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Author:

Mr Adib Khanafer
MBBS (India), FRCS (London), FRCS (Edinburgh), FRCS (General)

Institution & Department

Renal Unit
University Hospital of Wales
Heath
Cardiff

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Research Mentor

Mr A Asderakis
Consulant Transplant & Endocrine Surgeon
University Hospital of Wales

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Abbreviations

ACE	Angiotensin Converting Enzymes
ACEI	Angiotensin Converting Enzymes
ADMA	Asymmetrical dimethylarginine
AII	Angiotensin II
ADP	Platelets Derived Products
APKD	Adult Polycystic Kidney Disease
ARF	Acute Renal Failure
AST	Aspirtate Transaminase
ATN	Acute Tubular Necrosis
ATP	Adenosine 5-triphosphate
BPH	Benign Prostate Hypertrophy
CaM	Calmodulin
CAN	Chronic allogaft nephropathy
CE	Capillary electrophoresis
CMV	Cytomegalovirus
cNOS	constitutive Nitric Oxide Synthase
CNI	Calcineurin Inhibtors
CO	Carbon Monoxide
CSA	Cyclosporin
DGF	Delayed Graft Function
eNO	exhaled Nitric Oxide
ESRF	End Stage Renal Failure

FE_{Na}	Fractional Excretion of sodium
FSGS	Focal Segmental GlomeruloNephritis
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
GN	Glomerulonephritis
Hb	Haemoglogin
HbNO	Nitrosohaemoglobin
HO	Heme oxygenase
HPLC	High Performance liquid chromatography
HSP	Henoch Schonlein Purpura
iNOS	induced Nitric Oxide Synthase
IL-1	InterLeukin-1
IL-4	InterLeukin-4
IL-8	InterLeukin-8
IL-10	InterLeukin-10
IFN- γ	Interferon- γ
IRI	Ischaemia Reperfusion Injury
LPS	Lipopolysaccharide
MetHb	Methaemoglobin
MMF	Mycophenolate mofetil
MP	Methyl Prednisolone
mTOR	mammalian Target of Rapamycin
NANC	Nonadrenergic Noncholinergic

NMA	N-methyl-L-arginine
NF- κB	Nuclear Factor-κB
NO_2^-	Nitrite
NO_3^-	Nitrate
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NOx	Nitrite and Nitrate
O_2	Oxygen
O_2^-	superoxide
$OH^{\cdot}$	Hydroxyl
$ONOO^-$	Peroxynitrite
OxyHb	Oxyhaemoglobin
PD	Peritoneal Dialysis
PDP	Peritoneal Dialysis Peritonitis
PFB	Pentafluorobenzyl
PFB Bromide	Pentafluorobenzyl bromide
PGI2	Prostaglandin I2
PMN	Polymorphnuclear
PRTC	Post Renal Transplant Complications
RBF	Renal Blood Flow
ROS	Reactive Oxidative Species
ROI	Reactive Oxidative Intermediates
RRT	Renal Replacement Therapy

SDMA	Symmetrical Dimethylarginine
SGF	Stable Graft Function
SMC	Smooth Muscle Cell
TGF- β	Transforming growth factor-β
TGF- β1	Transforming growth factor-β1
Th1	T-helper 1
Th2	T- helper 2
TNF	Tumor Necrosis Factor
UTI	Urinary Tract Infection
UUO	Unilateral Ureteric Obstruction
VSMC	Vascular Smooth Muscle Cell

Abstract

Introduction

Renal transplantation is the treatment of choice for ESRF. The graft survival is determined by several factors. One of the most important factors is acute rejection (AR), which commonly occurs in the first 3 months following renal transplantation. The incidence of AR is 15–40%, but this depends on the immunosuppressive regime used. The early diagnosis and treatment of AR is mandatory to prevent long-term sequelae of AR. The gold standard test to diagnose AR is renal biopsy which is invasive, expensive, requires experienced personnel, with results reported after 6-8 hours.

Nitric oxide (NO) is one unique molecule with a proven role in AR. However, our interest in NO is whether it rises significantly enough in the peripheral blood that it can be measured and aid in the diagnosis of AR.

We aim to find out whether NO can be used as an alternative non-invasive test or marker to diagnose AR. We also measured NO with other post renal transplant complications (PRTC) (UTI, tacrolimus toxicity [TT] and peritoneal dialysis peritonitis [PDP]) which may mimic AR.

Methods

Ethical approval obtained. We recruited 50 consecutive renal transplant recipients between July 2002 and 2003. The only exclusion criterion was any recipient with previous failed renal transplants. A fasting blood sample was collected every other day and during outpatient follow-up.. Serum nitrate was measured using the Greiss reaction.

Results

Out of the 50 recipients, 6 were excluded. The remaining 44 recipients, 19 had AR and 13 had negative biopsies. There was a significant rise of serum nitrate (median 65.5±53 μmol/L) in AR compared to recipients with a negative biopsy group (41.5±23), UTI (36.5±38.75), TT (35.5±14.88), rise in serum creatinine >10% (44±10.5), or PDP (37.5±23.50) (P 0.001).

Conclusion

There is a significant rise in serum nitrate during AR. Such a rise is not seen with other PRTC.

Introduction

Renal transplantation is the treatment of choice for end-stage renal failure (ESRF). The introduction of new immunosuppressive agents, better understanding of the process of organ transplantation, and the improvement in organ preservation and retrieval have all been translated into longer graft and patient survival (Suthanthiran 1994).

One of the main issues in transplantation is organ shortage. In 2006/2007 over 3000 patients received an organ, but another 1000 died whilst waiting for one. The transplant waiting list rises by 8% per year. The true need for organ transplantation is 50% higher than the organs currently available, and this figure is rising rapidly with changing demographics in the UK, such as an ageing population and increased incidence of type 2 diabetes. This shortage of organs can only be overcome by increased organ donation from deceased and living donors: there has been an increase in living kidney donation of 21% in 2007 compared to 2006, and of 93% from 2000 to 2006. But kidney donation from heart-beating donors fell by 14% between 2006 and 2007 (UK transplant; organs for transplants, A report from the Organ Donation Taskforce, www.uktransplant.org.uk).

Renal allograft failure is the fourth most common cause of end stage renal failure after diabetes (35%), hypertension (23%) and glomerulonephritis (16%) (Agodoa 1997). In fact, allograft failure forms 25-30% of the waiting list for organ transplants (Li 2001, Vadivel 2007). Renal allograft failure is precipitated by a number of factors, the most common of which is acute rejection (Gjertson 2002).

Acute rejection is still a major risk factor for renal allograft failure, despite advances in immunosuppression, organ retrieval and surgical techniques. The incidence of acute rejection varies depending on the immunosuppressant protocol used, with reported incidence varying from 15–40% (Gulanikar 1992, Morrissey 2005, Moore 2008). The frequency and severity of acute rejection has a direct impact on renal allograft survival (Cecka 2000), therefore it is imperative to diagnose and treat acute rejection at an early stage to avoid the serious sequelae of acute rejection such as graft loss (Tomasoni 2008, Shrestha 2007). Renal core biopsy is still the gold standard test to diagnose acute rejection (Kokado 1998, Durkan 2006 Silva 2007); however, because it is an invasive procedure and is fraught with complications including loss of the transplanted kidney, clinicians may hesitate in performing it despite clinical suspicion of acute rejection (Rea 2000). There is also a group of recipients who develop subclinical rejection which is not associated with renal dysfunction and which can only be confirmed by performing protocol biopsies. This means that we are in need of a non-invasive test able to diagnose acute rejection with accuracy comparable to that of renal biopsy, which, in addition to diagnosing acute rejection (particularly subclinical rejection), avoids the risk and cost of protocol biopsies, thereby reducing the rate of graft loss and improving the outcome of renal transplantation (Li 2001). In addition, there is need of a test able to rule out other post

renal transplant complications such as urinary tract infection, immunosupressive toxicity and peritoneal dialysis peritonitis, which can also cause renal dysfunction.

Chapter 1

Nitric Oxide in General

Nitric oxide (NO) was first shown to be produced in mammals by Mitchell in 1916. He investigated the origin of nitrates in urine and found that the animal body excretes more urinary nitrate than it ingests in food (Mitchell 1916). His observation was ignored until the 1970s and 1980s when Tannenbaum demonstrated that endogenous nitrate is produced in healthy adult men and excreted in urine (Green 1981). Then, in 1980, Furchgott and Zawadzkiin showed that vasodilatation of a rabbit's descending thoracic aorta was dependent upon blood vessel endothelium and a mediator termed endothelial-derived relaxing factor (EDRF) (Furchgott 1980). A few years later both Palmer and Ignarro, working independently, showed that NO which was released from the endothelium of the artery and the vein had a similar action to EDRF (Palmer 1987, Ignarro 1987). They concluded that NO has similar biological and chemical properties to EDRF. A year later, Palmer demonstrated that L-arginine is the precursor for NO. Around the same period, Garthwaite identified the presence of NO in the brain cells (Garthwaite 1988), and Hibbs and Marletta showed that NO serves

as an intracellular signal within the activated macrophages when in a bactericidal or cytotoxic state (Hibbs 1988, Marletta 1988, Tayeh 1989). Others showed that NO is present in the lungs, liver, kidney and bowels (Suzuki 1991, Stark 1992). Because of the magnitude of its biological importance, this simple molecule was named the "Molecule of the Year" by Science Washington DC in 1992 (Morris 1994, Stark 1992). Ferid Murad, Robert F. Furchgott and Louis Ignarro were awarded the Nobel Prize in Physiology in 1998 for the discovery of the signalling properties of NO. Salvador Moncada, another notable contributor to NO research, also identified EDRF as an NO molecule , and, with his co-workers, showed that NO production was dependent on the presence of L-arginine (Moncada 1991). However, it was Hibbs in 1987 that reported that L-arginine was the substrate for NO production (Hibbs 1988, Morris 1994, Zhang 1999).

Synthesis of NO

NO results from conversion of L-arginine to citrulline where there is hydroxylation of one of the L-arginine guanidine nitrogen atoms (Suzuki 1991, Zhang 1999). This reaction is catalysed by a group of enzymes called Nitric Oxide Synthase (NOS), which incorporate oxygen into the guanidine group of L-arginine, yielding NO and L-citrulline (Wever 1999). Up to 50% of total body NO originates from L-arginine (Suthanthiram 1994). Arginine is derived from the diet or made endogenously by the kidney. Citrulline is the substrate for intra-renal arginine generation (Schmidt 1999). In the absence of the substrate L-arginine, or when it is in short supply, the same

group of NOS has the ability to produce free radicals [such as superoxide (O_2^-)] in addition to NO (Davies 1995).

L-arginine is the only substrate that forms NO, and this reaction can only be catalyzed by the NOS enzymes group; therefore an increase in NO production is an indirect indication of up-regulation of NOS enzymes.

Nitric Oxide Synthase (NOS) Enzymes

NOS enzymes belong to the cytochrome P450 enzyme group since it has the ability to use multiple oxidative cofactors. There are two distinct types of NOS: constitutive nitric oxide synthase (cNOS) and inducible nitric oxide synthase (iNOS). cNOS consists of two types: endothelial NOS (eNOS) and neuronal NOS (nNOS), of which nNOS is found in the peripheral and central neurons and also expressed in extra-neuronal sites such as the skeletal muscle, pancreas and kidney.

cNOS and iNOS share 50% of their amino acid sequence, but they vary in their sub-cellular location, regulation, sites and effect on NO production (Radomski1990, Weisbrodt 1996).

cNOS is a dimeric complex. It is activated by an increase in intracellular Ca^{+2}, which binds to calmodulin to form a complex that is important for enzyme activity. Calcium- and calmodulin-dependent cNOS is uniquely associated with the cell membrane. cNOS is tonically active and maintains a continuous production of basal NO by the vascular endothelium (Dedeoglu 1996). It is activated by humoral, chemical and mechanical forces acting on the cell and inhibited by cNOS translocation from the cell membrane and by NO. NO feeds-back on cNOS to inhibit

its activity. The activation of these enzymes is short lived and produces NO in small amounts (picomoles). NO mediates the physiological process by stimulating soluble guanylate cyclase. This NO is known as "basal-NO" and facilitates cytoprotective functions (Vos 2004, Castillo 1996, Beckman 1996).

iNOS is a tetrameric complex. It is calcium–dependent and in its active form tightly binds calmodulin. iNOS is inducible in many cells; macrophages, hepatocytes, cardiomyocytes, neurones, microglial cells, vascular endothelium and smooth muscle cells (Brovkovych 2001, Davies 1995, Morris 1994). iNOS is induced by endotoxin, IFN-γ, IL-1, IL-2, TNF-α and inhibited by endogenous hormones (dexamethasone and glucocorticoids), immunosuppressive drugs (cyclosporine and tacrolimus), and cytokines (IL-4, IL-8, IL-10, Transforming growth factor-β) (Morris 1994, Huang 1995) (Table 1.1).

Once induced, iNOS is active for up to 20 hours and produces NO in nanomolar concentrations, 1000 times greater than cNOS (Davies 1995, Morris 1994, Langrehr 1993), and NO is produced for the life of the active enzyme. The expression of iNOS is usually associated with pathological states and the produced NO is cytotoxic (Vos 2004, Castillo 1996). This NO is known as "stimulated NO". The iNOS-generated-stimulated-NO and cNOS-generated-basal-NO are differently regulated and have a different pathophysiological role however they interact (Morris 1994).

The functions of iNOS and eNOS are not only different, but imbalance between the expression and the activity of the iNOS and eNOS may affect cell viability in different ways and can contribute to pathological conditions such as ischaemia reperfusion injury (Pfeilschifter 2002). The loss of iNOS may make the cell resistant to hypoxic challenge, whereas the loss of cNOS and eNOS are lethally damaging to the cells when subjected to the same hypoxic challenge (Pfeilschifter 2002,

Goligorsky 2002). Therefore, selective inhibition of iNOS and sparing cNOS is cyto-protective against ischaemia (Ling 1998). Although all NOS produces the same NO molecule, their activity and function vastly differ due to differences in the temporary profile of NO output and the topography of NO release by each NOS. Moreover, the high output of NO production by iNOS may suppress/inhibit the activity of eNOS (Goligorsky 2002).

	Constitutive NOS	**Inducible NOS**
Source	Endothelial cells, platelets, macular densa, neurones	Leucocytes, macrophages, Kupffer cells, hepatocytes, mesangial cells
Membrane Bound	Yes	No
Calcium	Dependent	Dependent
Calmodulin	Dependent	Independent
Cofactor dependence	Independent	Dependent
Release	Short lived, picomoles	Sustained, nanomoles
Activators	Ach, BK, histamine, ADP/ATP, thrombin, excitatory amino acids, shear stress	Endotoxin, Cytokines
Inhibitors	Dimethylarginine	Glucocorticoids

Table 1.1: Features the difference between cNOS and iNOS. Ach; acetylcholine, BK; bradykinin. (modified from Suzuki 1991).

iNOS Expression in Different Species

Resting cells do not express iNOS but they have the capacity to do so when stimulated. This capacity is found both in immune cells such as macrophages and

non-immune cells such as hepatocytes, vascular smooth muscle cells, kidney cells and fibroblasts (Davis 1995, Morris 1994, Morrissey 1994, Nathan 1991).

Once iNOS is stimulated, there is a delay of several hours in NO synthesis and production. This is because of the time taken to form the essential cofactors needed to induce expression of iNOS and its synthesis. At the level of the mRNA this was seen 3 hours post-stimulation; the circulating nitrate (NO^-_3) levels peak at 12 hours and return to baseline levels after 24 hours (Morris 1994). In rodents, the sustained high levels of iNOS expression may last for a few days (Morris 1994). The stimulating factors that induce the expression of iNOS are different from cell to cell. iNOS pathways were first discovered in murine macrophages. They express high levels of iNOS upon exposure to lipopolysaccharide (LPS), but a strong synergy occurs when interferon-γ is added (Morris 1994). In rodents, macrophages respond vigorously when stimulated by LPS and IFN-γ. When the cultured rat hepatocytes are stimulated by the combination of LPS, IL-1, TNF and IFN-γ, there is an increase of iNOS mRNA levels and NO synthesis. Human cells demonstrate iNOS pathways but they require prolonged exposure to stimulating factors. Human cells show variability in their response to iNOS expression when compared with rodent-cells, which may indicate that human iNOS gene regulation is different from that of rodents (Miles 1995). Human pulmonary artery smooth muscle cells and human cardiac myocytes, when exposed to IL-1, TNF and LPS, show only a minor increase in NO production, whereas human hepatocytes exhibit a good iNOS activity similar to that seen in rodent macrophages. This response in hepatocytes enabled cloning of the human iNOS gene, which shares only 80% of the rodent iNOS, while human cNOS shares greater than 90%. This may explain the different response seen between the two enzymes (Morris 1994, Miles 1995). Therefore, the response of cells to stimulation

may vary from one to another, within the same cell type and from species to species, and there is a significant species difference in iNOS expression.

Types of NO

The type of NOS that produces NO will govern its effect. Up-regulation of eNOS generates NO in small amounts; this is the cytoprotective basal-NO produced during physiological functions. Up-regulation of iNOS generates NO in large amounts; this is cytotoxic stimulated-NO produced in pathological states (Davies 1995, Kroncke 1997). Cytotoxicity and cytoprotectivity are determined by the local concentration of NO, its final degradation products at a particular site and the redox state of the microenvironment.

Basal-NO is found in the endothelium and most cells of the body. It was found to be present in the brain cells, lungs, liver, kidney and bowels where it contributes directly or indirectly to organ function within physiological states (Suzuki 1991, Stark 1992, Garthwaite 1988). The majority of NO is generated from endothelial cells, transporting epithelial cells and the nervous system. It is produced under physiological conditions as a means of modulating blood pressure, renal haemodynamics and sodium excretion; hence it is also known as "Haemodynamically Renal/Active NO" (Baylis 1998). This endogenously-generated NO, in addition to the NO generated from diet and inhalation, is known as "total systemic nitric oxide" (Baylis 1998, Schmidt 2000).

The main source of stimulated-NO is phagocytic leukocytes such as polymorphonuclear leukocytes (PMN), monocytes and macrophages. During the

course of acute or chronic inflammatory processes or in acute immune response situations such as acute allograft rejection, activated macrophages are the main source of iNOS up-regulation and NO production (Suzuki 1991, Langrehr 1993, Vos 2004, Castillo 1996, Wheeler 1997, Miles 1995).

Mechanism of Production of NO

The basal-NO production is ongoing in the circulation depending on humoral, cellular and physical factors. NO interacts with the endothelial and blood cell elements and is responsible for blood vessel relaxation. The binding of NO to Guanylate Cyclase causes the production of cGMP, leading to protein phosphorylation and smooth muscle relaxation. Therefore, loss of endothelium and absence of NO would prevent the vessel from regulating its own tone. Physical force such as that caused by the circulation allows the cells to up-regulate or down-regulate NOS activity, resulting in NO production or inhibition respectively. Within the circulation, physical forces are converted into chemical signals in the form of NO that mediates the vessel wall reaction.

