients received one of three induction immunosuppressants; Simulect is administered one hour pre-operatively and so may be expected to exert any effect early on in the sampling period, Campath is administered during anaesthetic induction subcutaneously and so, like Simulect, effects may be seen in the early samples. ATG is administered as an infusion over 4 to 6 hours during the operative procedure. For this reason effects of ATG may not be fully apparent until at least sample F, taken 8 hours post reperfusion of the kidney. The following section will explore changes in NO_2^- and NO_3^- according to the induction agent administered.

6.2. Nitrite

The median concentrations of NO_2^- according to induction immunosuppression are shown in the line graph below Figure 6.1.

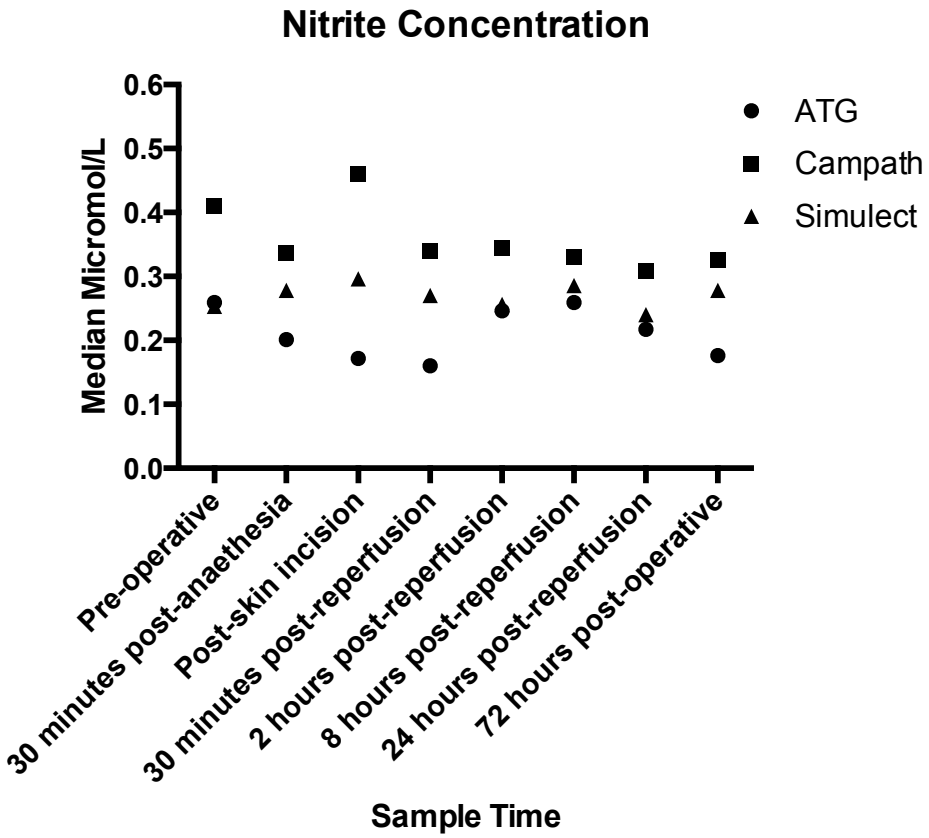


Figure 6.1: Median concentration of nitrite according to induction immunosuppression. *Graph demonstrating the median NO_2^- concentration in µmol/L over time, according to induction immunosuppression. At 30 minutes post-reperfusion the changes between immunosuppression group differed significantly ($n=35$, $\chi^2(2) = 6.38$, $p = 0.041$: Kruskal-Wallis)*

It is noticeable in Figure 6.1 above that the baseline NO_2^- concentration in the Campath group appears to be higher than the other immunosuppression groups; this is likely to reflect the relatively small sample size of the study. Although this was not found to be a statistically significant difference, it may have an influence on the other results and we will refer back to this observation throughout the

following analysis. Sub-analysis of induction regimes is at best exploratory given the small involved in each induction group. The following results should therefore be interpreted with caution.

The median distributions across the sampling period are shown for each induction therapy below, Figures 6.2, 6.3 and 6.4.

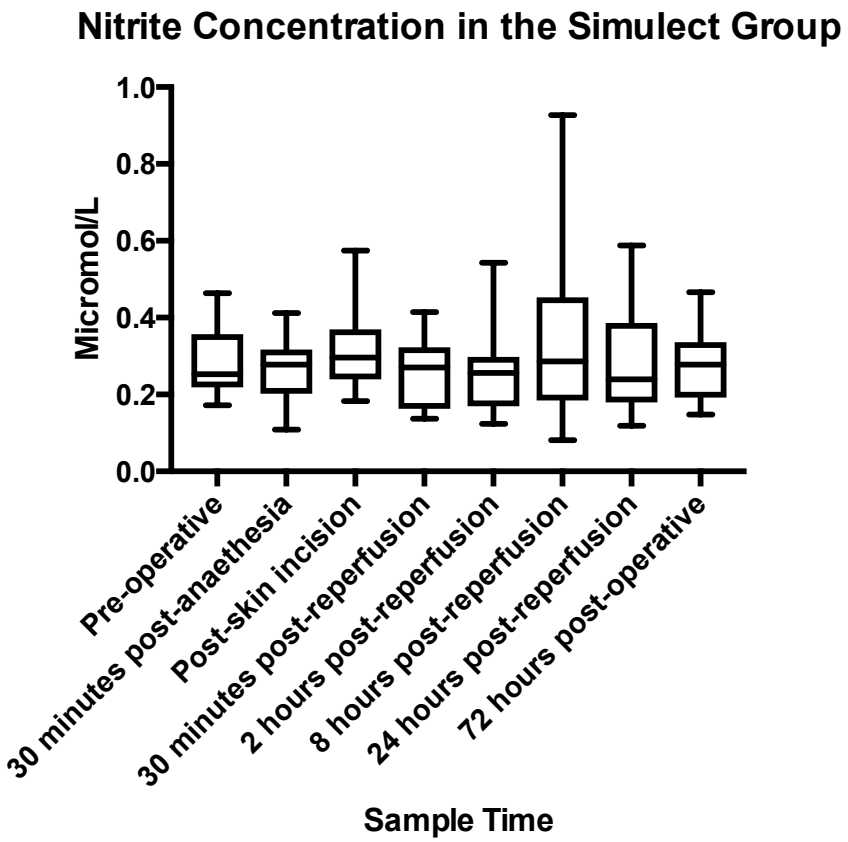


Figure 6.2: Box and whisker plots illustrating the median nitrite concentration in the Simulect group. *Box and whisker plots showing the median, interquartile and central range of NO_2^- concentration in μmol/L at each sample point in the Simulect group. There are no significant differences between the samples in the peri-operative period (n=12, $\chi^2(6) = 3.932$, $p = 0.686$: Friedman test).*

No statistical differences were found between sampling points in the Simulect group.