Platelets and leucocytes are the cell types which are responsible for the ongoing release of NO in the circulation. The interactions between NO and platelets make NO a thrombo-regulator in thrombus formation. It acts by preventing platelet adhesion to the endothelial cell surfaces by activating soluble guanylate cyclase. NO is also involved in leucocyte interactions with the vessel wall. It inhibits neutrophil aggregation and adhesion to the blood vessel wall and prevents migration and chemotaxis of mononuclear cells (Davies 1995, Kubes 1991).

The stimulated-NO production is triggered by immune response, infection and surgical stress. Immune responses such as acute renal rejection are a potent factor in stimulating up-regulation of iNOS, leading to an increase in NO production which is linked to the production of various types of cytokines (Brovkovych 2001, Heemskerk 2006, Ahren 1999, Hirabayashi 2005). Lipopolysaccharides (LPS) and endotoxins, both released in acute infectious conditions, are potent inducers of iNOS. LPS is a cell wall component of many bacteria such as E.coli, and can induce host defences for killing of bacteria, cytokine production and up-regulation of iNOS. Although during endotoxaemia most NO is generated by up-regulation of iNOS, some recent reports suggest that in the initial stages of endotoxaemia, up-regulation of eNOS may also contribute to NO production (Brovkovych 2001). The ongoing release of LPS and the impaired renal clearance of NO end-products which is usually seen in septic patients are the two other reasons for raised levels of NO (Heemskerk 2006). In sepsis there is a 40-fold increase in the up-regulation of iNOS within the kidney; this is associated with only a two-fold increase in serum NO but a significant increase of urinary NO, indicating that NO is produced mainly in the kidney. This rise in iNOS and NO radicals within the renal tissues could be responsible for renal injury during sepsis (Heemskerk 2006, Schrier 2004, Wan 2003). Septic shock per se leads to over-production of NO (Hirabayashi 2005, Endo 1996).

Stimulated-NO production could be related to the site of infection and type of pathogen. During gastroenteritis or respiratory tract infection there is an increase in local NO production that is translated into a rise in serum and urinary nitrate. NO production is higher in particular bacteria and viruses compared to others (Ahren 1999).

Surgery also induces the release of various types of cytokines including TNF-α, IL-1, IL-6, IL-8 (Hirabayashi 2005). The site of NO production is thought to be the "disordered organ", such as the liver, rather than the surgical wound itself. This is because it is the organ cells that have the ability to up-regulate NOS, while neutrophils, the cells present in the wound, cannot produce NO in significantly large amounts (Hirabayashi 2005, Denis 1994). The peripheral white cells have also been cited as the source of NO, particularly in sepsis (Miles 1995, Amin 1996, Tsukahara 1998). Whatever the final source of NO production, it eventually diffuses into the main bloodstream where it is detectable. NO production is thought to begin during the operative period, but it takes 2 days postoperatively to peak (Hirabayashi 2005), since this is the time needed for cytokines to be formed and produced.

Mechanism of Action of NO

NO has unique characteristics: it is a colourless gas, soluble in water, lipophilic and readily permeates biological membranes. It cannot be stored and does not require exocytosis to leave the cells. Furthermore, it does not act on specific extracellular receptors but instead diffuses freely and binds to intracellular receptors (Suzuki 1991, Davies 1995). Therefore, the ability of NO to diffuse across cell membranes and to bind to haem and non-haem iron proteins explains the mechanism by which NO reaches intracellular targets. This makes it a useful intracellular messenger since it diffuses through cells with little consumption or reaction with other intracellular elements (Beckman 1996, Davies 1995). NO has the ability to bind to a wide variety of proteins and enzymes, facilitating a large number of functions, some of which are

cytoprotective and others cytotoxic. It also binds with different enzymes during physiological or pathological conditions. Binding and activation of soluble guanylate cyclase produces guanosine 3,5 cyclic monophosphate (cGMP), which has myriad cellular effects such as smooth muscle relaxation and inhibition of platelet aggregation. Activation of soluble guanylate cyclase is the most relevant physiological action of NO (Davies 1995).

NO interacts with thiol groups such as cysteine, N-acetylcysteine and glutathione, forming S-nitrosothiols, which can influence the cellular functions of thiol-containing enzymes (Shah 2003). S-nitrosothiols are biologically active and perform the same functions as NO (Stamler 1992).

The other mechanism of action of NO is its interaction with superoxide and formation of peroxynitrite and peroxynitrite-induced nitration, leading to cellular and enzymatic injury (Shah 2003). This reaction is prevented by superoxide dismutase.

Degradation of NO

NO has a short half-life of 30 seconds, which arises from its reaction with superoxide in vitro, and its reaction with haemoglobin and other reactions in vivo (Beckman 1996). NO is an unstable molecule and rapidly oxidizes to stable end-products, namely nitrite and nitrate, both in vitro and in vivo. Nitrite itself is unstable in the presence of Fe^2 haem or transition metals when it is converted to nitrate.

NO is also converted to other intermediate metabolites such as nitrosoglutathione, nitrosoalbumin and nitrosohaemoglobin. The presence of intermediate metabolites in large quantities may exert biological effects (Wennmalm 1993); it is not known what

proportion of NO goes through which intermediary pathway, but most of these intermediaries eventually also yield nitrate. The reaction of NO with superoxide forms peroxynitrate, which also forms nitrate (Suthanthiran 1994). The final production of nitrate, an inactive molecule, is the most effective way of inactivating NO (Tsikas 2005). In tissues, once NO forms it diffuses rapidly out into the vascular compartment, where it is destroyed by haemoglobin (Beckman 1996). Within the circulation, NO is consumed by red cells or vascular smooth muscle cells (VSMC). It combines rapidly with oxyhaemoglobin (HbO2) to form methaemoglobin (MetHb) and nitrate, or with haemoglobin to form nitrosohaemoglobin (HbNO), which is converted to MetHB and nitrate. NO can be converted to nitrite before it diffuses into the red cells. However, nitrite follows the same pathway as NO within the red cells and forms HbNO, MetHB and nitrate. Nitrate is an inactive molecule that passes into the plasma and is cleared by the kidney (Figure 1.1) (Wennmalm 1993, Tsikas 2005).

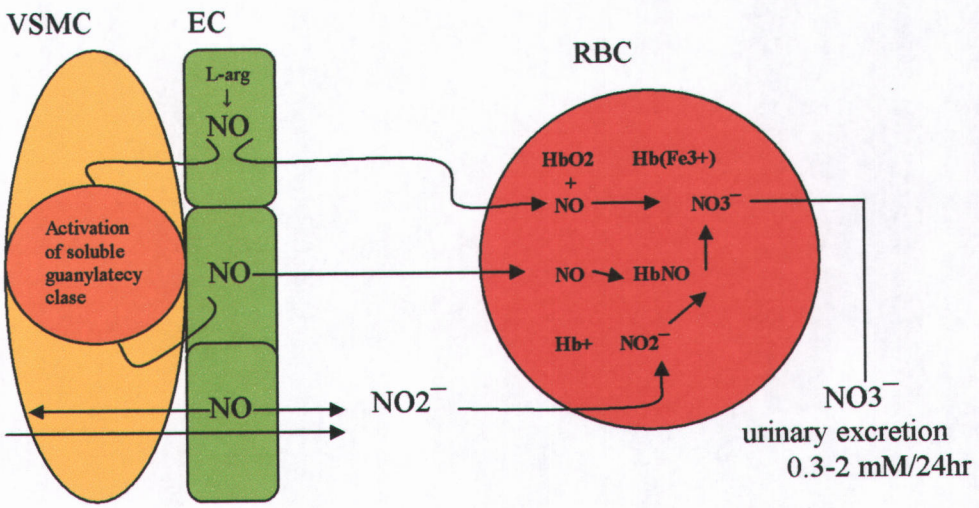


Figure 1.1: Metabolism of NO in vivo. Copied from Wennmalm 1993. EC (Extra Cellular)

NO is a free radical and interacts with other free radicals. Free radicals may also influence the functions of NO. Free radicals are any species capable of independent

existence and are chemically highly reactive (Weight 1996). Some of the free radical molecules are molecular oxygen (O_2), superoxide (O_2^-), hydroxyl ($OH^·$), peroxynitrite ($ONOO^-$) and transition metal-centred radicals such as perferryl radicals (Weight 1996). They are also known as reactive oxygen species (ROS). NO acts as a free radical scavenger and thus a cytoprotective factor (Cooke 1993, Weight 1996); on the other hand, NO can combine with superoxide and forms peroxynitrite, a potent oxidant, which decomposes to form a hydroxyl radical that can cause cellular injury (Trujillo 2008). Since free radicals are highly reactive molecules, their site of action must be close to their site of formation. Their targets are cell membranes, structural proteins, enzymatic proteins and DNA. The lipids found in cell membranes are peroxidised, and the proteins present in the structural and enzymatic proteins are denatured (Weight 1996, Ratych 1986).

Interactions between NOS, NO and ROS

The type of NOS enzyme up-regulated dictates the amount of NO released in the microenvironment. The final outcome of the effects of NO is determined by the concentration of NO, the site of release of NO and the duration of action of NO.

Transient spike-like NO generation caused by eNOS activation is critical for activation of haem-containing enzymes including soluble guanylate cyclase, as well as vasorelaxation, anti-apoptotic programme and protection against oxidative stress. Sustained high output of generation of NO caused by iNOS activation may turn on various functions such as apoptotic action, lipid peroxidation or DNA damage (Figure 1.2).

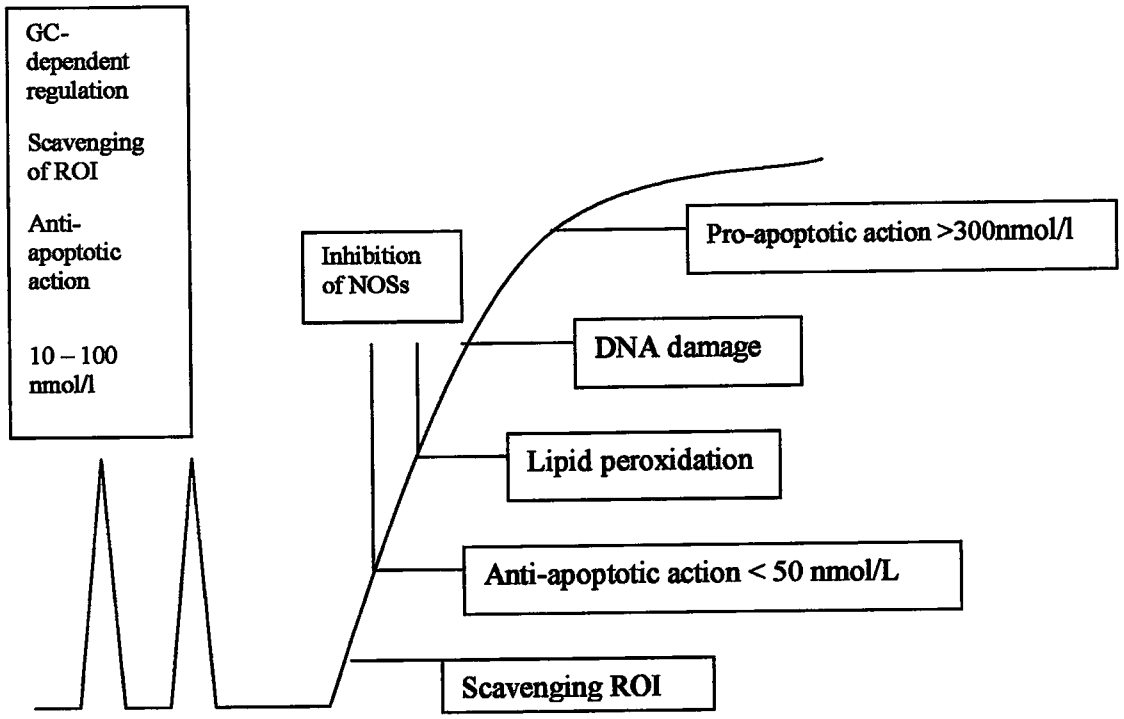


Figure 1.2: Various functions induced by eNOS-generated-NO and iNOS-generated NO (copied from Goligorsky 2002).

The stimulation of iNOS during inflammatory processes is associated with high production of NO and ROS. The interaction between NO and ROS produces potent nitrosating agents. Peroxynitrite ($ONOO^-$) results from the reaction of NO with superoxide (O_2^-) and dinitrogen trioxide (N_2O_3) results from the reaction of NO with molecular oxygen (O_2). These agents trigger signalling by altering protein kinases, protein phosphatises and transcription factors [nuclear factor-κB (NF-κB) and activator protein-1 (AP-1)] (Marshall 2000, Pfeilschifter 2000).

NO is thought to regulate an increasing number of genes. Once the cells are exposed to NO, there is activation or silencing of genes via transcription factors leading to up-regulation or down-regulation of groups of enzymes which include

protective antioxidant defence enzymes such as superoxide dismutase, haemoxygenase and pro-inflammatory mediators (chemokines IL-8) (Pfeilschifter 2002, Grisham 1999).

ROS are similar to NO; in small amounts they act as intra-cellular messengers, but in large amounts they are part of the defensive mechanism. ROS can be produced by a group of enzymes, such as NADPH-oxidase, xanthineoxidase, cyclooxygenase and notably NOS (Pfeilschifter 2002, Suh 1999). If ROS are produced in amounts larger than the cells can handle, a state of "oxidative stress" results. During oxidative stress, there is also up-regulation of protective genes and down-regulation of other genes (Pfeilschifter 2002, Pfeilschifter 2000).

The relative amounts of NO and ROS produced could determine the cellular outcome (apoptosis or necrosis). The simultaneous generation of radical molecules, and their interaction and opposing effect on certain genes, may result in quite dramatic changes in enzyme expression. This explains the various paradoxical biological effects of NO. Within physiological states, there is a low output of NO and low production of ROS, along with activation of soluble guanylate cyclase and generation of cGMP (Pfeilschifter 2002). In pathological conditions, the complexity of the multiple roles of NO and ROS could be multifactorial. The ROS-generating enzymes are constitutively expressed in tissues and produce ROS with minimal delay, while iNOS expression requires a period of several hours to elapse before NO production occurs (Kunz 1994, Cattell 2002). Moreover, the half-life and the range of diffusion of NO and ROS are also different (Radeke 1990, Kunz 1994).

NO and ROS have an impact on the expression of the enzymes that generate or metabolise them (feedback). This may alter their availability and, as a consequence, change the ratio of NO/ROS, which will cause a shift in gears with regards to

inflammatory gene expression. NO controls its biosynthetic machinery as it targets the transcriptional regulation of iNOS. NO functions in a positive feedback loop that speeds up and strengthens its own biosynthesis, and this forms the basis for the excessive formation of NO in acute and chronic inflammation diseases (Pfeilschifter 2002). ROS also potentiates the expression of iNOS and further amplifies the generation of NO. In contrast, NO inhibits ROS generation, leading to alteration of the NO/ROS ratio and thereby creating a dominating "NO state". NO-triggered amplification of iNOS expression could be limited simply by short supply of the substrate L-arginine. Reduction of L-arginine concentration can become rate-limiting for iNOS expression (Cuzzocrea 1998, Pfeilschifter 2002, Goligorsky 2002, Shah 2003).

Summary

Although NO is the smallest product of mammalian cells in terms of the amount produced, it mediates a diverse range of functions. The type of NOS, the amount of NO produced and the site of its synthesis determine the final function of NO within its environment, and this is crucial in determining the physiological and pathophysiological effects. NO contributes to the physiological function of most organs, including the kidneys, liver, gastrointestinal tract and brain, and it is also implicated in their dysfunction, which can lead to glomerulnephritis, hypertension and cirrhosis. Under physiological conditions, cNOS produces NO in small amounts and only trace amounts of ROS are available to scavenge NO, indicating that NO chemistry will dictate functional cell responses. Therefore, NO is beneficial and cyto-

protective when it is a free radical and a scavenger. Under pathological conditions, when a living cell is subjected to stress or change, it adapts to changes by expression of an inducible system of enzymes (iNOS) and mediators which provide an appropriate response. Inflammatory cytokines, endotoxins and LPS prime and activate iNOS, which generates the large amounts of NO that in turn cause nitrosation and oxidation of cellular proteins, and formation of toxic products (peroxynitrite). The deleterious effects of NO can also arise as a result of consuming large amounts of oxygen, since iNOS requires two oxygen molecules for every NO molecule produced (Davies 1995, Pfeilschifter 2002).

Role of NO in Organs During Physiological and Pathological Conditions:

Kidneys; Physiology

NO's role within the kidney is complex and diverse. The diversity of NO function can be explained by the various expressions and regulations of NOS within the renal tissues. NO effects are seen in relation to glomerular filtration rate (GFR), tubuloglomerular feedback and the juxtaglomerular apparatus (Figure 1.3) (Mundel 1992, Wilcox 1992, Ito 1993).

NO is an important mediator of a number of physiological processes within the kidney, including the homeostatic regulation of glomerular, vascular and tubular functions of the organ.

CNOS is present in the glomeruli, macula densa, the collecting duct and the inner medullary limb. The NO generated here regulates the glomeruli microcirculation by

modifying the tone of the afferent arterioles and mesangial cells. In the macula densa, NO causes vasorelaxation of the afferent arterioles, thereby modulating the tubuloglomerular feedback response by decreasing afferent arteriolar vascular tone and increasing glomerular capillary pressure (Wilcox 1992, Mundel 1992). Reabsorbed solutes by the tubular cells of macula densa influence the synthesis of NO within the macula densa (Wilcox 1992, Ito 1993, McKee 1994). This process also initiates the tubuloglomerular feedback response. NO's vasorelaxation effect is further enhanced by the fact that it is the natural antagonist of vasoconstrictive agents such as angiotensin II and endothelin I, which are continuously interacting and modulating renal haemodynamics. Therefore, NO is considered to be a very potent regulator of intra-renal haemodynamics (Blum 1998).

INOS is expressed tonically in the vascular smooth muscles, granular cells and proximal tubule (Roczniak 1996). At each site NO carries out a different role: in the vascular smooth muscle, found mainly in the afferent and efferent arterioles, NO plays an important role in the regulation of glomerular capillary blood pressure, glomerular blood flow and glomerular capillary ultrafiltration coefficient (Morrissey 1994). In granular cells, NO modulates the release of renin, which plays a role in the tubuloglomerular feedback and thereby controls glomerular haemodynamics. Finally, in the proximal tubule, NO is thought to be a regulator of Na transport.

Thus, it is obvious that NO modulates the vascular tone (Beierwalters 1992) and renin release (Schricker 1993), and influences sodium and water haemostasis within the kidney.