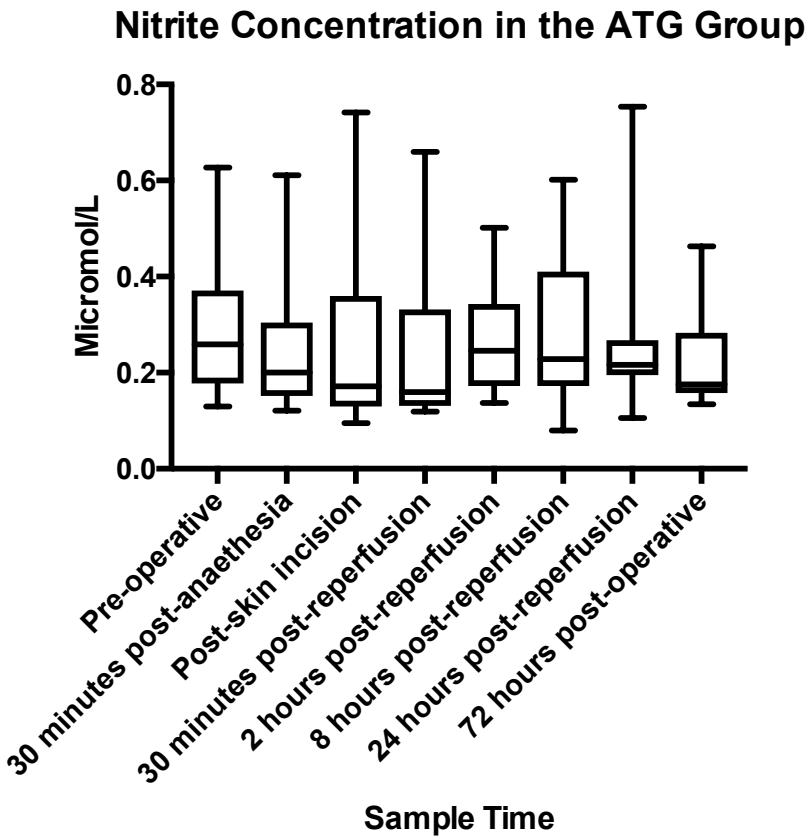


Figure 6.3: Box and whisker plots illustrating the median nitrite concentration in the ATG group. *Box and whisker plots showing the median, interquartile and central range of NO_2^- concentration in μmol/L at each sample point in the ATG group. There are no significant differences between the samples in the peri-operative period (n=13, $\chi^2(6) = 6.573$, $p = 0.362$: Friedman test).*

The effect of ATG on cytokine release is extensively reported; so much so that *cytokine release syndrome* is one of its documented side effects. However how this translates into an effect on NO has not been investigated (no relevant studies were returned in a PubMed search containing both terms). We may hypothesise that cytokine release, induced by ATG, would lead to up-regulation of iNOS and therefore NO synthesis however there is nothing in the published literature to confirm this theory. One study by Puschel et. Al looked at ATG and NO in the context of microvascular thrombus formation; this group found an increase in eNOS and iNOS after ATG administration however no detectable effect from NO as a result (they did not measure NO metabolites themselves). They

hypothesized that this may be as a result of post-translational changes influencing the enzymes ability to produce NO (266). There were no significant differences found between the samples post-reperfusion in this group in our data.

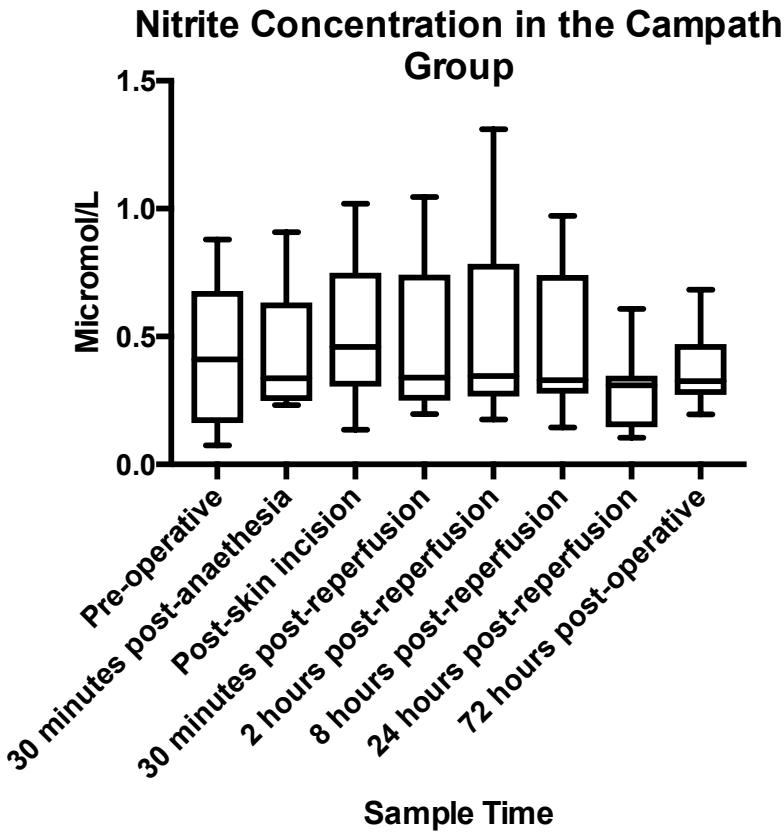


Figure 6.4: Box and whisker plots illustrating the median nitrite concentration in the Campath group. *Box and whisker plots showing the median, interquartile and central range of NO_2^- concentration in μmol/L at each sample point in the ATG group. There are no significant differences between the samples in the peri-operative period (n=10, $\chi^2(6) = 9.048$, $p = 0.171$: Friedman test).*

It is noticeable from Figure 6.3 and 6.4, that Campath appeared to have a different effect to ATG, with a peak of NO_2- found around the time of the skin incision followed by a plateau in concentration after reperfusion. This apparent difference in response may be as a result of both the selective nature of Campath (as a monoclonal antibody) in its mechanism of action against CD52 antibodies

and may also represent the pleiotropic nature of cytokines, that is to say that their action is dependent upon the physiological conditions of the tissues at the time. There were no differences found between individual samples in the Campath group but we were interested to see if there were differences between the immunosuppression groups.

Kruskal-Wallis H tests were used to see if there were differences in the change of NO_2^- concentrations between induction groups post reperfusion. At 30 minutes post-reperfusion the changes between immunosuppression groups were significantly different $\chi^2(2) = 6.38$, $p = 0.041$. Pairwise comparisons were performed using Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons. This post hoc analysis revealed that the statistically significant differences were between Campath and both the Simulect and the ATG groups, the p values are shown on the box plot below, Figure 6.5. No differences were identified at the other times.

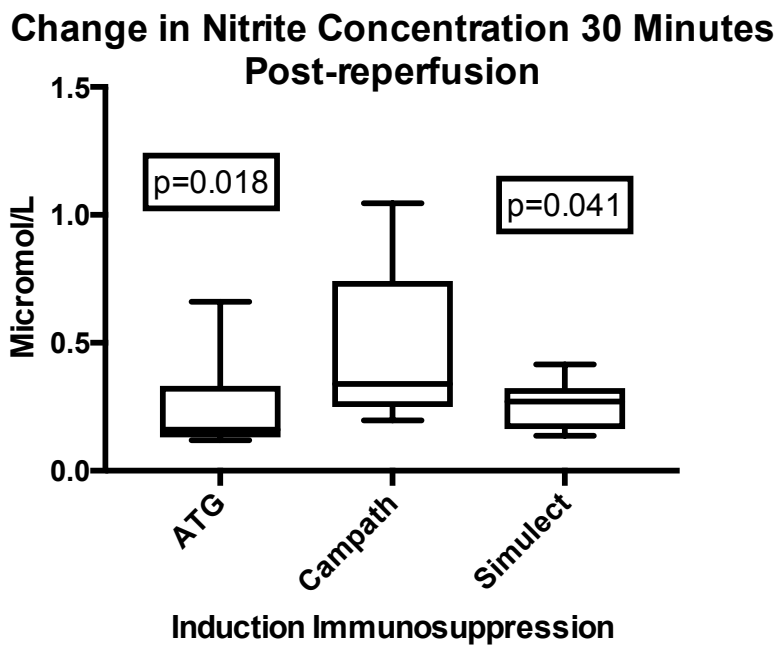


Figure 6.5. Distribution of nitrite at 30 minutes post-reperfusion, according to induction immunosuppression. *Box and whisker plots showing median, interquartile and central range of change in NO_2^- in μmol/L between induction groups at 30 minutes post perfusion, n=35. There was a significant difference in the median change between Campath (0.098 μmol/L) and ATG (-0.022 μmol/L) p = 0.018 and Campath and Simulect (-0.049 μmol/L) p = 0.041.*

6.2.1. Conclusion

We have found that the change of NO_2^- at 30 minutes post perfusion differed according to induction group. All of the agents used are known to have an effect on cytokine release although the pattern of cytokine release varies with the medication used. As discussed in the *induction immunosuppression* chapter of the introduction, ATG may cause a *cytokine storm* involving many pro-inflammatory cytokines; in particular IL-16 and TNF-α and this was reflected in the findings of this study. Both TNF-α and IL -1 act synergistically with IFN-γ to up-regulate NF-κβ which in turn causes up-regulation of NOS and NO production. We may have expected ATG to cause an increase in both NO metabolites via the classical pathway of iNOS up regulation and subsequent oxidation of NO to NO_2^- (catalysed in plasma by ceruloplasmin)(74) and by the reaction of NO with

oxyhaemoglobin to produce NO_3^- however this was not seen in terms of NO_2^- in our results. This could be a consequence of the duration of the ATG infusion causing us to miss the peak of its action on NO_{2-} which is less stable than NO_3^- in whole blood (75).

Campath is thought to suppress pro-inflammatory cytokines such as IFN-γ and IL-17 and increase anti-inflammatory cytokines such as IL-4 and IL-10 (242). Once again our results from IL-10 measurement support this. Theoretically we may have expected to find that this translated in to suppression of NO production. On review of the median change of NO_2^- within the Campath group (Figure 6.4) this theory was not confirmed, there was predominantly a positive change in NO_2^- in the first 24 hours after administration of the medication and we found that recipients in the Campath produced significantly more NO_2^- than those who received ATG in our early samples. This finding, which contradicts our theoretical knowledge of Campath and cytokines, may represent their *contextual* and *synergistic* nature; that is to say that the effects of cytokine release on a cell depend upon the conditions the cell is exposed to at the time and that varying combinations of cytokine release may cause varying biochemical effects. Our findings suggest that Campath may be influential, along with other factors, in changes in NO_2^- concentration.

Simulect, similarly to Campath, increases production of some cytokines whilst surpressing others; production of IL-10 is stimulated whist IFN-γ, TNF-α and IL-6 are suppressed (267, 268). We did not find that Simulect administration had any biochemical effect on NO_2^- concentration, we did find that IL-10 concentration rose after Simulect administration and we also found a reduction in TNF-α in this group.

6.3. Nitrate

Figure 6.6 shows the median concentration of NO_3^- measured according to induction immunosuppression group.