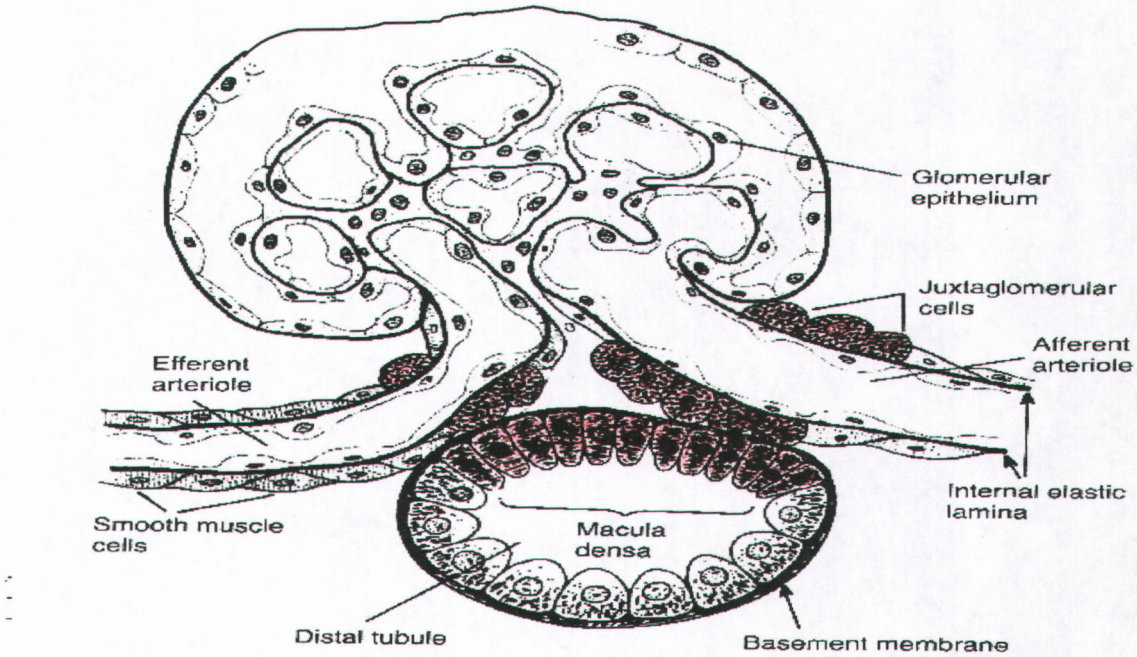


Figure 1.3: Anatomy of the nephron showing glomeruli efferent and afferent with distal tubule forming both juxtaglomerular cells and macula densa.

Morrissey localised iNOS to the outer medulla of a normal rat kidney. When the rat kidney was subjected to stimulation by LPS or cytokines, up-regulation of iNOS could be identified in the resident leukocytes found in the cortex, while macrophages like type II interstitial cells were found in the outer medulla, and endothelial cells were seen within the outer medulla and in tubular cells (Morrissey 1994).

NO production is not limited to the endothelium; it can also be generated by mesangial and inflammatory cells infiltrating the kidneys. Besides its vasodilatory function, it inhibits the proliferation of mesangial and smooth muscle cells. It can also act as a growth factor in modulating the growth of vessels and glomeruli. It is clear, then, that NO is a fundamental component of the cellular milieu in the kidney (Blum 1998).

NO and Renal Disease

The complex interaction between different NOS isoforms and the complexity of NO actions are contributing factors in inducing renal injury in ESRF, acute renal failure, glomerulonephritis and ischaemia reperfusion injury (Cattell 2002).

NO has been implicated in the pathogenesis of different renal pathologies, which include:

1. End stage renal failure (ESRF) and renal replacement therapy (haemodialysis and peritoneal dialysis)
2. Acute renal failure (ARF)
3. Glomerulonephritis
4. Renal ischaemic reperfusion injury
5. Obstructive Uropathy

End Stage Renal Failure (ESRF)

In ESRF, every step of NO generation is inhibited or deficient, creating a "state of NO deficiency" (Blum 1998, Vallance 1992). The deficiency of NO production in ESRF could be caused by a number of mechanisms, including an increase in endogenous NOS inhibitors such as Dimethylarginine (DMA), a loss of functional renal mass, an arginine deficiency or increased levels of oxidant stress (Davies 1995, Schmidt 1999, Wever 1999, Schmidt 2000, Cattell 2002). Both the "total systemic NO production" and the "haemodynamically renal/active NO production" are low in patients with ESRF, and this results in low urinary NOx (Schmidt 2000, Cattell 2002, Vallance 1992, Schmidt 1999). Plasma NOx was not significantly higher in the ESRF

group compared with the normal group because of reduced NO production and reduced renal clearance. ESRF patients on haemodiaylsis or peritoneal dialysis are found to have high levels of serum NOx despite low "total systemic NO production", and this is thought to be due to complete loss of renal clearance and high production of NO from other sites such as the cerebellum (Schmidt 1999). Therefore, plasma NOx alone may not be a true reflection of NO production. The rise in the levels of the NOS inhibitors asymmetrical dimethylarginine (ADMA) and symmetrical dimethylarginine (SDMA) cause the inhibition and reduction of "haemodynamically renal/active NO production" (Schmidt 2000). Vallance reported the presence of circulating methylatedarginine analogues (ADMA and SDMA) in ESRF patients; these can inhibit NOS and NO synthesis. Endogenous ADMA rises in proportion to the increase in serum creatinine, and is detectable in human urine and plasma (Blum 1998). N- monomethyl-L-arginine (L-NMMA), another endogenous methylatedarginine analogue, may also accumulate in ESRF patients.

The loss of renal mass has two impacts on NO production: firstly there is decreased intra-renal NOS activity, as demonstrated in rats with reduced renal mass (Blum 1998, Aiello 1997), and secondly there is low endogenous L-arginine generation due to the lack of functional renal mass, which is the main source of endogenous L-arginine supply after diet. Enzymes such as arginase and arginine decarboxylase may also compete with NOS for L-arginine, further reducing its availability in ESRF (Blum 1998, Vallance 1992). Citrulline is the main substrate for generation of intra-renal arginine, and this accumulates in the plasma supporting low endogenous arginine generation (Schmidt 1999, Schmidt 1999).

Haemodialysis (HD) may induce an inflammatory response and can stimulate iNOS up-regulation and increase NO generation. Despite this, there is a low production of

NO because HD increases the loss of arginine (Wever 1999). Plasma NO levels fall to control levels post-HD, suggesting that HD adequately replaces the kidney in clearing NO. However, HD does not clear the accumulated endogenous NOS inhibitors (ADMA) when measured pre- and post-HD (Schmidt 1999). Peritoneal dialysis (PD) does not stimulate the production of NO as HD does, nor does it not clear NO as well when compared with HD (Schmidt 1999). NO production is low in patients on PD, but they have higher levels due to an accumulation of NO arising from poor renal clearance. The peritoneum per se does not stimulate iNOS except in the presence of peritonitis where iNOS is stimulated (Schmidt 1999).

Acute Renal Failure

Acute renal failure is characterised by an abrupt and reversible kidney dysfunction. The causes are broad, and include ischaemia, a nephrotoxic agent and sepsis syndrome. The insult to the kidney can result in vascular, glomerular and tubular dysfunction (Goligorsky 2002). NO has variable effects depending on the stimulated NOS, the site of its production, the duration of its effect and the associated presence of ROS.

There is an imbalance between the expression and the activity of iNOS and cNOS in ARF. Inhibition of cNOS is one of the hallmarks of endothelial cell dysfunction seen during acute renal injury. The endothelial cell dysfunction is followed by induction of iNOS, increased production of NO and ROS and generation of peroxynitrite. This will lead to the destruction of tubular epithelial cells. The high output of NO will inhibit cNOS, causing further insult to the kidneys (Goligorsky

2002). NO also inhibits complex I respiratory chain enzymes, adding to its cytotoxicity (Clementi1998). The renal tubular cells are resistant to hypoxic insult when depleted of iNOS, but the same cells sustain lethal damage when depleted of cNOS. This reno-protective effect caused by selective inhibition of iNOS against ischaemia is possibly due to inhibition of NO production that, although shared by cNOS, may have a different outcome as a result of its action (Ling 1998).

Glomerulonephritis

In glomerulonephritis there is stimulation of iNOS and increased production of NO and other ROS (Joles 2002). In the early stages of glomerulonephritis, NO peaks; this is associated with macrophage infiltration (Jansen 1994). At later stages of the disease, there is a decline in NO levels despite the persistence of macrophage infiltration. This phenomenon is due to the biphasic expression of iNOS where high levels of NO will down-regulate iNOS by inactivation of nuclear factor-κB (NF- κB) (Cattell 2002). Selective inhibition of iNOS is reno-protective, however non-selective inhibition of NOS can have deleterious effects on the kidney. Therefore, when the functions of all NOS are blocked, the deleterious effects of inhibiting eNOS will prevail over the benefits of inhibiting iNOS. This is an indication that NO may have some protective roles within the kidney, such as opposing the increased vasoconstriction in injured glomeruli (Goligorsky1999). This is well demonstrated in animal models: glomerulonephritis in humans shows up-regulation of iNOS and suppression of eNOS. iNOS is expressed within parenchymal tissues but not in infiltrating macrophages. The initial response to injury is rapid induction of iNOS and

increased generation of NO, which is cytotoxic and cytostatic (Cattell 2002). Suppression of eNOS and loss of eNOS-generated NO will further aggravate tissue damage and promote inflammatory reactions in acute glomerulonephritis (Heeringa 2002).

Ischaemia Reperfusion Injury (IRI)

IRI may be precipitated by sepsis, shock and organ transplantation. IRI in the kidneys causes impairment of glomerular and tubular function. Impairment of glomerular function results in a rise in serum creatinine and urea, while tubular dysfunction causes a rise in fractional excretion of sodium (FE_{Na}) and serum levels of aspirtate transaminase (AST) (Chan 1999). In the post-ischaemic phase, the endothelium becomes refractory to the vasodilatory action of NO. At the same time, there is an increase in the secretion of endothelin. This leads to alteration of the "nitric oxide: endothelin ratio". Both actions cause reduction in renal blood flow (RBF) and GFR (Weight 1996). Renal injury can be further accelerated by other factors, including PMN attraction and activation, generation and formation of ROS, formation of pro-inflammatory cytokines (IL-1β, IL-6, IL-8, TNF-α), expression of endothelial and neutrophil adhesion molecules [vascular cell adhesions molecules (VCAM-1) and intercellular cell adhesion molecule-1 (ICAM-1)], overproduction of vasoactive mediators and formation of RNS. All factors contribute significantly to ischaemic acute tubular necrosis (ATN), leading to acute renal failure.

The exact contribution of NO to changes in renal function during injury is poorly understood, though it is thought that NO effects are produced at the cellular and

microvascular levels. All 3 isoforms of NOS are widely expressed intra-renally. They produce NO, which is a potent vasodilator, and are natural vasconstrictor antagonists. Inhibition of all 3 NOS isoforms has a deleterious effect, which will cause excessive vasoconstriction, exacerbate organ ischaemia and lead to microvascular thrombosis. However, selective inhibition of iNOS could be reno-protective. Weight postulated that NO has a biphasic role in experimental renal warm IRI. During early stages of the ischaemic phase, there is up-regulation of iNOS, and production of high levels of cytotoxic iNOS-generated-NO which generates large amounts of peroxynitrite and hydroxyl radicals. This oxidative stress causes injury by direct oxidant and protein tyrosine nitration, DNA damage, depletion of NAD and ATP and ultimately cell death (Weight 1996, Chatterjee 2002). At a later stage, NO becomes cytoprotective. NO inhibits the sequestration of activated neutrophils and maintains RBF, which is facilitated by up-regulation of cNOS and the generation of cytoprotective eNOS-generated-NO. Inhibition of iNOS expression and the release of large quantities of iNOS-generated-NO, which further reduces the levels of peroxynitrite, will reduce renal injury by IRI (Chatterjee 2002, Weight 1998, Weight 1999).

The up-regulation of eNOS and iNOS takes place at two different periods with different effects. At two hours of ischaemia there is up-regulation of eNOS and a production of NO in small amounts that lowers the perfusion pressure of the organ. However, six hours after perfusion there is up-regulation of iNOS, which results in the production of large amounts of NO and the generation of free radicals. The production of free radicals may compromise the vasodilatory effect of NO. Subjecting the kidney to short periods of ischaemia (before ischaemia reperfusion occurs) may make the organ more tolerant of ischaemia; this is called "ischaemic preconditioning". This tolerance to ischaemia reperfusion injury damage could be attributed to an

increased release of NO (Torras 2002). Ischaemic preconditioning is a complex process which involves activation of the protein kinase C (PKC) directly or indirectly (Downey 2007). Indirect activation of PKC involves up-regulation of NOS. Evidence shows that both eNOS-generated-NO and iNOS-generated-NO play an important role in the protective effect of ischaemic preconditioning (Torras 2002, Yamasowa 2004). Jefayri demonstrated that an increase in NO production was due to an increased expression of eNOS (Jefayri 2000). The ability of the kidneys to produce NO even when exposed to long periods of ischaemia, and the ability of the NO to be a vasodilator and inhibit leukocyte adherence and platelet interaction with endothelium, indicate the cytoprotective role of NO in IRI (Kin 1995, Pryor 1995, Chan 1999, Chaterjee 2002).

Obstructive Uropathy

Obstructive uropathy leads to renal injury and fibrosis. In obstructive uropathy there is an increase in NO activity and generation. NO and NOS play an anti-fibrotic protective role after the onset of ureteral obstruction. eNOS is the predominant source of NO within the obstructed kidney and the eNOS-generated NO confers the anti-fibrotic and protective role (Chevalier 1992, Morrissey 1996, Wheeler 1997, Chang 2002). The absence of iNOS leads to a compensatory increase in the production of eNOS-generated NO (Huang 2000). Chuang thought that both iNOS and eNOS are protective molecules which protect against tissue damage, as he showed using obstructed ureters in rats (Chuang 2005).

Urinary outflow obstruction (UOO) can be transplant- or non transplant-related. Transplant-related UOO is of of two types: ureteric ischaemic stenosis and ureteric anastomotic stenosis. Non transplant-related UOO is mainly caused by benign prostate hypertrophy.

In UOO there is an increase in serum and urinary nitrite for a period of time, as well as up-regulation of eNOS and iNOS during ureteric obstruction. The initial rise in serum and urinary nitrite is due to a rise in eNOS; a subsequent rise, which may last for 21 days post ureteric obstruction, is secondary to up-regulation of iNOS, which then declines (Huang 2000, Chang 2002, Zhou 2003, Chuang 2005). Since bladder epithelium contains NOS, they may contribute to the levels of urinary nitrate (Heemskerk 2006). Chevalier et al demonstrated that during unilateral ureteric obstruction there is an increase in NO production in the obstructed kidney, and this is partly to counteract the release of vasoconstrictive agents such as angiotensin, thromboxanes and endothelin (Chevalier 1992).

NO in the Lungs

Both cNOS and iNOS are expressed in the lung tissues and NO is detectable in exhaled air, localising its production to the airways (Asano 1994, Asano 1995). NOS and NO contribute to the bronchial and vascular tone and to the defence mechanisms (Schmidt 1994). The coexistence of cNOS and iNOS in human alveolar and bronchial epithelial cells confers a defensive barrier at the air/surface interface and prevents airway hyperreactivity (Asano 1994). The airway epithelium has the capacity to

tonically express iNOS under basal conditions, but not the peripheral human lung tissues or the resident macrophages (Guo 1995).

NO is thought to have a bronchodilatory effect by acting on the nonadrenergic noncholinergic (NANC) pathway. Intravenous or inhaled nitrates are effective relaxants of airway smooth muscle cells (Guo 1995). NO also acts as a vasodilator in the lungs, however the pulmonary circulation is less influenced by NO when compared to the systemic circulation (Schmidt 1994). NO is implicated in the pathogenesis of lung inflammatory diseases. Asthmatic patients have high levels of NO in their exhaled air. Inhaled corticosteroids down-regulate iNOS and decrease NO production, therefore losing the bronchodilatory effects of NO as well as its cytotoxic effects against mycobacterial and fungal infection. This supports the protective role of iNOS and NO in the airways, which mediates cytotoxicity against bacteria, viruses, fungi and protozoa (Guo 1995).

NO in the Brain

In the brain, neuronal cNOS is localised to 2% of cerebral cortical neurones, and is also found in the vasculature of the dendrites and axons. NO acts as a neurotransmitter but it differs from other neurotransmitters within the brain (Figure 1.4). It lasts longer than acetylcholine. The diffusibility of NO means it is able to diffuse to several million synapses and take part in various functions (Beckman 1996).

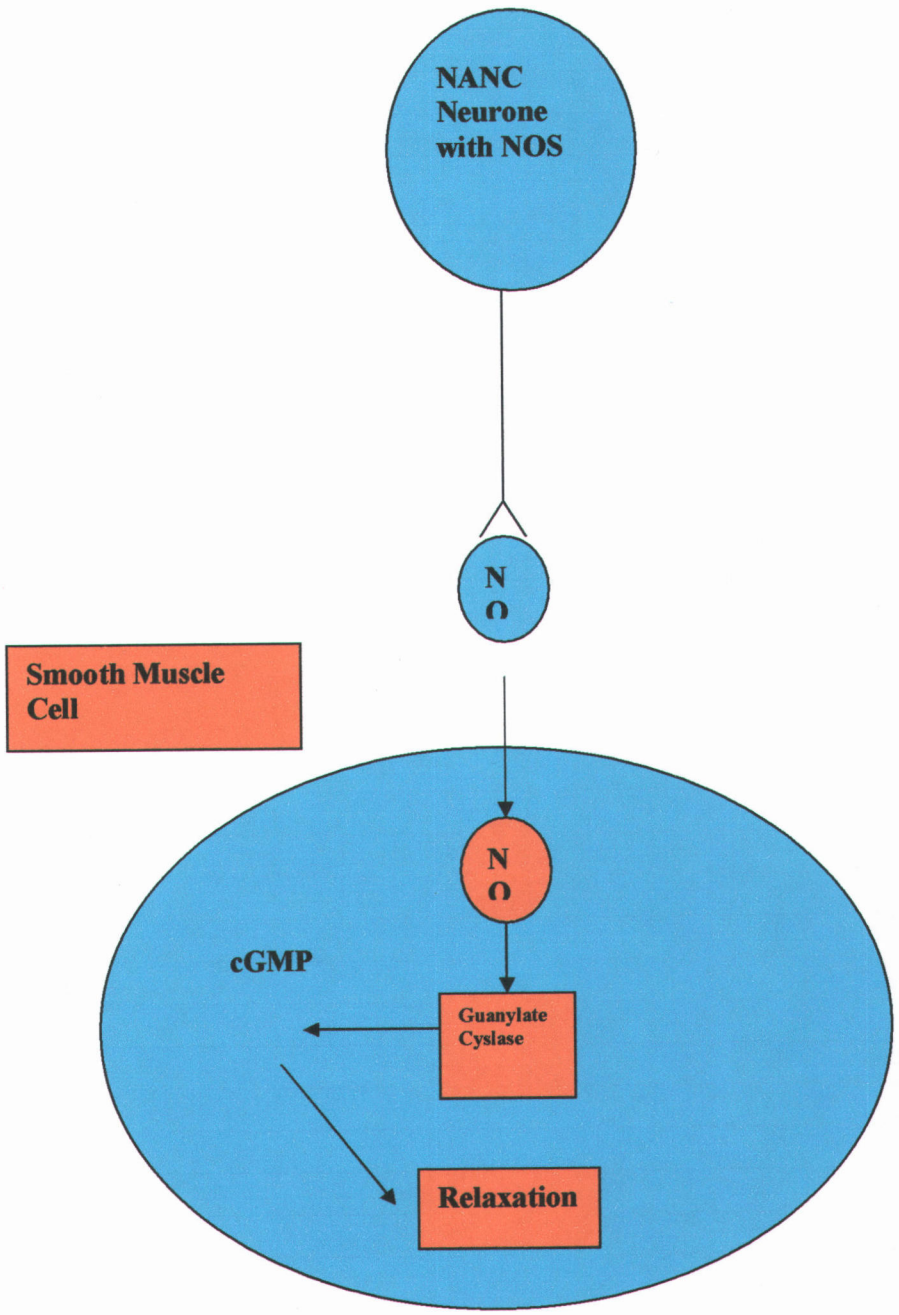


Figure 1.4: NO in brain as a neurotransmitter.