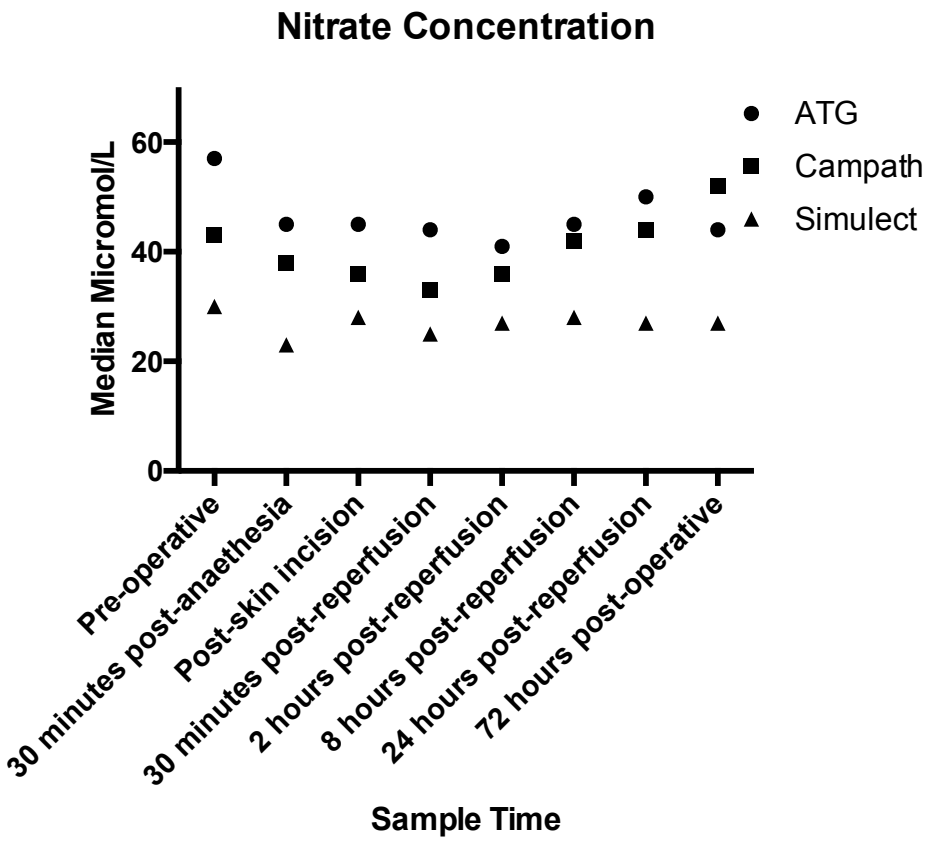


Figure 6.6: Median concentration of nitrate according to induction immunosuppression. *Graph demonstrating the median NO_3^- concentration in µmol/L over time, according to induction immunosuppression. Using a Kruskal-Wallis analysis there were no significant differences between induction groups at any time point, n=35.*

It appears that NO_3^- concentration is highest in the ATG group, with a general trend of an initial fall in concentration, rising again back towards the baseline post reperfusion. As we observed in 4.5.2., baseline values appear different between immunosuppression groups; to correct for this our subsequent analysis looks at change in nitrate levels as opposed to absolute values. The following three Figures, 6.7, 6.8 and 6.9, depict the ranges found for each induction group.

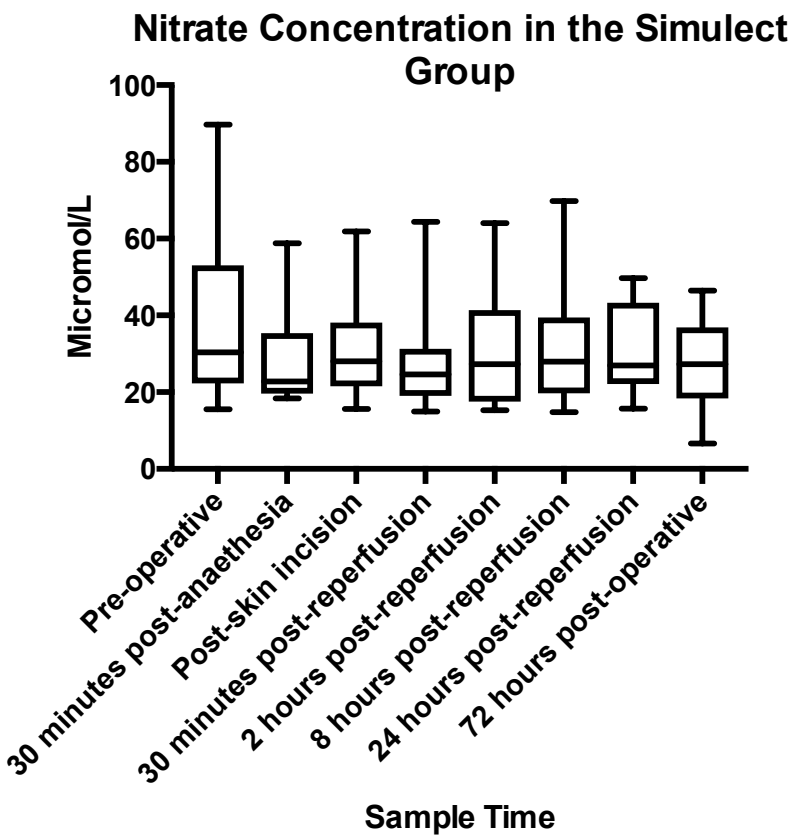


Figure 6.7: Box and whisker plots illustrating the median nitrate concentration in the Simulect group. *Box and whisker plots showing the median, interquartile and central range of NO_3^- concentration in μmol/L at each sample point in the Simulect group. There are no significant differences between the samples in the peri-operative period (n=12, $\chi^2(6) = 1.3$, $p = 0.972$: Friedman test).*

There were no statistical differences between the sample times in the Simulect group.

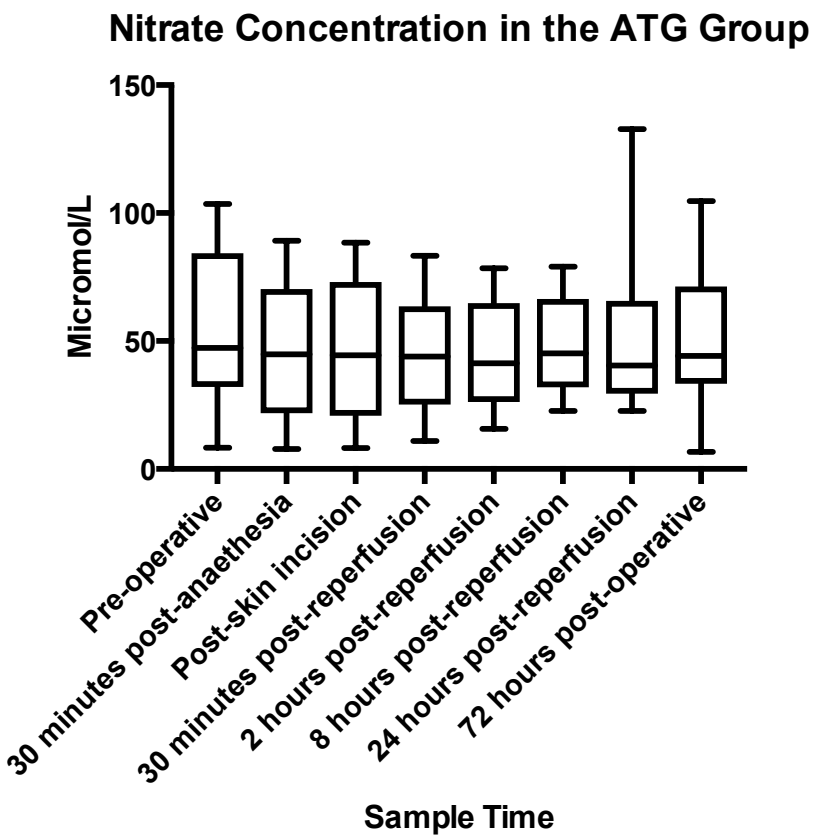


Figure 6.8: Box and whisker plots illustrating the median nitrate concentration in the ATG group. *Box and whisker plots showing the median, interquartile and central range of NO_3^- concentration in μmol/L at each sample point in the ATG group. There are no significant differences between the samples in the peri-operative period (n=13, $\chi^2(6) = 6.641$, $p = 0.465$: Friedman test).*

There were no statistical differences between the sample times in the ATG group.

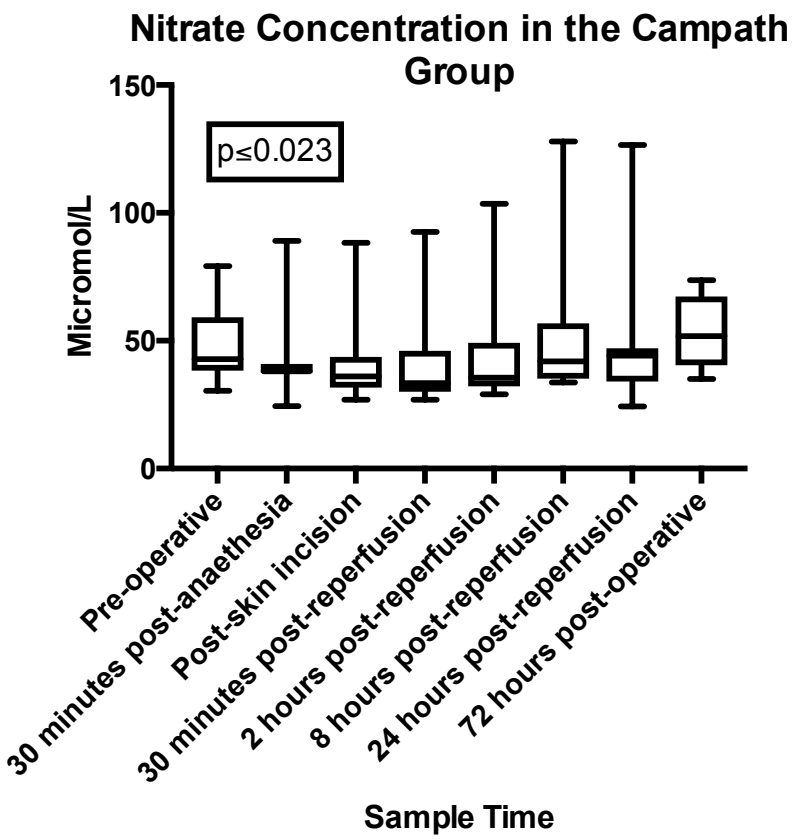


Figure 6.9: Box and whisker plots illustrating the median nitrate concentration in the Campath group. *Box and whisker plots showing the median, interquartile and central range of NO_3^- concentration in µmol/L at each sample in the Campath group. The change in NO_3^- concentration in µmol/L varried significantly across the sampling period (n=10, $\chi^2(6) = 14.714$, $p < 0.023$: Friedman test). Median (range): Sample A = 42.8 (30.4 – 79.2), Sample B = 38.1 (24.4 – 89.1), Sample C = 36.1 (26.9 – 88.3), Sample D = 33.5 (27 – 92.6), Sample E = 35.6 (29.1 – 103.6), Sample F = 42 (33.6 – 127.9), Sample G = 44.2 (24.3 – 126.6) and Sample H = 51.8 (35 – 73.7).*

The change of NO_3^- concentration was statistically significantly different at the different time points in the Campath group, $\chi^2(6) = 14.714$, $p < 0.023$. Post hoc analysis revealed statistically significant differences in change in NO_3^- between samples B (taken 30 min post-induction of anaesthesia (and Campath administration), C (taken post skin incision but prior to clamping of the vessels), and D (taken 30min post reperfusion of the transplanted kidney) and sample H (taken 72 hours post operatively) (p = 0.05), (p = 0.006) and (p = 0.003)

respectively. There was also a significant change between samples C and D and sample F (taken 8 hours post reperfusion of the transplanted kidney) ($p = 0.022$) ($p = 0.012$) respectively.

Nitrate concentration appears to be affected by Campath induction. Kruskal-Wallis H tests were used to see if there were differences in the change of NO_3^- concentrations between induction groups post-reperfusion. This analysis did not show any significant differences between these groups.

6.3.1. Conclusion

In this section we have found that changes in NO_3^- concentration were evident in the Campath group only, we also found that changes in NO_2^- at 30 minutes post reperfusion were significantly higher in the Campath group in comparison to both ATG and Simulect. We have found differences in both nitrite and nitrate concentrations related to the induction therapies; in order to check that our previous findings were not purely attributable to the induction the regression analysis was repeated including induction immunosuppression as a variable, the recipient age and 1st WIT were still found to be significant so we are confident that the results are not just down to the immunosuppression, although from the subsequent analysis it is likely that it also plays a role.