NO in the Gastrointestinal Tract

In the gastrointestinal tract, NO is synthesized at multiple sites in the muscularis. The NOS is localised to the myenteric plexus and neuronal processes of the circular smooth muscle layer. NO mediates its action via the nonadrenergic noncholinergic (NANC) system. It causes relaxation of the longitudinal and circular smooth muscles of the lower oesophagus, stomach, duodenum, small intestine and internal anal sphincter (Stark 1992).

The stimulation of NANC nerves leads to the release of neurotransmitters which stimulate the production of NO within gastrointestinal smooth muscles. Therefore, NO is considered to be a secondary messenger. NO could be stored as stable precursors called "nitrosointermediates", which are released by nerve stimulation or by synthesis at a pace in line with release, thus avoiding the need for storage. Both mechanisms could be operative depending on the site and type of physiological response. Ongoing release of NO may be the preferred mechanism in sphincters or smooth muscles with active tone, while it is not needed in the ileum and jejunum smooth muscles with predominantly phasic activity (Stark 1992).

NO is one factor of many in maintaining the integrity of the mucosal barrier. The role that NO plays in mucosal blood flow (it increases it), and the interaction with prostaglandin and NANC, may indicate that NO is an important factor in the physiology of mucosal protection (Stark 1992, Salzman 1995, Wallace 2000). The source of NO within the mucosa could be the vascular endothelium, epithelial cells, white cells, or neurons. It is the balance between vasodilatory substances (NO) and vasoconstrictors (thromboxane and endothelin-1) which may affect the mucosal blood flow, its integrity and defences (Chang 2005). Imbalance between these substances may lead to mucosal protection or injury, depending on the prevailing substances. Therefore, inhibition of NO production may cause mucosal haemorrhage, ulceration

and loss of integrity, while the application of NO donors such as nitroprusside protects the mucosa (Payne 1993, Fink 2003). In endotoxaemia, there is a release of a large number of vasoactive mediators, which can compromise the intestinal mucosa; a problem worsened by stimulating the release of oxygen radicals such as superoxide anions. The formation and release of NO maintains mucosal microvascular integrity and protects against the harmful effects of endotoxic shock. NO is a vasodilator and anti-oxidant. It interacts with the superoxide anion, forming a less toxic species (antioxidant effect), and inhibits propagation of endotoxic-induced damage by inhibiting neutrophil adhesion and migration (Crouser 2000, Stark 1992).

NO in the Liver

NO mediates changes in the hepatic blood flow and will influence the overall function of the liver. Under normal conditions, eNOS-generated NO is expressed to maintain normal hepatic blood circulation. However, during liver injury such as sepsis, cirrhosis, or ischaemia reperfusion injury, NO is produced in large amounts due to up-regulation of iNOS (Kučera 2004). There is growing evidence to support the role of NO synthesis in liver injury (Davies 1995). The complexity of the NO molecule is seen within the liver environment where NO may be cytoprotective or cytotoxic. During endotoxinaemia and sepsis, the stimulated Kupffer cells up-regulate iNOS, leading to an increase in the production of NO (Moncada 1991, Morris 1994) (Figure 1.5). The amount of NO production and the redox state of the liver determines the role of NO. During oxidant stress where there is an excessive number of free radicals, NO is considered to be hepatoprotective, while overproduction of NO in

normal free radical production may produce peroxynitrite, which is cytotoxic (Stark 1992, Kuo 1994, Davies 1995, Beckman 1996, Chen 2003).

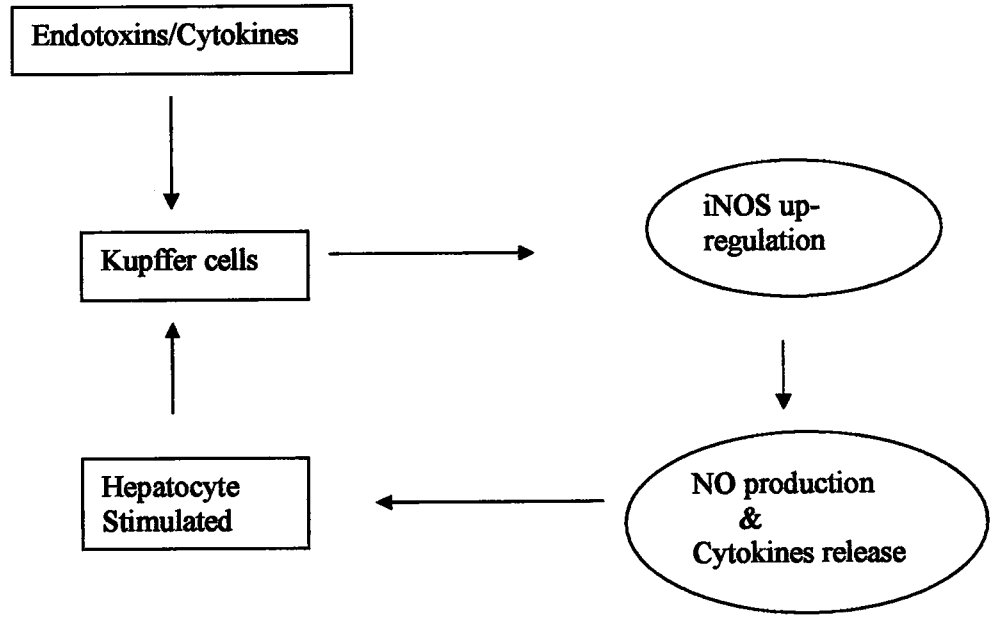


Figure 1.5: NO in liver during sepsis.

Increased production of NO is seen in Cirrhosis of the liver, and has a number of clinical consequences. NO overproduction causes systemic vasodilatation and hyperdynamic circulation, which contributes to formation of decreased protein synthesis, ascites, oedema and portal hypertension (Turkay 2004, Stark 1992, Davies 1995).

Summary

Endothelial cell dysfunction is central to various pathological processes. It reduces the bioavailability of NO by decreasing NO production, increasing NO degradation or

both. NO and endothelial cell dysfunction could be one of many mechanisms contributing to the development and progression of pathological conditions (Figure 1.6). The physiological role of NO in many organs has been established, but its role in the pathophysiology of diseases is yet to be fully elucidated.

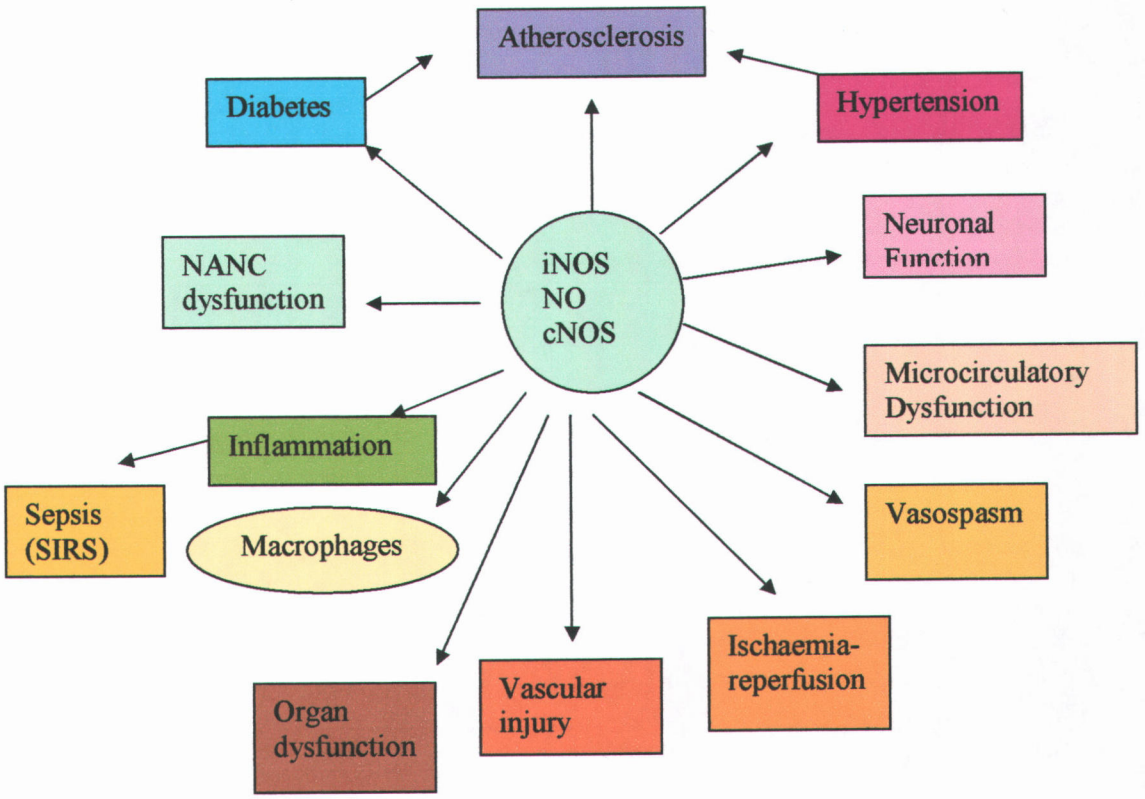


Figure 1.6: Diverse role of NO in Pathological conditions (Davies 1995).

Chapter 2

Renal Transplantation

History of Organ Transplantation

The first experimental animal renal transplant was performed by Ullman in 1902 when he transplanted a kidney to a dog neck (Ullman 1902). But it was Jaboulay who first transplanted goat's and pig's kidneys into humans in 1906 (Jaboulay 1906). It was not until 1933 that Voronoy performed the first renal transplant from a human donor. Although he performed seven more transplants between 1933 and 1949, all failed due to mismatch (Voronoy 1936). Hufnagel, Hume and Landsteiner in Boston achieved transient renal function, but their success was limited by immunological barriers. The lack of understanding of the pathophysiology of the immune response in transplantation was the main cause of loss of the transplanted organ. Peter Medawar was the first to document the immunological nature of rejection and tolerances. At first an attempt was made to control rejection by using total body irradiation before the beginning of pharmacological immunosuppression. Calne introduced 6-

mercaptopurine A to prolong canine renal transplants and Zukowski introduced the use of corticosteroid based on its anti-inflammatory effect. Between 1966 and 1978, azathioprine and corticosteroids formed the basis of immnosuppression in renal transplantation. In 1979, Calne introduced cyclosporine A, a fungal metabolite, as a new immunosupressive agent. This resulted in a significant increase in the rate of graft survival (Calne 1979, Senda 1995). CsA and tacrolimus formed the cornerstone of immunosuppressive therapy, and improved the survival rate of solid organ transplants by 15-20%. The one-year survival rates for renal allografts from living and cadaveric donors are >93% and >87% respectively. The half-life of renal allografts has improved from 12.7 to 21.6 years in live donated renal allograft transplants, and from 7.9 to 13.8 years for cadaveric allografts (Hariharran 2005). The availability and judicious use of various immunosuppressants, better understanding of acute rejection, improved retrieval and preservation of organs, better organ preservation, improvement in surgical techniques and anti-infectious therapy all contributed to recent improvements in renal transplantation (Suthanthiran 1994, Matas 2001, Oberholzer 2004, Orsenigo 2004, Lee 2005, Hazzan 2005).

HLA Matching and Cross-Matching

The genes encoding the HLA antigens are located on the short arm of chromosome 6. HLA-A, B and C antigens are called class I and are displayed on all nucleated cells and platelets (Figure 2.1). HLA-DR, DP and DQ antigens, class II, are expressed on B-cells, monocytes-macrophages and dendritic cells. T-cells and non-lymphoid cells

(such as renal tubular epithelial cells) display class II proteins only when activated by cytokines (Suthanthiran 1994).

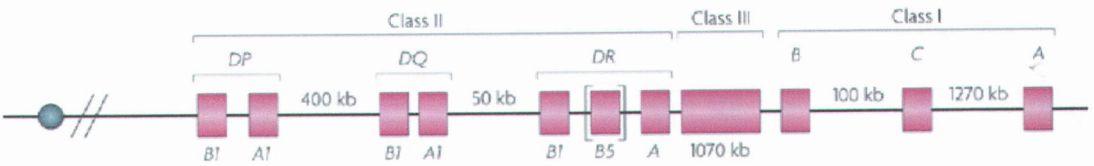


Figure 2.1: Short arm of chromosome 6 (adapted from *Nature Reviews Genetics* 2008; 9, 516-526).

The matching has clinical benefits. The one-year graft survival rate was 94% in recipients with HLA-identical kidneys matched for two haplotypes, while it is 89% for one haplotype matching. The Collaborative Transplant Study (CTS) demonstrated that the graft-survival rate of HLA-identical recipients is superior to that of grafts matched for one haplotype (Oplez 1992). The estimated half-lives of matched kidneys are 26.9 years compared to the 12.2 years of those that matched for one haplotype (Suthanthiran 1994, Oplez 1992). The one-year graft-survival rate of matched kidneys is 88% compared to 79% in mismatched allografts, and the estimated half-life of HLA-matched grafts was 17.3% compared to 7.8% in mismatched allografts (Takemoto 1992). The HLA-matched allografts have a better graft survival rate, with longer cold ischaemic time when compared with mismatched allografts and a short cold ischaemic time. Allografts matched for four or more HLA antigens have a better outcome than those with fewer than four matches. The matching of HLA A, B and DR has been shown to be beneficial to the graft survival rate, while this is less so for HLA-C and DQ; based on this, some policies make it mandatory to allow no mismatches in the HLA A, B and DR, which is justified by the positive results outlined above (Cicciarelli 1991).

Renal transplantation is associated with increased expression of HLA class II on the tubular epithelial cells and endothelium by day five post transplant. This is an indication of graft rejection (Loutfi 1991). Down-regulation of MHC class II by monoclonal antibodies is associated with prolonged graft survival. The monoclonal antibodies also up-regulate iNOS (in the macrophages) and increase nitrate production. This production increases on day five post renal and liver transplantation and increases significantly during acute rejection (Weight 1996).

The Role of NOS and NO in Renal Transplantation

During renal transplantation, a number of events inevitably take place which precipitate endothelial cell injury: up-regulation of NOS and production of NO. These events occur from the time of retrieval and for the life of the organ or the recipient. The kidney is subjected to warm ischaemia in live donation or cold ischaemia in cadaveric donation during organ retrieval followed by transplantation surgery. Vascularisation of the transplanted organ induces ischaemia reperfusion injury. Following surgery, acute rejection and opportunistic infection are the main post-transplant complications. These events may occur individually or together, and one may lead to another or facilitate its happening. In addition, these events may favour rejection episodes.

The events affecting the transplanted kidney during organ transplantation may induce iNOS up-regulation and NO production, an oxidative stress state and other cellular events including cytokine release and expression of adhesion molecules and MHC antigens (Bulkley 1994, Cattell 1994). The up-regulation of NOS and the

production of NO may occur with every event and may contribute, directly or indirectly, to each event. The events may also favour rejection episodes, since the released factors (such as cytokines) that play a part during acute rejection can up-regulate NOS and increase the production of NO (Devlin 1994). The induction of iNOS and NO production during these events are potentially detrimental to the graft survival (Salahudeen 1996).

Both isoforms of NOS exist in the recipient and in the donor kidney and they have different effects on the transplanted organ. The effects of NOS and NO in renal transplantation depend on the type of the activated NOS, amount of NO produced and the redox state of the microeniverоment.

The effects of cNOS-generated NO are different from the effects of iNOS-generated NO. Native cNOS and cNOS-generated NO has a protective property during pathological conditions, and its loss may precipitate hypertension and allo-arteriosclerosis (Vos 2004).

iNOS is "constitutively" expressed in the transplanted kidney's tubules, glomeruli, and arteries (Mattson 2000, Albrecht 2000). In rat kidneys, iNOS exists as macrophage NOS and vascular smooth muscle cell NOS (Cattell 1994, Agarwal 1996, Suzuki 2004). Constitutively expressed iNOS is only up-regulated during inflammation or acute rejection. iNOS is also up-regulated in the invasive inflammatory cells which are the recipient-derived activated macrophages that infiltrate the transplanted organ during acute rejection. The deleterious effects of recipient-derived-iNOS would overshadow the beneficial effects of donor-derived-iNOS found in the resident parenchymal cells of the renal allograft (Vos 2004, Joles 2002). The deleterious effects of iNOS modulate and promote rejection, and therefore inhibition of recipient-derived iNOS which is expressed on the infiltrating

macrophages in the kidney graft and could protect the transplanted kidney from the infiltrating macrophages that cause renal transplant injury (Albrecht 2000). The function of iNOS within the allograft is diverse: it is cytoprotective when it acts as an NO-producing enzyme, while it is cytotoxic when acting as a peroxynitrite-producing enzyme. The biological effect of iNOS is not only influenced by the radical species it releases, but also by the antioxidant capacity of the cellular microenvironment and ROS production (Joles 2002). The bioavailability of NO is critically dependent on the sufficient antioxidant capacity of the microenvironment. When the antioxidant capacity is insufficient, the reaction of NO and O_2^- and the formation of peroxynitrate will result. However, if the antioxidant capacity is sufficient, NO production is beneficial (Joles 2002). The site of this oxidative stress is the activated macrophages that infiltrate the graft. They up-regulate iNOS and activate NADP(H) oxidase, which produces NO and O_2^- (Grimm 1999). Massive production of ROS species also influences the availability of NO. ROS increases degradation of NO and reduces NO bioavailability. The end result of reduced NO bioavailability is endothelial dysfunction and leukocyte recruitment, hence providing a key element for rejection. ROS can also reduce NO production by inhibiting the activity of NOS (McCord 1985). Deficiency of the NOS substrate (L-arginine or co-factor BH_4) may alter the function of NOS and ROS production. The low supply or availability of L-arginine or BH_4 will cause "NOS uncoupling", where NOS will produce superoxide instead of NO (Vos 2004, Xia 1996). The responses of NOS to substrate deficiency vary: eNOS depends more on L-arginine binding, whereas iNOS depends more on BH_4 binding. Therefore, uncoupled eNOS would generate a large amount of O_2^- while uncoupled iNOS would generate a large amount of peroxynitrite. NOS uncoupling is associated with early acute rejection (Huisman 2002).