Concluding remarks: Induction immunosuppression is clearly influential on the results of this study; the concentration of cytokines in our samples seems to be particularly affected. We have not been able to demonstrate any relationship between NO and the cytokines that we were able to measure. The lack of a direct relationship between NO and the cytokines found in this study may represent the differing effect of induction immunosuppression on cytokine production, but there may also be a contributing effect of the two pathways of NO production that were reviewed in Chapter 1; we would expect both the *classical* pathway, which results from up-regulation of iNOS in response to inflammatory cytokines, and the *alternative* Nitrate – Nitrite - NO pathway, which is activated at times of

hypoxia and releases NO from NO_2^- and NO_3^-, to play a role in NO release in the context of renal transplantation. It is beyond the scope of this project to identify the mechanism of production in our recipients however the inability to link the cytokine production with the concentrations of NO_2^- and NO_3^- implies a significant role for the alternative mechanism. A future extension of this project may be to look at iNOS up regulation and also investigate the patient's genetic predisposition to produce cytokines. In the context of this study endothelial dysfunction as a result of IRI may also limit the production of NO by NOS pathways. IRI is a complex phenomenon but one of clinical importance to transplant physicians and their patients; this project has studied one element of a multifactorial process and identified some significant associations, in the following chapter we will summarise and collate our findings.

CHAPTER 7: DISCUSSION AND CONCLUSIONS

Discussion and Conclusions

Renal ischemia reperfusion injury plays a significant role in the short and long term outcomes post kidney transplantation. A shortfall between supply and demand of organs for transplantation has resulted in an expansion of the boundaries of acceptability in terms of ECD and DCD kidney donors accepting that these organs are more susceptible to IRI. Research into the mechanisms and therapeutic options to attenuate this injury is important to facilitate expansion of transplant programs within the health service. NO production is undoubtedly modulated by IRI owing to the dysfunction caused by the injury on the endothelium. This study set out to investigate NO levels around the time of renal transplantation by quantifying NO metabolites found in the blood of renal transplant recipients peri-operatively and correlating them with factors known to influence IRI.

From our review of the literature and previous research done in the department we found that NO, NO_2^- and NO_3^- exist in a dynamic axis and NO_2^- and NO_3^- may have differing roles with NO_2^- serving as reservoir of NO to be utilised in hypoxic conditions. The method of quantifying NO metabolites used by our unit (and also found frequently in the literature in the field of transplantation) was the Griess reaction however a broader literature review revealed that other medical sub-specialties (mainly cardiology) were utilising and recommending different, more sensitive methods. Given our hypothesis that NO_2^- and NO_3^- may play differing roles and also have differential effects depending on concentration it was important to this study that an accurate method was used and so in my initial experiments I quantified NO_2^- and NO_3^- using both the Griess reaction and ozone chemiluminescence. As reported in section 2.4, I found that when measuring NO_3^- in the plasma of renal transplant recipients the Griess reaction is an acceptable method and has the advantage of being cheaper, quicker and less labour intensive than ozone chemiluminescence. If, however, NO_2^- measurement is required the Griess reaction is not sensitive enough for accurate quantification of the levels found in human plasma and a more sensitive technique such as

ozone chemiluminescence should be employed. Given the aims of this project I chose to use ozone chemiluminescence for the rest of my NO analysis.

As well as measuring NO metabolites I was also interested in levels and changes of cytokines in my samples, specifically, to see if they could be correlated with the changes seen; a Luminex based assay, was chosen in conjunction with staff at the WTAIL who are experienced with the technique and equipment, as discussed in section 2.5.2. I encountered difficulties with three of the cytokines I chose to investigate and a future extension of this project may be the addition of this data by a different method. The results obtained were verified by another, more experienced, scientist, checked by the manufacturers of the kit and in the case of IFN-γ, confirmed with a different test and whilst disappointed not to have a complete set of data the whole group were satisfied that the reported results for the remaining cytokines were accurate.

At the time of starting my thesis our group had previously found that total NO_2^- and NO_3^- levels are elevated during times of acute rejection and that levels of NO metabolites differ according to type of transplant. As my pilot study I was able to add samples from my first 11 subjects to this data and demonstrate a significantly greater change in NO_3^- in the recipients of DCD kidneys when compared to kidneys from DBD or live donors; within the DCD group I found that the cold ischemic time and the donor age were also influential. Having confirmed that risk factors for IRI i.e. DCD donation, donor age and cold ischemic time seemed to be important and that for the purposes of this study accurate quantification of both NO_2^- and NO_3^- was most appropriate with ozone chemiluminescence the next objective was to look at each NO metabolite and identify any factors affecting its production.

When reviewing the NO_2^- data, as expected, we saw a rise in concentration following reperfusion of the kidney, on further analysis of the data I demonstrated that increasing age impairs the ability of the body to mount a NO_2^- response. This if, as is believed NO_2^- exerts a protective effect early after reperfusion (145, 146) it may explain, in part, why a greater incidence of DGF is

found in older recipients. One limitation of this study is that we have not investigated NOS expression as this may assist us in explaining this finding. An Elisa method of quantifying NOS, tested previously by our group, proved unreliable for frozen samples and its use in fresh, centrifuged samples is prohibitively impractical. My next step was to see if any of the known risk factors for IRI, namely WIT, CIT and donor and recipient age influenced and changes in NO_2^-; I found that in the 8 hours after graft reperfusion both recipient age and 1st WIT affected changes in NO_2^- concentration. Possible explanations as to how WIT may be influential include:

1. It reflects damage to the graft as a result of IRI therefore stimulating iNOS up-regulation and NO production.
2. It reflects the production of NO via the Nitrate-Nitrite-NO axis.

A further extension to this work may be to look at eNOS and iNOS up-regulation in the same samples. As part of my analysis I looked at other variables including storage method and donor/recipient sex, none of these investigations revealed any associations with NO_2^- changes.

The next stage was to see if NO_3^- behaved in the same way as NO_2^-; I found that NO_3^- levels rose significantly in the 8 hours after reperfusion and I would deduce that this represents NO production. I found the same association with recipient age i.e. changes in NO_3^- post reperfusion are negatively correlated with recipient age and this supports the theory that increasing age blunts the NO response via its detrimental effect on the endothelium. No other significant relationships were identified. It seems that only NO_2^- was influenced by WIT, which may be a protective response to the injury of ischemia and reperfusion and supports the theory that NO_2^- is mobilised via the Nitrate-Nitrite-NO axis during hypoxia as opposed to the classical pathway. To explore that idea further I looked to see if the ratio of NO_2^- to NO_3^- was important however my analysis did not reveal any significant associations further to those seen already. A interesting expansion of this project would be measurement of peroxynitrite in the samples as its production, which occurs as a result of the reaction of NO and superoxide, causes damage to renal tubular structure and may therefore be contributing to the

deleterious effects that have been associated with NO. This reaction is more likely to take place during pro-inflammatory states, such as IRI, when superoxide is in abundance and may go someway to explain the "conflicting roles" of NO that were discussed in the introduction. It may be that NO_3^- was not increased enough to have a detrimental effect and a threshold might need to be crossed before the reaction is driven towards peroxynitrite and deleterious effects. One of the aims of this project was *to establish whether NO is protective or detrimental in IRI,* because of the limitations previously discussed this thesis was unable to answer that question and I believe that extra data regarding eNOS/iNOS expression and peroxynitrite concentration may be required in order to do so.