The effects of ROS are further potentiated by the decrease in the antioxidant capacity of the transplanted kidney (MacMillan-Crow 1996). The enzymatic activity of endogenous antioxidants such as catalase, glutathione peroxidase and superoxide dismutase is low. Superoxide dismutase is one enzyme found to be inactive during acute rejection, and as a result an increase is seen in the levels of O_2^- and $ONOO^-$ (Vos 2004).

During human renal rejection, the inflammatory and non-inflammatory cells in the renal allograft produce ROS which activate the transcription factor NF- κB, and in its turn it up-regulates iNOS in the interstitium, the glomeruli and the invasive cells infiltrating the graft. The deleterious effects of large amounts of iNOS-generated NO by the invasive cells infiltrating the graft are more important than the beneficial NO produced by the native cells and inhibition of all isoforms of NOS, resulting in a loss of beneficial cNOS-generated NO, which outweighs the beneficial effects of inhibition of iNOS. During acute rejection, the increased iNOS expression appears to be deleterious, while in chronic rejection the iNOS upregulation is beneficial and compensatory (Joles 2002). Inhibition of NO production from all NOS can decrease the chances of renal graft survival by causing the loss of protection against ischaemia or by aggravating the allo-immune response (Vos 2004).

The renal injury caused by acute rejection involves the endothelium, leukocytes, cytokines, adhesion molecules, NF-κB, NO and NOS (Figure 2.2). All these factors inter-relate. Endothelial cells are the first cells to be affected and are the main target of the inflammatory process. The modifications of the normal biological functions of the endothelium, such as vascular tone, coagulation and inflammation control, lead to acute rejection (Castillo 1996). Endothelial injury is associated with recruitment, adhesion and extravasation of leukocytes, which are assisted by adhesion molecules

(Vos 2002). NF-κB has a diverse role: it recruits leukocytes, activates cytokines and free radicals, and up-regulates iNOS. It also up-regulates MHC-II expression. Therefore, NF-κB enhances graft immunogenicity and promotes acute rejection (Baeuerle 1997). Interestingly, NO tends to inhibit NF-κB (De Caterina 1995) (F 2.2).

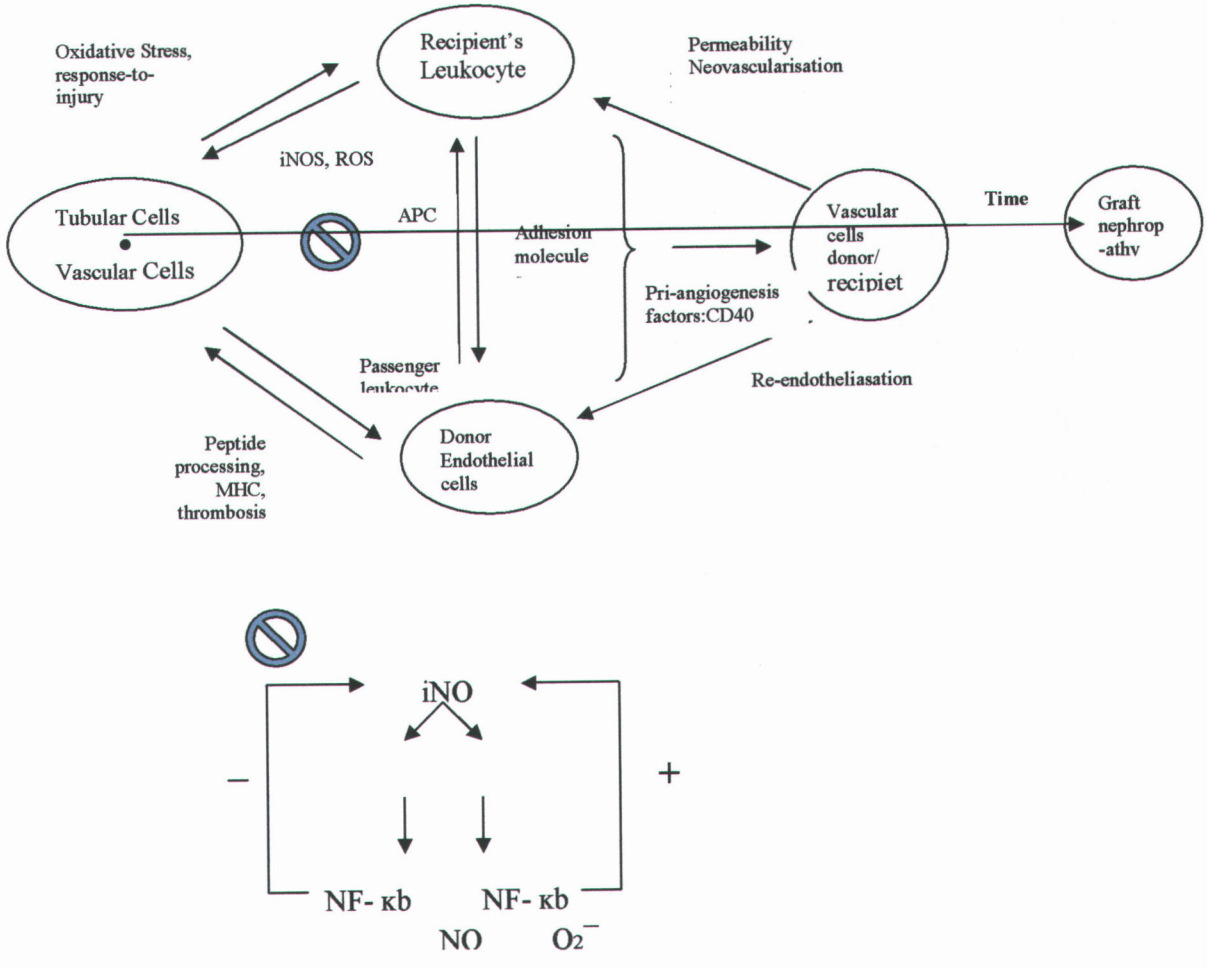


Figure 2.2: The cell types and compartments involved in graft nephropathy. Many factors, including pretransplant condition of the organ, ischaemia reperfusion injury in the transplant process and leukocytes- and antigen-mediated inflammation, interact in the different compartments (copied from Vos 2004).

NO in Acute Rejection

Acute rejection creates a stress situation in a stable environment, subjecting normal living cells to an inflammatory process. The living cells react by expressing inducible enzymes and mediators and the inflammatory process is a complex, tightly regulated sequence of events, with initial production of pro-inflammatory mediators that recruit inflammatory cells. A balance between defensive or offensive factors would determine the outcome of the process: progression or resolution.

Acute rejection is a complex immunological process involving both cellular and humoral components of the immune system, and it depends on the coordinated activation of alloreactive T-cells and antigen presenting cells (APC) (Pelzl 2003). Acute rejection is a T-cell dependent process causing the destruction of the allograft, which is orchestrated by the release of cytokines, cell-to-cell interaction and the assembly of lymphocytes (CD4+ T-cells, CD8+ cytotoxic T-cells, antibody-forming B-cells and leukocytes) (Suthanthiran 1994).

The incompatibility of antigens (MHC) between the donor and the recipient of a graft leads to an immune response against the graft. The passenger leucocytes migrate rapidly out of the tissue following transplantation, traveling to the recipient lymphoid organ where they are able to interact with and stimulate the host's immune response. These passenger leucocytes rapidly mature into antigen presenting cells (APC) with the ability to stimulate T-lymphocytes. The APC expresses major histocompatibility complex (MHC) on its surface (Krensky 1990). A foreign protein then embeds itself in the MHC groove; this is recognised by T-cells forming T-cell-antigen recognition complex. T-cells may recognise the antigen directly or following processing by the APC. The T-cells express CD4 and CD8 proteins that combine with HLA class II and class I, respectively, which is expressed on the APC or the renal allograft. The T-cell-

antigen recognition complex stimulates the intracellular protein tyrosine kinase, which increases the activation and expression of genes central to the T-cell growth. Interleukin-2 is secreted by the activated T-cells. There is a proliferation of autocrine T-cells arising from the binding of IL-2 to IL-2 receptors on the T-cells. IL-2 triggers the activation of tyrosine kinase, leading to the expression of several DNA binding proteins (c-jun, c-fos, c-myc) and to the progression of the cell cycle. The outcome of this cytokine production is the emergence of antigen-specific, graft-infiltrating, destructive T-cells (Suthanthiran 1994, Storm 2000). Cytokines also cause the activation of other inflammatory cells (e.g. macrophages), B-cells (producing anti-donor antibodies) and up-regulation of the expression of HLA on the graft cells and APC (Suthanthiran 1994, Storm 2000). Calcineurin participates in signal transduction and blocks it by cyclosporine, while tacrolimus appears to be central to immunosuppression activity (O'Keefe 1992).

While donor leucocytes can migrate into the host lymphoid organs, the recipient leucocytes migrate into the graft. The activated T-cells are able to recruit and activate other cells. They do so by producing cytokines, which direct the proliferation and differentiation of effector cells. IFN-γ and interleukins are particularly involved in the generation of activated macrophages. During the process of acute rejection, the renal allograft is infiltrated by the activated macrophages; in addition to forming part of the immune response, they also show an increased expression of iNOS, leading to production of NO (Vos 2004, Devlin 1994). The presence of iNOS is usually associated with a sustained need for large amounts of NO. This need is usually associated with cytotoxicity. Although the mechanism of NO production in acute rejection is clear, it is unclear what role it plays. Immunologically-released NO from endothelial cells or macrophages brings about increased blood flow, hypotension,

modulation of leukocytes (Suzuki 1991), platelets and other immunological reactions (Radomski 1990).

The discovery that iNOS-produced NO by monocytes-macrophage lineage in response to inflammatory cytokines (IL-1, TNF, IFN-γ) prompted Jan M. Langrehr and Rosemary A. Hoffman to investigate the significance of NO synthesis during the course of immune response to alloantigens and whether it has possible immunomodulating effects on allografts (Langrehr 1992). They demonstrated that NO production in rat and mouse splenocyte MLR promoted a profound inhibitory effect on cytotoxic T-lymphocyte (CTL) induction (Langrehr 1992, Shiraishi 1995). When N-monomethyl-L-arginine (NMA), a competitive inhibitor of oxidate L-arginine metabolism, was added to the cultures, the nitrite/nitrate levels decreased markedly, resulting in antigen-specific proliferation and CTL induction (Langrehr 1991). Based on this, they looked at whether NO production could be shown in an in-vivo allograft system. To do this they used a sponge matrix allograft, injected it with syngenic or allograft splenocytes and analysed the surrounding fluids for the type of cells infiltrating the sponge, in vivo NO production and the interactions between the infiltrating cells and the ongoing NO synthesis. There was an early increase in the number of macrophages in both syngenic and allogenic sponge grafts by days six and eight after grafting in an allogenic compared with a syngenic graft. The sponge fluid analysis showed higher $NO2^-/NO3^-$ levels in the allogenic graft on days four, six and eight after grafting, compared with the syngenic graft. The cultured and harvested graft-infiltrating-cells were primarily made up of macrophages, which produce significantly more NO. They produce more NO when cultured with L-arginine. The effects of NO on the function of the infiltrating macrophages were evident when the cells were cultured in the presence and absence of N-methyl-L-arginine (NMA).

When NO production was inhibited by NMA, the allograft-infiltrating cells were cytotoxic. In the absence of NMA, high NO production was associated with the failure of allograft-infiltrating cells to show cytolytic function. The conclusion was that there was a significantly higher nitrite/nitrate level in the allogenic graft sponge fluid at day six after grafting, which correlates with the point in time where there is evidence of alloreactivity – this is when the donor-specific cytotoxic T-lymphocytes (CTL) function was first detected. Allograft responses are accompanied by NO production that is mediated by macrophages, which are the main graft-infiltrating cells and the major source for the inducible NOS and NO production. Re-exposure of the activated macrophage cells to the specific donor allo-antigen resulted in a surge of NO synthesis; therefore, the magnitude of the immune response with alloantigeneic stimulation correlated well with the amount of NO produced. NO may also lead to inhibition of T-cell proliferation, therefore down-regulating the immune response. However, NO production is limited by the availability of the substrate (L-arginine) (Langrehr 1993). Langrehn et al hypothesized that cytokines produced by allo-activated T-cells initiate the NO pathway in the graft-infiltrated-macrophages. The NO in turn inhibits lymphocyte proliferation and the development of allo-sensitivity. Therefore, macrophages play a central role in both initiating the allo-immune response (processing the antigen, presenting it to lymphocytes and secreting IL-1) and inhibiting the response (by secreting arginase to decrease arginine levels) and by using arginine to produce immuno-modulatory substances such as NO (Figure 2.3) (Langrehr 1991).

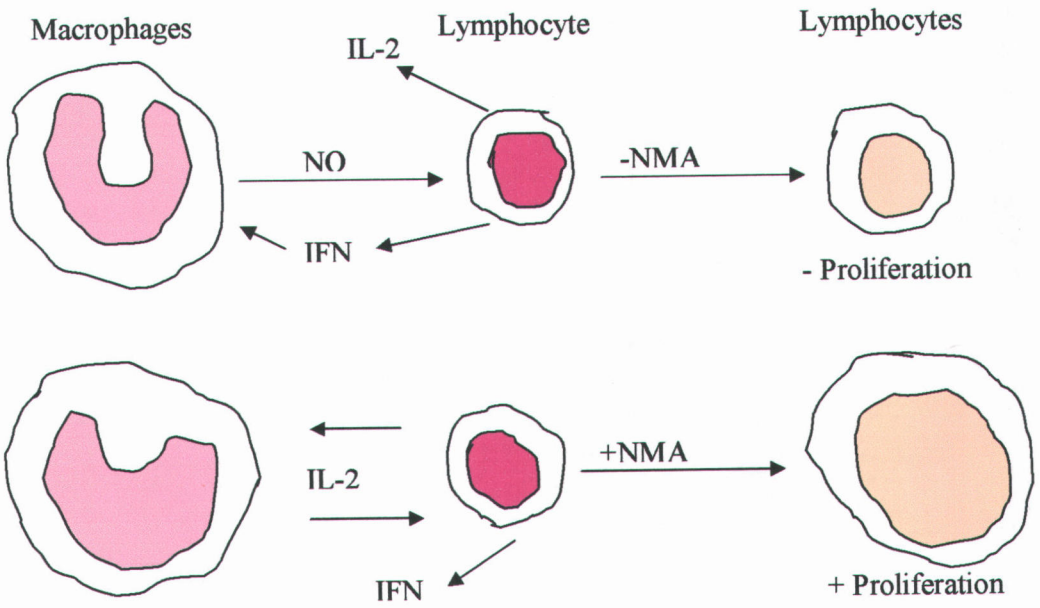


Figure 2.3: NO synthesis during allo-immune response. Macrophage presents alloantigen to lymphocyte which secretes IL-2 and INF. INF stimulates macrophage NO, resulting in inhibition of lymphocyte proliferation. NMA (N-methyl-L-arginine) (copied from Langrehr 1993).

Worral suggested that the net role of NO in allograft transplants is to promote rejection. NO can lead to up-regulation of class II major histo-compatibility (MHC) complex or increase local production of cytokines, thereby increasing allograft alloantigenicity (Worrall 1995).

A further experiment was conducted by Langrehr to determine whether NO production is associated with organ allograft rejection and graft-versus-host disease (GVHD) in vivo, and whether NO could be a marker for this immune response. The end-products of NO (nitrite/nitrate) were measured in untreated and immunosuppressed rats receiving liver, heart, small bowel, skin, sponge matrix and bone marrow/spleen cell allografts (Langrehr 1992). Recipients with organ allografts who developed acute rejection had a significantly high level of serum nitrite/nitrate. However, recipients with syngenic grafts and those immunosuppressed with FK506 or CsA had normal serum nitrite/nitrate levels. Recipients with skin grafts and sponge

matrix grafts did not show changes in the level of serum nitrite/nitrate during the rejection process. This experiment showed for the first time that there is an increase in NO production during the rejection of a vascularized organ (Langrehr 1992). This was a significant finding by Langrehr, and linked NO production to acute rejection and therefore suggested the possibility of using NO as a diagnostic test in solid organ transplantation.

Shirashi reported an increase in NO production during the early stages of acute rejection in rat lung allograft transplants. He suggested that serum nitrite and nitrate can serve as an early marker for acute rejection (Shiraishi 1995). In pancreatic transplants in rats, Tanaka found a rise in serum nitrate level on the fifth day post transplant in allograft transplants, but not in syngenic grafts. The elevation of NO in the blood was detected before the elevation of the blood glucose level (Tanaka 1995). Ohdan studied NO during acute rejection in rat liver transplantation, demonstrating that acute rejection in liver transplantation is associated with a rise in serum nitrite and nitrate (Ohdan 1995). Lu examined the role of NO in acute renal rejection in rat models. By the eighth day post transplant, all animals had severe degrees of acute rejection. There was a rise in serum nitrite levels from day two, which remained high up to the eighth day, and also a positive correlation between the level of nitrite in the serum and the graft. His final conclusion was that there was an increase of serum nitrite earlier than the pathological manifestation of acute rejection. He suggested that NO could be used as an early indicator of acute rejection (Lu 1999). Another study by Suzuki measured the levels of serum and urinary NO in rats during renal acute rejection. He found a significant rise in serum NO levels during acute rejection in allografts when compared with isograft models, while the urinary NO was found to be lower in allograft models (Suzuki 2004).

In humans, Albrecht used serum and urinary nitrite and nitrate as non-invasive markers to diagnose acute rejection in renal allograft recipients. The results showed a gradual increase in serum nitrite and nitrate and a significant decrease in urinary nitrite and nitrate two days before the diagnosis of acute rejection. In cyclosporine toxicity, serum NO was elevated, but this was not significant compared with recipients with acute rejection or an uncomplicated course; however, urinary NO was significantly higher compared with recipients with acute rejection. Albrecht suggested that serial measurement of urinary and fasting serum NO can be of clinical value as a non-invasive marker in acute renal allograft rejection (Albrecht 2000). In a similar study, Dedeoglu et al looked at serum and urinary NO in paediatric renal allograft recipients. The results of the study showed that serum NO did not change significantly during acute rejection episodes. This is because in renal transplants there was an intra-renal production of NO, which contributes largely to the quantity of NO excreted in the urine of renal allograft recipients and decreases the levels of serum NO. Urinary NO decreased by 74% of the baseline value during acute rejection. During acute rejection there is also a reduction in GFR and tubular injury, which contributes to the diminished urinary NO excretion (Dedeoglu 1996). Takahashi measured serum nitrate in eight renal allograft recipients, and saw an increase in serum nitrate during acute rejection. Recipients receiving tacrolimus have markedly reduced serum nitrate levels in the early postoperative period when compared with those receiving cyclosporine (Takahashi 1998). Smith measured nitrite and nitrate in urine during acute rejection and urinary tract infection in 25 renal allograft transplant patients (11 cadaveric and 14 live). Urinary nitrate levels were higher during acute rejection, with no change in nitrite levels, while there was an increase in the levels of nitrite in urinary infection, with no change in urinary nitrate. This is because bacteria converts nitrate to nitrite in

patients with UTI (Smith 1996). Buben also used urinary nitric oxide/urinary creatinine (U-NOx/U-Cr) to diagnose acute rejection in human recipients receiving cadaveric kidney transplantation. He also used the same test in cyclosporine toxicity. He concluded that U-NOx/U-Cr decreases two days before acute rejection, but there were no changes in U-NOx/U-Cr in cyclosporine toxicity (Buben 2001). Mügge looked at the NO production during acute rejection in cardiac transplant patients. He measured urinary nitrate in the recipients, since it is nitrate that is excreted by the kidney. He used a single urinary test and did not interfere with the patients' diets. He examined the results with regards to urinary nitrate and urinary nitrate excretion (quotient urinary nitrate to urinary creatinine concentration). He found a significant increase in urinary nitrate and in urinary nitrate excretion in acute rejection. The degree of increase in urinary nitrate and in urinary nitrate excretion was in keeping with the severity of rejection. However, measuring urinary nitrate excretion was associated with a wide inter- and intra-individual variation, possibly due to dietary variations and immunosuppressive regimes (Mügge 1996). In heart transplant patients, Paul showed an increase in serum nitrate in acute rejection (Paul 1996). A similar study was conducted by Worrall, who reported that there was increased NO production during early acute rejection, and that NO production persisted throughout the unmodified rejection process in experimental heart transplants (Worrall 1997).

Silkoff measured the exhaled NO (eNO) in lung transplantation as a non-invasive marker. ENO is thought to increase during acute rejection but not with infection (Silkoff 1998). Others used NO as a marker of lung allograft dysfunction and chronic rejection (Gabbay 1999, Fisher 1998). This further supports the role of NO in acute rejection in solid organ transplantation.

Following the various studies providing evidence supporting the use of serum or urinary NO in diagnosing acute rejection, others exploited the role of NO in organ transplantation. For instance, Koyama used NO to predict the allograft outcome following acute rejection, stating that chronically rejected organs might produce NO due to an ongoing latent immunologic reaction. Recipients with chronic rejection who went on to lose the allograft were found to have twice the level of NO when compared with recipients without chronic rejection. Thus it was concluded that serum NO can predict the progress of chronic rejection and the fate of the allograft (Koyama 2000).

Castillo's work concentrated on the role of NO in the biological mechanisms during hyperacute vascular rejection in xenotransplantation (pig-to-dog). He reported a rise in serum nitrite in the renal vein five minutes after perfusion, therefore representing an increase in NO levels in the blood coming out of the graft, while serum nitrite in the renal artery remained unchanged. These two observations indicated that the source of NO production is the transplanted kidney and is unlikely to be of systemic origin. Therefore, the rise in serum nitrite in the renal vein is most likely to be due to hyperacute vascular rejection and not to surgical injury during the transplantation procedure. Castillo also found that iNOS activity in the renal parenchyma at 30 minutes post-perfusion was greater than that of cNOS. As a result of this, large amounts of NO are produced; this is cytotoxic and not a vasodilator. Endothelins are also produced, and may precipitate the intense vasoconstriction that is seen in hyperacute vascular rejection (Castillo 1996).

Immunosuppression and its Effects on NO and NOS

Immunosuppressive drugs have an effect on the microvascular endothelium. Most immunosuppressive drugs significantly enhance the production of NO, with the exception of tacrolimus (Trapp 2005). The quality and quantity of immunosuppression modify endothelial function and lead to a dose-dependent and oxygenation-state-related endothelial activation. MP and MMF induce minor changes in endothelial function compared with cyclosporin, rapamycin and tacrolimus (Trapp 2005). Rapamycin has also been found to decrease the expression of eNOS in the endothelial cells (Chen 2006). CNI inhibits iNOS expression and reduces NO production (Hämäläinen 2008, Tuñón 2003, Strestikova 2003, Strestikova 2001, Watarai 2004, Kim 2003, Dawson 1993). Tacrolimus inhibits iNOS at the transcriptional level in the macrophages, while down-regulation of iNOS expression by cyslosporin occurs post-transcriptionally (Strestikova 2001, Chen 2006). CNI nephrotoxicity is thought to be mediated by a vasconstrictive effect arising from the enhanced activity of vasoconstrictor endothelin and a decrease in production of intra-renal NO, a vasodilator (Watarai 2004). Rapamycin and mycophenolate mofetil (MMF) also inhibit NO production and iNOS expression and decrease the intracellular generation of ROS (Tuñón 20031, Lui 2001).

Corticosteroids

Coricosteroids inhibit T-cell proliferation and the expression of cytokines (IL-1, IL-2, IL-6, IFN-γ, TNF-α). They also form a heterodimeric complex which blocks the transcription of the IL-2 gene (Suthanthiran 1994). Corticosteroids can inhibit both iNOS and cNOS and inhibit the production of NO. Corticosteroids can inhibit iNOS

directly or indirectly (by induction of a protein that inhibits the enzyme iNOS) or inhibit endogenous cytokine synthesis. This could be the basis of the anti-inflammatory action of glucocorticoids (Radomski 1990, Huang 1995, Fuhua 1995) (Table 2.1).

Azathioprine

Azathioprine is an anti-metabolite and a derivative of mercaptopurine, and inhibits the entire purine biosynthetic pathway. Azathioprine causes inhibition of iNOS and this is the basis of its anti-inflammatory action. Mercaptopurine has no effect on iNOS (Moeslinger 2006, Grisham 1994).

Calcineurin Inhibitors (CNI)

Cyclosporin blocks calcineurin (playing the part of a signal transducer) and hence blocks T-cell activation and T-cell activation genes (IL-2, IL-2 receptors, c-myc, c-fos). It is a potent inhibitor of IL-2-stimulated T-cell proliferation and generation of cytotoxic T-cell lymphocytes (Suthanthiran 1994). Cyclosporin is known to inhibit NO production (Morris 2000, Zhang 1999) (Table 2.1).

Tacrolimus (FK506) inhibits calcineurin by binding it to a heterodimeric complex consisting of FK506 and an FK-binding protein, inhibiting the phosphatase activity of the calcineurin (Suthanthiran 1994). It also inhibits iNOS expression and NO

production (Hämäläinen 2008, Tuñón 2003, Strestikova 2003, Strestikova 2001, Watarai 2004, Kim 2003, Dawson 1993) (Table 2.1).

CNI reduces NO production by modifying the endothelial function or increasing the rate of decay of iNOS (Watarai 2004, Trapp 2005). Tacrolimus inhibits iNOS at the transcriptional level in the macrophages, while iNOS expression down-regulation by cyslosporin occurs post-transcriptionally (Strestikova 2001, Chen 2006). CNI nephrotoxicity is thought to be mediated by a vasconstrictive effect arising from the enhanced activity of vasoconstrictor endothelin and a decrease in production of intra-renal NO, a vasodilator (Watarai 2004).

Mycophenolate Mofetil (MMF)

MMF selectively inhibits the de novo pathway for purine biosynthesis and is converted into an active compound known as mycophenolic acid (MPA). MPA inhibits the proliferation of T- and B-lymphocytes by blocking inosine monophosphate dehydrogenase, the rate-limiting enzyme for purine synthesis during cell division. MPA also inhibits the generation of cytotoxic T-cells and antibody production. MPA has no effect on cNOS but it inhibits iNOS in a dose-dependent fashion. iNOS is a cofactor-dependent enzyme (Table 2.1). These cofactors include tetrahydrobiopterin (BH4), FAD, FMN and NADPH. BH4 is the rate limiting factor. iNOS depends on BH4 biosynthesis, and MPA inhibits biosynthesis of BH4, therefore suppressing cytokine-induced NO production (Senda 1995).

Sirolimus (Rapamycin)

Sirolimus prevents acute rejection by forming a complex with FKBP12, and then targets an intracellular protein called "mammalian target of rapamycin" (mTOR). mTOR is a serine-threonine kinase which is important in several signal transduction pathways that lead to stimulation of T-lymphocytes and other non-immune cells following cytokine or growth factor stimulation. It therefore inhibits the progression of the cell cycle and interferes with the proliferation of T-lymphocytes and other cells.

Sirolimus inhibits NO production by inhibiting up-regulation of iNOS at the mRNA levels (Tuñón 2003, Pham 1998) (Table 2.1).

Both rapamycin and mycophenolate mofetil (MMF) decrease the intracellular generation of ROS (Tuñón 20031, Lui 2001).

Immunosupressive Drug	**Mechanism of Action**	**Anticipated Effects**
Calcineurin inhibitors	Inhibit iNOS transcription Induce eNOS transcription Inhibit eNOS activity	May limit NO production
Corticosteroids	Inhibit iNOS transcription Induce eNOS transcription Increase eNOS activity	May limit NO production
Rapamycin	Inhibits iNOS transcription	May limit NO production
Mycophenolate mofetil	Limits BH4 availibility	May limit NO production

Table 2.1: Summary table of immunosuppressive effect on NO and NOS (Shah 2003).

Other Post Renal Transplant Complications

Urinary Tract Infection

This is the most common opportunistic infection in renal transplant recipients. Patients with urinary tract infection have an elevated urinary nitrite level when compared with healthy controls (Smith 1994). In large quantities, NO can modulate the inflammatory response. The source of NO can be from the inflammatory cells or from the infected uroepithelium (Poljakovie 2003). The NO released by the inflammatory cells is bactericidal while that released by the uroepithelium is involved in shedding the infected and damaged uroepithelium (Poljakovie 2003). During the course of UTI, large numbers of neutrophil are recruited by cytokines (IL-6 and IL-8), which are released by the infected epithelial cells in the urinary tract (Wheeler 1997). These urinary neutrophils show an increase in the activity of iNOS: the inflammatory response promoted by iNOS is responsible for the symptoms of UTI (dysuria and frequency). The production of large quantities of NO and ROS and the formation of peroxynitrite make iNOS bactericidal. The activity of iNOS is 43 times higher in neutrophils in urine from patients with UTI. There were no increases in the activity of eNOS in the urine from patients with UTI. Patients on immunosuppressive therapy showed no increase in iNOS activity despite persistent bacterial and fungal infection (Wheeler 1997, Smith 1994, Olsson 1998). eNOS could be responsible for an increase in NO production in the first hour of urinary sepsis, and iNOS activates four hours after infection. eNOS phosphorylation is involved in the early response to UTI, and

iNOS is involved in the later response since it requires transcription and translation (Kang 2004).

Peritoneal Dialysis Peritonitis

This is one of the complications that may occur in recipients on peritoneal dialysis post renal transplantation. Its symptomology is abdominal tenderness and pain and infected discharge from the peritoneal fluids. It is treated by surgical removal of the peritoneal catheter. It is a local inflammatory process which involves recruitment and extravasation of phagocitic leukocytes in the peritoneal cavity, release of cytokines and stimulation and up-regulation of iNOS, and large-scale production of NO. In animal experiments, injection of LPS into the peritoneal cavity is associated with increased systemic NO production (Olsson 1998, Kang 2004). Therefore, an increase is anticipated in serum NO during PD catheter peritonitis, as seen with other infections (Duranay 2007, Ni 2005, Davenport 2004, Plum 1999, Choi 1998, Yang 1996, Douma 1995).

Chapter 3

Renal Biopsy and Non-invasive Tests Used in Diagnosing Acute Rejection

Renal Biopsy

Renal core biopsy is the gold standard technique used in diagnosing acute rejection. It is performed whenever there is abnormal renal function in a renal transplant recipient. In a safe, experienced hand, it yields a 96.4% adequate histological diagnosis, and 97% of recipients are discharged within 48 hours, though it can be performed as a day case procedure (Hussain 2003). Haematuria is the commonest complication, occurring in 4% of recipients, while 1.5% encounter major complications requiring blood transfusion or other interventions (Tang 2002, Hussain 2003). Schwarz et al looked at 2170 renal biopsies; all were treated as outpatients using a 16- or 18-gauge needle yielding an adequate specimen. The reported complications were gross haematuria (3.5%), perirenal haematoma (2.5%), arterio-venous fistulae (7.3%) and a major complication requiring blood transfusion (1%). The hospitalisation rate was 1.9% and the four-hour recovery period post biopsy was adequate (Schwarz 2005). Preda et al reviewed 550 renal biopsies in transplanted and native kidneys. They had 95% adequate tissues. The complication rate in the transplanted kidney was 8.7%, while the major complication rate in the transplanted kidney was 2.9% – higher than that in the native kidney (2.4%). The major complications included death and loss of transplanted renal allograft. The minor

complication rate in the transplanted kidney was 5.8%, while this was higher in the native kidney biopsies (Preda 2003). Furness looked at 3613 biopsies and assessed them for major and minor complications, concluding in his study that the benefits outweigh the risk in recipients with stable graft function undergoing protocol biopsy, although he admits that they did not assess the direct benefits to the recipients (Furness 2003).

Besides complications, the other drawbacks of renal biopsy are:

- The need for an experienced ultrasonographer
- The need for a trained doctor to perform the procedure
- The need for an experienced histopathologest
- The time-consuming nature of the procedure
- The long reporting time following the procedure
- The requirement for hospitalisation
- The frequent delay in treatment
- The high cost of the procedure

Non-invasive Tests

Interest in non-invasive methods continues to attract attention because of the invasive nature and the complications associated with renal true-cut biopsy; hence, clinicians are hesitant in recommending protocol renal biopsies unless there is a strong clinical indication (Schwarz 2005). Avoiding renal biopsy means subclinical acute rejection is being missed, which in itself could have an effect on the long-term results of the renal allograft.

Acute rejection ignites a cascade of a complex network of cellular interactions, cytokine release and various gene and related surface protein (CD80, CD28, CTLA-4) up-regulation (Suthanthiran 1994). Cytokines also up-regulate the expression of adhesion molecules (a co-stimulator), facilitating the function of T-cells. These proteins are expressed in the peripheral blood cells, excreted in the urine or within the urinary cells or up-regulated within the graft tissues. Various techniques and methods have been developed and used to measure these proteins in blood, urine and graft tissues in an attempt to understand the mechanism of acute rejection, and to be simultaneously explored as a non-invasive method to diagnose acute rejection. The continuous advances of new technology in molecular biology have enabled us to investigate and study the complicated process of acute rejection, and the same new technology is being used as a non-invasive method to diagnose acute rejection.

Blood, tissues, urine and radiological tests have been used in searching for an alternative test to renal biopsy to diagnose acute rejection.

1. Blood

Peripheral blood is the first line of interest by researchers since it is easy to obtain and readily available. Simon looked at the "peripheral blood" gene expression measurement for perforin and granzyme B using a real-time polymerase chain reaction (PCR). During acute rejection there is a higher transcription rate of perforin and granzym B and a decrease in their expression by anti-rejection treatment. The best diagnostic results were obtained from samples taken on days 8-10, with a specificity of 90% and a sensitivity of 82% for perforin, and a specificity of 87% and sensitivity

of 72% for granzyme B (Simon 2003). The same group used another marker (soluble CD30) in an attempt to predict which of those recipients would be at risk of acute rejection (Pelzl 2003). Aquino also looked at perforin, granzym B and Fas ligand during subclinical acute rejection. He concluded that there is an increase in the expression of protein-encoding genes (which are involved in the cytolytic attack against the allograft) during subclinical acute rejection, confirming it as an active immune process potentially deleterious to renal allografts. However, there was no significant difference when compared with the results from normal functioning allografts (Aquino 2004).

Akalin et al used a high-density oligoarray technique, which is a quantitive test to study the expression of thousands of genes in the small tissue of the renal biopsy. Up-regulation of a specific gene transcripted during acute rejection was considered to be significant if transcript abundance increased four-fold or more relative to control biopsy samples. Of these transcripts, only four (human monokine induced by interferon-gamma, T-cell receptor active beta-chain protein, interleukin-2 stimulated phosphoprotein, and RING4 (a transporter involved in antigen presentation)) were consistently up-regulated in each acute rejection sample relative to at least two of three control biopsy samples (Akalin 2001). A flow cytometery technique was used to quantify production within peripheral blood mononuclear cells (PBMCs) of multiple cytokines. The technique is sensitive enough to detect differences but more research needs to be done in relation to acute rejection (Magee 2004).

2. Urine

The cellular content of urine may show some changes during acute rejection. Urine cytology may show an increase in the lymphocyte count (58%) when compared with neutrophils (35%) during acute rejection and this can be used as a simple diagnostic test (Tatomirovic 2003). The measurement of the mRNA encoding cytotoxic proteins (perforin and granzym B) in the urine cells using a polymerase chain reaction (PCR) has a good level of sensitivity and specificity and can offer a non-invasive means to diagnose acute rejection (Suthanthiran 1994). A large number of expressed proteins excreted in the urine cells were measured during acute rejection and most studies showed up-regulation of proteins such as chemokines [CXCR3-binding chemokines, monokines (Mig/CXCL9), IFN-gamma-induced proteins of 10 kDa (IP-10/CXCL10), IFN-inducible T-cell chemoattractants (I-TAC/CXCL11), CD103, CD103 mRNA, adhesion molecules (sICAM-1, sVCAM-1), and complement degradation products (C4d) (Ding 2003, Lederer 2003, Hu 2004).

3. Radiological Tests

Haemodynamic changes occurring within the rejected renal allograft can be picked up radiologically, so duplex ultrasound has been used to diagnose acute rejection. It was reported by Kahraman that a high resistive index (RI) and pulsatile index (PI) would indicate impaired allograft function. Performing duplex at a repeated interval may predict early and long renal dysfunction (Kahraman 2004). Quresh used an isotopic renogram and showed that during renal perfusion an increased uptake would indicate acute rejection, advocating anti-rejection treatment (Qureshi 2005). The latest advances in radiological techniques have also been used, for instance magnetic

resonance imaging (MRI) has been used to determine the feasibility of using blood oxygen level to differentiate between acute tubular necrosis (ATN), acute rejection, and normal function. It was found that the R2* measurements (which measure the rate of signal loss in a specific region and are related to the amount of deoxyhemoglobin present) in the medullary regions of transplanted kidneys with acute rejection were significantly lower than those in normally functioning transplants or transplants with ATN. These results suggested that marked changes in intra-renal oxygenation occur during acute transplant rejection (Sadowski 2005).

Chapter 4

Methods of Nitric Oxide Analysis

NO analysis and measurement is achieved using various methods and techniques. The advantage of an automated analysis is a short analysis time, and high selectivity which avoids both false positive and false negative results due to interfering substances such as phosphate and citrate in physiological concentrations, which could be found in plasma and urine. Urine in particular is a complex sample, and removing interfering substances could be associated with the loss of a small amount of nitrate (Green 1982).