Clarification of the role of NO and its metabolites in renal IRI may in the future allow its protective effects to be isolated and utilised therapeutically by either augmentation of NO or inhibition of any harmful metabolites. Neither NO_2^- nor NO_3^- demonstrated any direct relationship with DGF, this reflects the limitation of study size and was partly expected; DGF represents the clinical manifestation of one end of the continuum of IRI, whilst it is important in terms of patient outcomes and a useful way of defining injury, IRI is not exclusive to the DGF group and it is unlikely that molecules which are so ubiquitous in human physiology would exhibit a simple relation with such a complex problem.

The final aim of this work was to study the role of cytokines in NO production; cytokines are produced in varying quantities depending on genetic predisposition within the population, previously our group had shown that the UK population may be either high or low producers of IFN-γ depending on genotype and subsequently the highest producers have been shown to be more prone to acute rejection. We were interested to see if cytokine production correlated with any changes in NO metabolites in the context of renal transplantation or if the effects of the induction immunosuppression would override any differences. I saw an increase in all of the quantifiable cytokines after reperfusion, although in the case of TNF-α the change was not significant. At 30 minutes and 2 hours post reperfusion I found changes in IL-6 and 10 were significant and whilst this could be an effect of the surgical stimulus I was

interested to see whether induction medication had an effect on them. There are two limitations to my work in chapter 5 and they are that we were unable to measure cytokines in all of the samples and therefore we may be missing the peak of their production and also that when the induction immunosuppression sub groups are analysed the number of participants in each group is very small therefore the results should be taken in this context and interpreted with caution.

This study found that cytokine responses varied between induction immunosuppression groups with the largest changes seen in the ATG group (as would be expected from our review of the literature), these finding were evident despite variation in administration timings of induction; with what is known about the mechanism of action of the induction therapies, (reviewed in section 1.6. of the introduction) this was to be expected however to the best of my knowledge, this is the first study to report this directly. I was not able to establish any correlation between the changes in cytokine levels that were measured and changes of NO_2^- and NO_3^-, or any association between cytokines and risk factors for IRI. We used a Luminex based assay to measure cytokine levels; the fact that this proved unreliable for IFN-γ that is directly related to the production of NO is disappointing. Further information regarding eNOS/iNOS up-regulation may help answer this question.

Whilst I didn't find any specific statistical links between NO metabolites and the cytokines measured (as mentioned IFN-γ gave unreliable results) I did find that induction immunosuppression was influential on cytokine production and so looked to see if there was also any effect on NO; I found differences in change of NO_2^- between immunosuppression groups at 30 minutes post perfusion and was surprised to see that the Campath group displayed the largest concentration change. In the NO_3^- data, once again, it was within the Campath group that differences were evident, I found that NO_3^- concentration rose after reperfusion; in the samples taken at 24 and 72 hours there is likely to be a dietary influence as the patients may well have taken nitrates in orally however the increase in

concentration found in the early post reperfusion samples warrants further investigation. As has been mentioned previously these conclusions should be interpreted with caution due to the size of the groups and the fact that the ATG infusion was on going for several of the post perfusion samples

Overall the findings of my study were: changes in NO_2^- were influenced by factors effecting renal IRI and also by Campath induction. Increasing age was associated with dampening of the NO_2^- and NO_3^- response. Cytokines rise after reperfusion of the kidney and changes in them were modified by induction immunosuppression. A useful extension to this project would be an investigation of eNOS/iNOS up regulation and peroxynitrite production.

Appendices

Appendix 1: Cytokine Families

Receptor family	Members	Shared function
Class 1 cytokine receptors or haematopoietin family receptors.	IL-6 subfamily GM-CSF subfamily IL-2 subfamily IL-13 subfamily and IL-12, G-CSF, Erythropoietin, Growth hormone and prolactin	T/B cell activation Haemaotpoiesis
Class 2 cytokine or interferon family receptors	IFN-α/β, IFN-ϒ, IL-10, IL-20, IL-22	Antiviral (with the exception of IL-10)
TNF receptor family	TNF-α, TNF-β, TRAIL, BAFF	Pro inflammatory
IL-1/ Toll like receptors	IL-1α, IL-1β, IL-1RA, IL-18	Pro inflammatory
Tyrosine kinase receptors	TGF-β, Bone morphogenic proteins, Activins, Inhibins	Growth factors
Chemokine receptors	CXC, CC, CXC3 and C chemokines	Chemotactic

Appendix 2: Griess Reaction

Reaction diluent was made up (see below) and samples prepared before moving on to step 2 of the reagent preparation.

Sample preparation:

1. Add 100 µl (microlitres) of sample and 100 µl of reaction buffer.
2. Ultra-filtrate through a 10,000 kDa (kilo-Daltons) molecular weight cut-off filter to eliminate protein.

Reagent preparation:

1. Reaction diluent (1X) - dilute 30 mls (10X) of reaction diluent concentrate into deionised water to prepare 300ml of reaction diluent (X1).
2. Prepare standards using the appropriate 2000 µmol/l standard stock and reaction diluent (X1), (100 µmol/l, 50 µmol/l, 25 µmol/l, 12.5 µmol/l, 6.25 µmol/l and 3.12 µmol/l concentration) vortex thoroughly prior to use.
3. Reconstitute NADH with 5.0ml of deionised water, allow to sit for 3 min, with gentle agitation then place on ice until needed.
4. Reconstitute nitrate reductase with 1 ml nitrate reductase storage diluent, vortex and allow to sit for 15 minutes at room temperature. Vortex again and allow to sit for a further 15 minutes then use immediately for step 5.
5. Dilute Nitrate reductase immediately before use. Using following equation:
    1. Nitrate reductase (µl) = (# wells + 2) x 5 µl.
    2. Reaction buffer (µl) = volume from step a x 4.
    3. Add volumes from step a and b to a tube and vortex.
       Place in ice and use within 15 minutes.