A limitation inherent to all techniques used to measure NO production is that they do not discriminate between the various isoforms of NOS (eNOS, nNOS, iNOS). Within renal diseases, iNOS expression may vary at different stages of the disease and this could reflect accordingly on the NO production and plasma nitrite and nitrate levels (Wever 1999).

NO is an unstable molecule and can be converted to nitrite, nitrate and other intermediaries (nitrosoglutathione, nitrosoalbumin and nitrosohaemoglobin; all converted to nitrate). Nitrate and nitrite, however, are stable molecules and can be used for quantitative measurement (Tsikas 2005, Tsikas 2006). Their concentrations in biological fluids are less prone to changes caused by alterations in metabolism or decay during sample collection or storage. It is worth noting that endogenous nitrate generation is not limited by the L-arginine-NO pathway, and therefore nitrate level is not a steady-state situation. Nitrate is an inactive metabolite and its concentration is constant (Baylis 1998). Nitrite concentration in humans and animals reflects endothelial-dependent NO synthesis (Tsikas 2005).

Based on an automated analysis system, nitrite and nitrate concentration were measured in various biological fluids (thousands of samples of human and rat urine, human saliva and plasma) (Table 4.1) (Green 1982).

Biological fluid	**Nitrate (NO_3^-) µmol**	**Nitrite (NO_2^-) µmol**
Urine	250 – 2000	Not determined
Saliva	200 – 600	30 – 210
Plasma	15 – 60	Not determined
Gastric juice	50 – 85	0.40 – 60
Milk	20 - 30	Not Determined

Table 4.1:The range of nitrite and nitrate concentration in humans.

However, it is worth noting that nitrite and nitrate are measured by various techniques and the outcome may vary greatly (Table 4.2).

Method	Nitrate	Nitrite	NO2+NO3	Reference
Batch methods:				
Griess	4 – 45	1 – 13		Moshage et al (143)
Fluorometry			34	Marzinzig et al (132)
Instrumental Methods				
Griess (automated)	15 – 60	N.R.		Green et al (126)
GC-MS	38	1.8		Tsikas et al (127)
HPLC-UV	26	1.3		Wennmalam et al (131)
CZE	52	3.3		Ueda et al (146)
HPLC-UV			1.1	El Menyawi et al (133)

Table 4. 2: Various methods of NO analysis. NR: Not Reported; GC-MS: Gas Chromatography-Mass Spectrometry; HPLC-UV: High Performance Liquid Chromatography-UltraViolet; CZE: Capillary Zone Electrophersis.

Nitrite and nitrate in the body fluids originate from dietary intake, inhalation of NO (minor source) and endogenous NO synthesis.

Dietary nitrate and nitrite is mainly nitrate found in green vegetables and in some water, and nitrite is used as a preservative in processed foods. Therefore, nitrite and nitrate intake is variable between individuals. The excretion of oral nitrate is via faeces (1-3 %) and urine (60-70 %), and the rest (29-39 %) is lost within the body through bacterial degradation of NOx in the lower gastrointestinal tract (Baylis 1998). Nitrate is actively transported into the salivary gland and is then secreted in saliva. Salivary nitrate is converted into nitrite by micro-organisms on the tongue, and once it reaches the stomach some of the nitrite is converted to NO within the acidic environment. The NOx that passes through the stomach may be reabsorbed into the gastrointestinal tract (entero-salivary cycling), lost in the faeces or degraded by bacteria. NOx is also lost via sweat and exhaled air (Baylis 1998, Blum 1998, Schmidt 1999).

Urinary nitrate cannot be used as a quantitative measurement of endogenous NOx production in uncontrolled NOx dietary intake (Baylis 1998). If the oral intake of NOx is high, the urinary NOx excretion is usually less than the intake; therefore the net NOx synthesis cannot be demonstrated (Green 1981). To achieve a meaningful and accurate urinary NOx measurement, samples must be obtained under controlled low nitrate intake. In the case of blood samples, it is recommended that the patient should fast for 12 hours prior to the sample being taken, and the sample reflects mainly endogenous NO production. If blood samples are collected randomly without dietary restriction, the plasma nitrate measurement could be variable. Plasma nitrate peaks 45 minutes after oral intake and remains high for several hours. However, balance is achieved within 24 to 48 hours after a low-nitrate diet, and urine and blood samples can then be collected. It would take four to five half-lives for nitrate to reach steady-state (Baylis 1998).

The other factors that may have an impact on urinary or plasma nitrate levels are atmospheric pollution, heavy exercise, phosphate, citrate, nitroso-containing substances, acid pH and Vitamin C (Green 1982). Heavy exercise causes increased NO generation and NOx excretion via sweat; therefore activities should be limited before obtaining a sample, to obtain a true estimate of basal production (Shen 1995). Drug therapy such as isosorbide dinitrate may also affect plasma NO, particularly in NO donors, however it contributes little to the NOx levels (Baylis 1998).

Thus, the sources of nitrate and nitrite can be either endogenous or exogenous. Nitrate and nitrite results endogenously from a breakdown of NO, and exogenously from diet. Therefore, measurement of plasma nitrite and nitrate cannot differentiate the source of nitrite and nitrate; hence, intake of nitrite and nitrate must be controlled when using nitrite and nitrate to measure NO production (Schmidt 1999).

In humans, NO oxidizes in the plasma to nitrite:nitrate in a ratio of 1:5. However, this depends on the oxygenation of red cells (Hb). In venous blood, NO exists more as nitrite because of lower levels of oxygenation, and it is formed by autoxidation in the plasma. In arterial blood, NO exists more as nitrate and it is oxidised by OxyHb. Oxidation by OxyHb is 450 times faster than autoxidation. Furthermore, oxidation of nitrite to nitrate occurs in red cells. Nitrite and nitrate are stable for 60 minutes if blood samples are stored on ice and plasma incubated at 37°C (Tsikas 2006, Tsikas 2005), and both are stable for several months if stored at -18°C (Wennmalm 1993). The changes in the levels of endogenous endothelial NO production arising from causes such as pharmacological drugs are best reflected by serum nitrite, which can be used as quantitative indices of NO production, while urinary nitrate is a more reliable indication of "whole body NO synthesis" under basal or stress situations (Tsikas 2006, Tsikas 2005) (Figure 4.1). If the intake of NO is increased by NO inhalation there is an associated increase in the levels of nitrate and MetHb, but not in the level of nitrite. The inhaled NO is taken up by the red cells and converted to nitrate and MetHb. This is the major metabolic pathway for endogenously-formed NO (Tsikas 2005, Wennmalm 1993). Incubating arterialised blood with nitrite results in dose-dependent increases in nitrate and MetHb formation, and incubating nitrite with venous blood increases the formation of nitrate, MetHb and nitrosohaemoglobin (HbNO), which is converted to nitrate (Wennmalm 1992, Tsikas 2005).

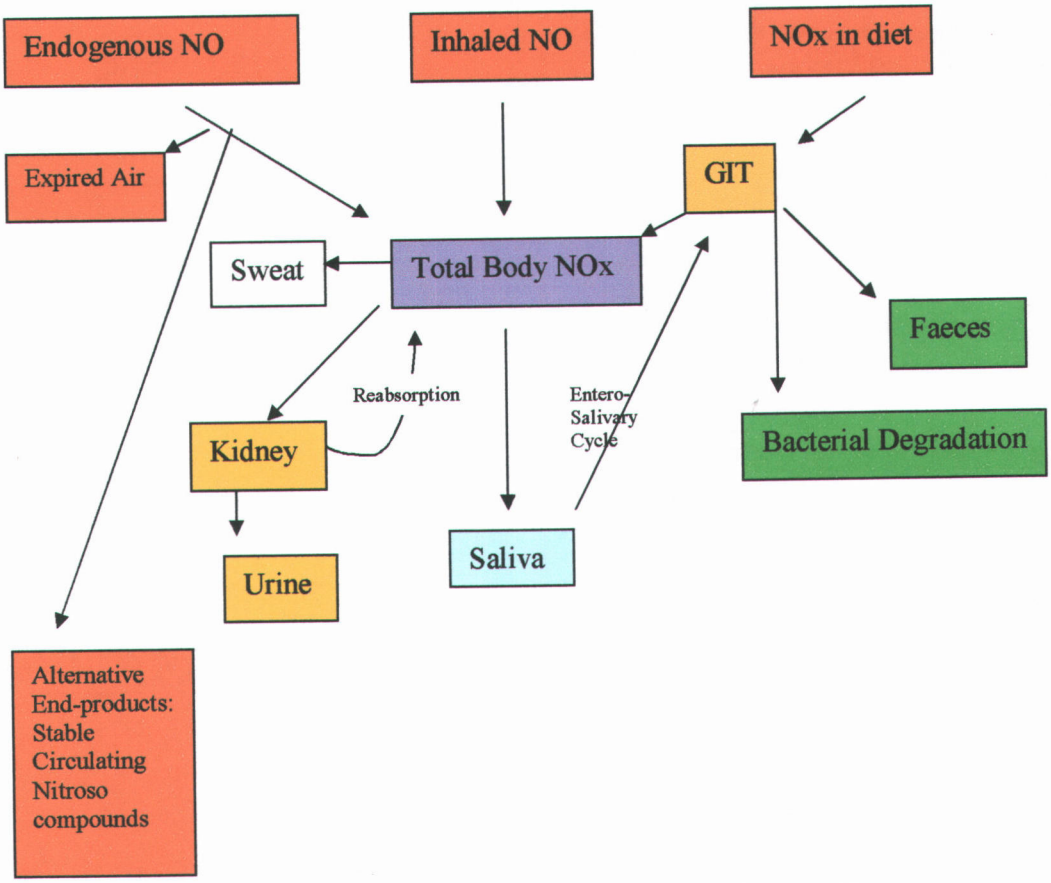


Figure 4.1: NO absorption, secretion and metabolism (copied from Baylis 1998).

Measurement of plasma NOx reflects the level of renal function, plasma volume and indirect index of systemic NOx production. Measurement of urinary NOx is uninformative or not a true quantitative indicator of NO production, due to a number of reasons:

1. The proximal tubule contributes to the plasma nitrate by its substantial tubular reabsorption of NOx; therefore a urinary sample is not informative about either renal or total systemic NO production.

2, Acute changes in the urinary NOx could be due to epithelial handling of NOx or NO generation (Suto 1995).

3. NOx can be exerted by other routes; faeces, exhaled air (Westfelt 1995).

4. NO can be converted into other end-products.

5. NOx cannot be eliminated completely from the diet (Baylis 1998).

The acute changes in urinary excretion of nitrite and nitrate do not necessarily predict renal vascular NO production. Under normal conditions nitrite and nitrate are extensively reabsorbed and generated by the proximal tubule epithelial cells (Suto 1995).

The endothelial cells, neuronal sources, epithelial tissues or all these in combination generate NO in very small amounts with a large impact on the haemodynamics (haemodynamically active NO), but this does not contribute a significant amount to the total NOx level. Increased production of haemodynamically active NO is trivial and not detectable in the circulating systemic concentration of NO. The increase in the total body NOx generation does not reflect biologically active NO, but it reflects an increase in the production of NO during pathological states such as immunological stimulation (Baylis 1998, suthanthiran 1994).

Methods of Quantatitive Measurement of NO in Biological Fluids

NO end-products can be measured by various methods which include:

1. Colorimeteric spectrophotometric
2. Ultraviolet spectrophotometric
3. Fluorometric assays
4. Chemiluminescence
5. High Performance Liquid Chromatography (HPLC)

6. Capillary Electrophoresis (CE)

7. Gas Chromatography (GC)

8. Gas Chromatography-Mass Spectrometry (GC-MS)

Each method gives a different serum and urinary nitrite and nitrate value in humans. The reasons for this could be dietary intake, methodological problems or pre-analytical factors. The pre-analytical factors depend on the NO end-products measured. Although nitrite and nitrate are stable for 60 minutes in blood samples when placed on ice, and stable in plasma when stored at 37°C, nitrite has a short life in whole blood samples since it is oxidised to nitrate. Laboratory materials (pipettes, pipette tips, monovettes) may contain nitrite and nitrate. Anticoagulants used in blood sample monovettes such as EDTA also contain nitrite and nitrate (Tsikas 2005).

The principle behind measuring nitrite and nitrate in serum and urine is based on two types of reaction; derivatization reactions and diazotization reactions.

Derivatization Reactions

In a derivatization reaction, nitrite or nitrate is converted to derivatives that can absorb light in the ultraviolet or fluorescent range. GC, GC-MS and chemiluminescence are based on derivatization reactions. In GC and GC-MS, volatile and thermally stable derivatives are generated. GC-MS is a commonly-used test to determine nitrite and nitrate based on two derivatization reactions:

First reaction: nitration reaction where nitrite is oxidised to nitrate and then forms a nitroaromatic derivative. The drawbacks of nitration reaction are:

1. In the presence of high levels of nitrate such as in plasma, serum and urine, nitrite cannot be accurately determined.

2. Derivatization reactions cannot measure nitrite and nitrate simultaneously.

Second reaction: nitrite reacts with pentafluorobenzyl bromide (PFB Bromide) to form nitro-PFB and then nitrate reacts with PFB to form nitric acid ester PFB. This is the sole reaction, which can measure both nitrite and nitrate simultaneously. Furthermore, samples do not need to be pretreated (Tsikas 2006, Tsikas 2005).

Diazotization Reactions

In diazotization reaction, sulfanilic acid reacts with nitrite to form a diazonium cation, which reacts with α-naphthylamine to form a diazo compound that absorbs light around 540 nm. This reacton is specific for nitrite. This method can be carried out in batch or automated assays. This is the basis of the Griess reaction. There are a few drawbacks associated with diazotization reactions:

1. Unspecificity of the results in batch assays, hence automated tests are more reliable because they eliminate interfering substances.

2. Incomplete reduction of nitrate to nitrite by the nitrate reductase.

3. Reduction of nitrate to nitrite may vary from sample to sample.

4. Calibration curves are used to quantitatively determine nitrite and nitrate in the sample. Incomplete and variable reduction of nitrate to nitrite may result in different calibration curves; therefore quantification methods based on calibration curves may give different values.

Greiss Reaction

In 1879, Greiss described the diazotiation of an aryl amine by nitrite and coupling of the product to form an azochromophore (Green 1982). This assay determines NO based on the enzymatic conversion of nitrate to nitrite by nitrate reductase. The reaction is followed by a colorimetric detection of nitrite as an azo dye product of the Griess reaction. The Griess reaction is based on the two-step diazotization reaction in which acidified NO2- produces a nitrosating agent which reacts with sulfanilic acid to produce the diazonium ion. This ion is then coupled to N-(1-naphthyl) ethylenediamine to form the chromophoric azo-derivative which absorbs light at 540 nm (Figure 4.2).

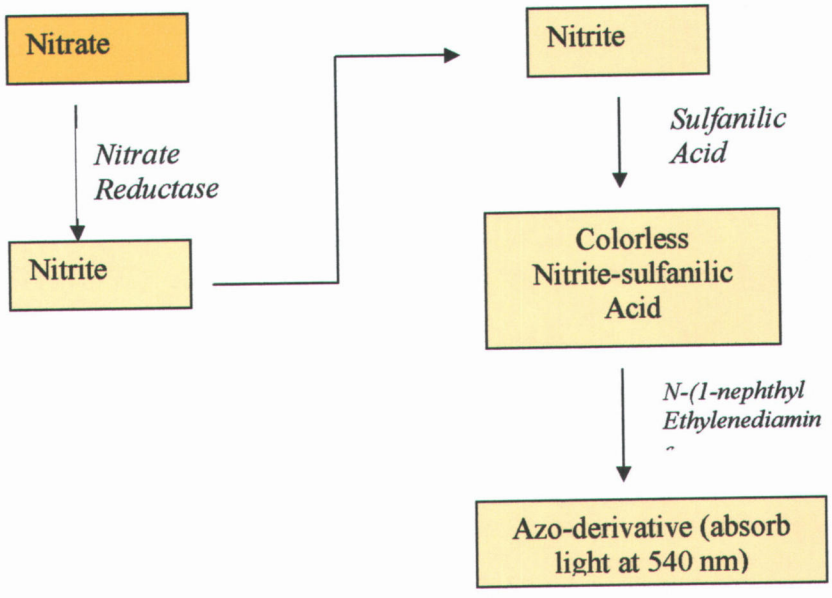


Figure 4.2: Greiss Reaction

The advantages of the Greiss reaction are that it is simple to perform, cheap, precise and accurate (Tsikas 1997). The disadvantages are:

1. Interference: Since it is conducted in an acidic reaction, this can lead to interference by reduced thiols such as cysteine, N-acetylcysteine and glutathione, which then reduce the absorption to 540 nm. Reduced proteins in the acidic media may also reduce absorption.

2. Nitrite and nitrate cannot be measured directly in the plasma but the reaction has to be performed in the ultrafiltrates (Tsikas 1997).

3. Nitrite cannot be measured, firstly because reduced thiols (cysteine, N-acetylcysteine and glutathione) interfere with nitrite determination, and secondly because nitrite may react with sulfhydryl groups and other plasma proteins and plasma constituents (Tsikas 1997).

4. Recoverabiltiy of nitrate after reduction to nitrite is unknown and it is difficult to standardise (Tsikas 1997).

5. During the conduction of the Greiss reaction to measure urinary nitrate, the urinary sample is usually dilated (in our study it was in the order of 1:400). As a result this may dilate the nitrate and give inaccurate results (Tsikas 1997).

Other Methods Used to Measure NO

NO can be measured using a number of other techniques, including immunochemical (Albrecht 2000), oscillopolarography (Lu 1999), fluorometric assay (Tsikas 2006), high performance liquid chromatography (Tsikas 2006, Tsikas 2005), near-infrared spectroscopy (Ohdan 1995), and gas chromatography-mass spectrometry (Tsikas 1997, Tsikas 1998).

Fluorometric assay (Tsikas 2006) is one of the most common assays used. It is a derivatization reaction, and there are a number of modifications that can be made to the test itself to improve speed and sensitivity. It can measure NO metabolic products in serum, plasma and urine. It is 50-100 times more sensitive than the Greiss reaction. It can also be used to monitor the kinetics of nitrite production from cells treated with LPS or endotoxins. However, fluorometric assay is too sensitive for routine measurement because of its limited linear range.

High performance liquid chromatography (HPLC) is another test used for NO measurement. It is easy to apply and can measure NO in water, urine and serum. HPLC can detect both nitrite and nitrate simultaneously. The test is very sensitive, rapid and accurate but it is time-consuming and needs a large and expensive setup. HPLC is not practical for routine use (Tsikas 2006, Tsikas 2005).

Near-infrared spectroscopy is rapid, simple and less invasive. The results are not affected by the kidney functions (Ohdan 1995). Gas Chromatography-Mass Spectrometry is also precise and accurate, and is free of interference. This is because nitrite can be measured in the plasma without interference from thiol groups, sulfhydryl groups, plasma proteins (S-nitroso compounds) or plasma constituents (free amino acids such as tyrosine and tryptophan); there is therefore a good recovery of nitrite and nitrate. In this method a stable isotope-labeled analogue of nitrite and nitrate can be used as a reliable internal standard (Tsikas 1997, Tsikas 1998).