Nitrite Assay procedure:

1. Prepare reagents, standards and samples as described.
2. Add 50 µl of reaction diluent to any blank wells of the supplied 96 well plate
3. Add 50 µl of nitrite standard or sample to remaining wells.
4. Add 50 µl of reaction diluent to all wells
5. Add 50 µl of Griess reagent I to all wells.
6. Add 50 µl of Griess reagent II to all wells and mix by tapping at side of plate.
7. Incubate for 10 minutes at room temp.
8. Determine the optical density for each well using a microplate reader set at 540-570 nm

Nitrate Assay procedure:

1. Prepare reagents, standards and samples as described.
2. Add 50 µl of reaction diluent to any blank wells
3. Add 50 µl of nitrite standard or sample to remaining wells.
4. Add 25 µl of NADH to all wells.
5. Add 25 µl of nitrate reductase into all wells, mix by tapping on the side of plate and cover with adhesive tape.
6. Incubate for 30 min at 37°C.
7. Add 50 µl of Griess reagent I to all wells.
8. Add 50 µl of Griess reagent II to all wells and mix by tapping at side of plate.
9. Incubate for 10 min at room temp.
10. Determine the optical density for each well using a microplate reader set at 540-570 nm.

Appendix 3: Study Consent Form

Role of Nitric Oxide and Interferon Gamma Polymorphisms in the Development of Delayed Graft Function in Non Heart Beating Donor Transplants. (IFNO)

Please initial box

1. I confirm that I have read and understood the information sheet dated 14.01.2012 for the above study (Version 4) and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving reason, without my medical care or legal rights being affected. ☐

3. I understand that sections of any of my medical notes may be looked at by the investigators or by individuals from regulatory authorities where it is relevant to my taking part in the research. I give permission for these individuals to have access to my records. ☐

4. I understand that samples will be taken to test my genetic material that affects the production of cytokines in relation to the study ☐

5. I understand that I will not benefit financially if this research leads to the development of new treatment or test. ☐

6. I agree to take part in the above study. ☐

7. I agree to my GP being informed of my taking part in this study. ☐

Name of Patient Date Signature

Name of Person taking consent Date Signature
(If different from researcher)

Researcher Date Signature

(1 for patient; 1 for researcher; 1 to be kept with hospital notes)

Appendix 4: Luminex Assay

Reagent preparation

1. Wash buffer. Add 20 ml of wash buffer concentrate to 480 ml of deionized water.
2. Make up the standards using 3 fold dilutions of calibrator diluent. Leave to sit for 15 minutes.
3. Reconstitute standard cocktails 1 and 2 with calibrator diluent RD6-40. Referring to the standard value cards supplied with the kits (varies according to lot. and analyte).

Assay procedure

1. Pre wet the plate with 100 µl of wash buffer and take the liquid off using a vacuum manifold.
2. Prepare the micro-particle cocktail by adding microparticles to the microparticle diluent. Volumes used will depend on the number of wells on the plate being utilised on a particular run according to the manufacturer's instructions.
3. Vortex the diluted micropartlcle cocktail and add 50µl to each plate
4. Add standard or samples to the plate (this must be done within 15 minutes to avoid degradation of the light sensitive micro-particle cocktail.
5. Cover the plate with foil and incubate for 3 hours on the microplate shaker at room temperature.
6. Use the vacuum manifold to remove the liquid and wash a further 3 times using 100 µl of wash buffer each time, taking the buffer off at the end.
7. Prepare the biotin antibody cocktail by diluting biotin antibodies in the biotin antibody diluent 2. Volumes used will depend on the number of wells on the plate being utilised on a particular run according to the manufacturers instructions.
8. Add 50 µl of diluted biotin antibody cocktail to each well, cover with foil and incubate for 1 hour on the microplate shaker.
9. Repeat wash step 6.

10. Dilute the 100x Streptavidin PE to a 1X concentration using wash buffer. Volumes used will depend on the number of wells on the plate being utilised on a particular run according to the manufacturers instructions.
11. Add 50 µl of diluted Streptavidin PE to each well. Cover with foil and incubate for 30 minutes at room temperature on the microplate shaker.
12. Repeat wash step 6.
13. Add 100 µl to each well and Incubate on the microplate shaker for a further 2 minutes.
14. Read within 90 minutes using Bio-Rad® analyser.

Appendix 5: ELISA Assay

Reagent preparation

All reagents should be brought to room temperature before use.

1. Reconstitute the IFN-γ standard using calibrator diluent RD6-21, referring to the standard value cards supplied with the kits for the volume required, as this varies according to lot. Allow to stand with gentle agitation for 15 minutes.
2. Wash buffer. Add 20 ml of wash buffer concentrate to 480 ml deionized water to prepare 500 ml of wash buffer.
3. Make up the standards using serial dilutions of the prepared IFN-γ standard, undiluted this serves as the highest standard, to produce a dilution series.
4. Substrate solution. *This should not be prepared until immediately before use in step x below.* Equal amounts of colour reagents A+B should be mixed together. The absolute amount should be calculated on the day depending on the number of wells being used on the plate.

Assay procedure

1. Add 100 µl of assay diluent RD1-51 to each well of the plate.
2. Add 100 µl of standard or sample to the wells, after this point the reagent MUST be added to the plate within 15 minutes.
3. Cover the plate and incubate at room temperature for 2 hours with gentle agitation.
4. Wash step. Aspirate the contents of the wells and add 400 µl of wash buffer. Repeat the aspiration and wash for a total of 4 washes.
5. Add 200 µl IFN-γ conjugate to each well, cover and incubate for a further 2 hours, with gentle agitation at room temperature.
6. Repeat the wash as in step 4.
7. Add 200 µl of the substrate solution (which should be made up immediately before use)
8. The plate must now be protected from the light. Incubate the plate for 30 min at room temperature in a dark cupboard.

9. Add 50 µl stop solution to each well. N.B. The colour should now change from blue to yellow.
10. Using the Bio-Rad® analyser, determine the optical density within each well. The wavelength frequency should be set to 540nm and the plate should be read within 30 min.

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