Most methods discussed, and others not mentioned, can be suitable for detecting the products of NO with a similar degree of accuracy, but each assay has its own limitations.

Other NO-related Markers used as Diagnosed Tests

Besides NO, other substances have been used as markers to diagnose acute rejection. These include iNOS (Albrecht 2002, Joles 2002, Albrecht 2000) and cGMP (Smith 2000, Castillo 1996). Measurement of iNOS is a complex procedure; it can be measured in cells or biopsies. Other substances such as cGMP, citrulline and NO by-products have been found to be affected by other factors and are less reliable than NO.

Chapter 5

Methods

Population

Ethical committee approval was obtained. Verbal and written consent was also obtained from all patients on admission. All patients receiving their first renal transplant were included in our study. The only exclusion criterion was the presence of a previous renal transplant. Recruitment started on the 1st July 2002 and concluded in July 2003 in the University Hospital of Wales where we recruited 50 renal allograft recipients (mean age 46 ± 1.93 years, 35 males and 15 females). Twelve recipients (24%) were Live-related Allograft Donors (LAD) and 38 recipients (76%) were Cadaveric Allograft Donors (CAD). Transplant nephrectomy was performed in 3 recipients; 2 recipients had renal vein thrombosis and 1 recipient had life threatening intra-operative bleeding from the renal allograft. These were excluded from further follow-up. Out of the 47 recipients, 3 recipients had previous failed renal tranplants, of which 2 recipients (6.4%) were receiving their second transplants and 1 recipient (2.1%) was receiving a third transplant. We continued to collect blood and urine

samples and followed them up for 3 months. The remaining 44 recipients (91.5%) were primary renal transplant patients.

Recipients were on different types of Renal Replacement Therapy (RRT) in the pre-transplant period, as shown in Table 5.1.

Type of Renal Replacement Therapy (RRT)	Number of Recipents	Percent
Haemodialysis	20	42.6
Peritoneal Dialysis	14	29.8
Pre-dialysis	10	21.3
Haemodialysis and Peritoneal Dialysis	3	6.4
Total	47	

Table 5.1: The type of renal replacement therapy the recipients received prior to renal allograft transplantation.

Follow-up Policy

Recipients were followed up for 3 months. During follow-up, we collected the following data:

1. Renal function test
2. Urine culture and sensitivity
3. Tacrolimus or cyclosporin trough levels
4. Other complications (PD peritonitis, UTI infection)

During this 12-week period, the following post-renal transplant complications were observed:

1. Acute rejection proved by renal biopsy confirming acute rejection according to Banff 97 classification.
2. Urinary tract infection defined as positive urinary culture or colonies count >100,000 per HPF or WCC count >100.
3. Tacrolimus and cyclosporin toxicity. Tacrolimus toxicity defined as serum tacrolimus > 15mg/dl and cyclosporin toxicity defined as serum cyclosporine >250 mg/dl.
4. Increased serum creatinine >10% defined as increase of serum creatinine >10% compared to previous levels of serum creatinine.
5. Peritoneal dialysis catheter peritonitis diagnosed from the presence of two of three criteria: signs and symptoms of peritonitis, positive peritoneal culture or microscopy showing WCC >100 cells or >50% neutrophils.

Recipient Demography and Database

We developed two systems for data collection: the first system was to collect the demographic information of the recipients and the donors and the second system was devised to collect the laboratory, clinical, sample and biochemistry data.

First System

We collected data on all recipients and donors (Table 5.2 and Table 5.3), which included the following information:

1. Study number
2. Age of recipient
3. Gender of recipient
4. Cause of ESRF
5. Type of dialysis: Haemodialysis or CAPD
6. Duration of dialysis
7. Previous transplants
8. Total urine output before transplant
9. Date of transplant
10. Type of transplant: live or cadaveric
11. Age of donor
12. Gender of donor
13. Tissue typing
14. Cause of death of donor
15. Recipient tissue typing cross match
16. Kidney side recipient
17. CMV status; donor CMV status
18. Cold ischemia time (hours:mins)
19. Pre-transplant medication

Second System

A database was created to collect all the laboratory, clinical, sample and biochemical data. Laboratory data included number of blood and urine samples and site of storage. Clinical data included number of acute rejections, date of rejections, histology of biopsies, treatment administered for acute rejections, number of episodes and dates of urinary tract infections and results of culture and sensitivity and microscopy. Biochemical data included serum creatinine and tacrolimus and CsA trough level

Data entered on SPSS spread sheath as shown in Appendix 1 and 2

Causes of ESRF in Recipients	No.
APKD	7
Unknown	5
Hypertension	5
IgA Nephropathy	5
Diabetic Nephropathy	4
FSGS	2
Hypoplastic Kidneys	2
Glomerulonephritis	1
Nephrotic syndrome	1
Acute nephritis	1
Reflux Uropathy	1
Renal Calculi	1
HSP	1
Congenital	1
Rhabdomyolysis	1
Goodpasture Syndrome	1
Fitchner`s Syndrome	1
Missing	7

Table 5.2: Causes of ESRF in recipients. APKD: Adult Polycystic Kidney Disease; FSGS: Focal Segmental Glomerulonephritis; HSP: Henoch Schonlein Purpura.

Demography of Recipients and Donors	No.
Number of patients	47
Age	46 ± 14
Sex	
Males:Females	34:13
Transplant	
Primary	44
Secondary	2
Tertiary	1
CAD	35
LAD	12
Ischaemic Time	
CAD	19 ± 1.1 hrs
LAD	5 ± 1 hrs
Donor Age	44 ± 14
Donor Sex	
Males:Females	27:20
Causes of Donor Death	
SAH	16
ICH	10
Head injury	3
Others	6

Table 5.3: Demography of recipients. CAD: Cadaveric Allograft Donor; LAD: Live Allograft Donor; SAH: Subarachnoid Haemorrhage; ICH: Intracerebral Haemorrhage.

Immunosuppression

Triple therapy (tacrolimus, azathioprine and prednisolone) was received by 34 recipients: 3 recipients received low dose tacrolimus, sirolimus and predniosIone; 7 recipients were recruited in the ELiTe Symphony Trial (gp-A: 2 received cyclosporine, MMF and prednisolone, gp-B: 1 received zenapax (5 doses over 8 weeks) low dose tacrolimus, MMF, prednisolone, gp-C: 2 received zenapax (5 doses over 8 weeks) low dose sirolmus, MMF, prednisolone, gp-D: 2 received zenapax (5 doses over 8 weeks) low dose cyclopsorin, MMF, prednisolone).

The 3 recipients with previous failed renal allograft transplants were given tacrolimus, MMF and prednisolone.

Management of Acute Rejection

Biopsy-proven acute rejection was treated with three doses of methyl prednisolone. Persistent acute rejection proven by repeat biopsy and failure to respond to steroid therapy was treated with OKT3. Out of the 19 recipients with biopsy-proven acute rejection, 3 recipients had persistent acute rejection and required OKT3 therapy.

Blood Sample Collection

A total of 1178 blood samples were collected over the period of 12 weeks. **Blood samples were collected in the morning before the recipients broke their fast.**

Following discharge the recipients were instructed to remain fasting until the blood sample was obtained.

On admission, 10 mls of bloodwere collected from the patients (pre-transplant/day 0).

Blood and urine samples were also collected on the first day post renal transplant. Thereafter, samples were collected on alternate days. Once discharged, the recipients were followed up and blood and urine were collected at every outpatient visit. Any associated complications were noted. If the recipient was admitted then samples were collected on alternate days.

Blood samples were collected in blood bottles that contained lithium-heparin as an anti-coagulant (Sarstedt Monovette®) and urine samples were collected in sterile empty universal containers. Blood and urine samples were placed immediately on ice and transported to the laboratory and processed within two-four hours following collection.

Blood samples were centrifuged at 800 G for 20 minutes and the serum collected and then stored at -80°C to be analysed at a later date.

rine sample was sent to be tested for culture and sensitivity, which is an in-house policy for all recipients. During the first week, urine was collected from the catheter (urinary catheter are taken out 7 days post renal transplantation) and subsequently MSU whenever possible. Another sample of urine (5 mls) was sent to be measured for urinary creatinine.

Blood Sample Collections from Healthy Volunteers

We recruited 10 healthy volunteers: 4 doctors, 2 laboratory technicians and 4 staff nurses. The group included 5 males and 5 females with a mean age of 45 years. We collected blood samples without any dietary restrictions. The blood samples were processed as mentioned above.

Renal Biopsies

Ultrasound-guided core renal biopsies were carried out by transplant registrars. The biopsies were conducted in the presence of a histopathology technician. The biopsy specimen was fixed in formalin, embedded in buffer paraffin and microwaved, and stained with haematoxylin and eosin, periodic acid-schiff and aldehyde fushsin stain. The specimens were reviewed and classified with the use of Banff 97 classification by a single pathologist (DG) who did not know the result of our NO analysis.

In our study, 30 recipients (mean ± SD age 45 ± 2.07, 24 men and 6 women) underwent a core needle biopsy, yielding 68 biopsies of which 41 showed acute rejection in 19 recipients (mean age 42 ± 1.72, 15 male and 3 female) and 27 showed no rejection in 13 recipients (mean age 42± 2.68, 9 male and 3 female).

The 41 acute rejection-proven-biopsies were classified using Banff 97 as follows:

1. Borderline rejection	16
2. Tubulointerstitial with moderate tubulitis (IA)	5
3. Tubulointerstitial with severe tubulitis (IB)	6
4. Cellular with mild to moderate arteritis (IIA)	10
5. Cellular with severe arteritis (IIB)	1
6. Severe vascular rejection (III)	1

7. Antibody mediated acute rejection	2
Total	**41**

Of the 16 biopsies which showed borderline changes for acute rejection, only 11 of those were treated with methyl prednisolone; 5 episodes were not treated.

The other 27 biopsies showed the following histology:

1. Normal	10
2. Acute tubular necrosis	9
3. Recurrence (FSGS)	1
4. Diffuse non-specific tubular changes	1
5. Infarcted tissues	1
6. Non-specific focal inflammation.	1
7. Atrophic changes	1
8. Recovering from acute rejection	2
9. Suspicoun of acute rejection	1
Total	**27**

Nitric Oxide Colorimetric Assay

Instruments

The instruments used to prepare the reagent and perform the test are:

1. Centrifuge

2. Micro-centrifuge

3. Pipettes

4. Tubes used to store the specimens

5. Tubes used to dilate the specimens and perform the test

6. Incubator: two types; small and large

7. Spectrophotometery

8. Software used to read the assay

9. Printer

10. Refrigerator used to store the specimens at -80°C

Principle of the Assay

The assay determines NO based on the enzymatic conversion of nitrate to nitrite by nitrate reductase. The reaction is followed by colorimetric detection of nitrite as an azo dye product of the Greiss reaction (Figure 5.1).

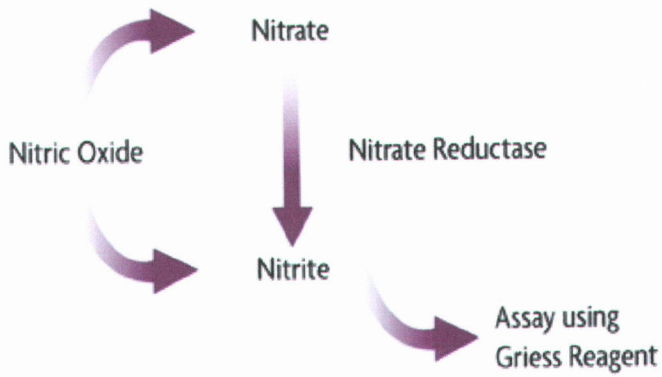


Figure 5.1: NO end-products and Greiss reaction

On the day of the analysis, the samples (both blood and urine) were thawed at room temperature. The plasma sample was diluted two-fold and the urine diluted five-fold.

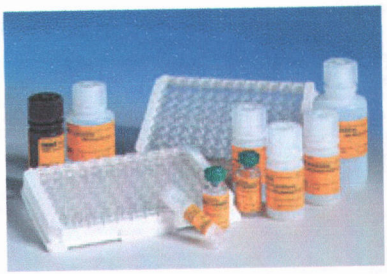
The blood sample was diluted two-fold with a reaction buffer, vortexed, and 400 µmol of the prepared mixture pippetted out into a micro-centrifuge filter. We used Ultrafree®-MC microcentrifuge filters, and NMWL 10,000 Dalton (supplied by Sigma-Aldrich); this was to deproteinise the serum to avoid artefact. The sample was centrifuged at 8000 G for 40 minutes, yielding the 100 microml of serum and 300 microml of urine that were used in nitric oxide assay. For every new assay a new sample was thawed, diluted and filtered.

All of the NO assays were conducted under the same ambient conditions and the same steps and procedures were used. The results of the plasma assay were multiplied by two and the urine assay multiplied by five to correct for the dilution factor. The tests were carried out in duplicate; it was decided that if the results were not identical or differed by more than 20%, the assay of that sample was repeated, however this did not prove necessary and none of the tests were repeated. All of the samples were repeated four times for inter-assay and intra-assay accuracy.

Reagents (R&D SYSTEM)

The nitric assay involves the following reagents:

1. Nitrate Reductase
2. Nitrate Reductase Storage Buffer
3. NADH
4. Nitrite Standard



5. Nitrate Standard

6. Reaction Buffer Concentrate

7. Griess Reagent I

8. Griess Reagent II

Bring all reagents (except the Nitrate Reductase) to room temperature before use. Use deionized or distilled water when reconstituting or diluting the reagents, in order to avoid nitrite/nitrate contamination.

Reaction Buffer

Dilute 30 mL of Reaction Buffer Concentrate (10X) into deionized or distilled water to prepare 300 mL of Reaction Buffer (1X).

NADH Reagent Reconstitution and Dilution:

Reconstitute the NADH with 1.0 mL deionized or distilled water. Allow the NADH to sit for 3 minutes with gentle agitation prior to use. Keep tightly capped on ice for the duration of the assay. Immediately before use, dilute 900 mL of NADH with 1.8 mL of deionized or distilled water. Keep on ice for the duration of the assay.

Nitrate Reductase Reconstitution and Dilution

Reconstitute the Nitrate Reductase with 1 mL Nitrate Reductase Storage Buffer. Vortex vigorously and allow to sit for 15 minutes at room temperature. Vortex again and allow to sit for an additional 15 minutes at room temperature. Vortex again. Keep on ice for the duration of the assay.

Immediately before use, dilute the Nitrate Reductase using the below equation.

Determine the number of standard and sample wells to be used (do not include blanks). All samples and standards should be assayed in duplicate. Use the following formula to dilute the Nitrate Reductase reagent:

a. Nitrate Reductase (_L) = (# wells + 2) x 10 _L.

b. Reaction Buffer (_L) = volume from step a x 1.5.

c. Add volumes from steps a and b to a tube, vortex.

d. Place on ice and use within 15 minutes of dilution.

Since we used the whole kit to measure the nitrate and ceased to measure the nitrite, we contacted the manufacture and a new formula was supplied to dilute nitrate reductase; this could then be used for both trays supplied in the kit. The new formula is as follows:

a. Nitrate Reductase (_L) = (# wells + 2) x **5** _L.

b. Reaction Buffer (_L) = volume from step a x **4**.

c. Add volumes from steps a and b to a tube, vortex.

d. Place on ice and use within 15 minutes of dilution.

Preparation of Nitrite and Nitrate Standards

Figure 5.2:

Nitrite Standard - Pipette 900 µL of Reaction Buffer (1X) into the 200 µmol/L tube. Pipette 500 µL of Reaction Buffer (1X) into the remaining tubes. Use the 2000 µmol/L standard stock to produce a dilution series (below). Mix each tube thoroughly and change pipette tips between each transfer. The 200 µmol/L standard serves as the high standard and the Reaction Buffer (1X) serves as the zero standard (0 µmol/L).

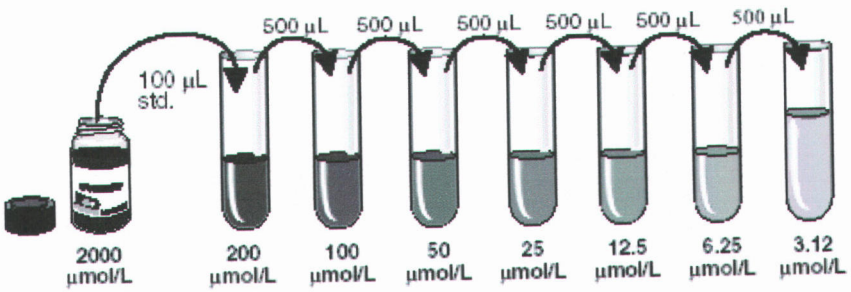
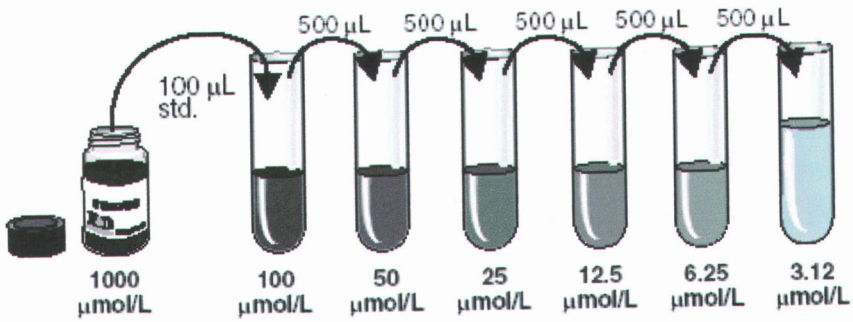


Figure 5.3:

Nitrate Standard - Pipette 900 µL of Reaction Buffer (1X) into the 100 µmol/L tube. Pipette 500 µL of Reaction Buffer (1X) into the remaining tubes. Use the 1000 µmol/L standard stock to produce a dilution series (below). Mix each tube thoroughly and change pipette tips between each transfer. The 100 µmol/L standard serves as the high standard and the Reaction Buffer (1X) serves as the zero standard (0 µmol/L).



Nitrite Assay Procedure

Bring reagents to room temperature before use. It is recommended that all samples and standards be assayed in duplicate. This assay procedure measures the concentration of endogenous nitrite present in the sample.

1. Prepare all reagents, working standards and samples.

2. Remove excess microplate strips from the plate frame, return them to the storage bag.

3. Add 200 µl of Reaction Buffer to the blank wells.

4. Add 50 µl of Reaction Buffer to the zero standard wells.

5. Add 50 µl of Nitrite Standard or sample to the remaining wells.

6. Add 50 µl of Reaction Buffer to all standard and sample wells.

7. Add 50 µl Griess Reagent I to each well except the Blank wells.

8. Add 50 µl Griess Reagent II to each well except the Blank wells. Mix well by tapping the side of the plate gently.

9. Incubate for 10 minutes at room temperature.

10. Determine the optical density (OD) of each well using a microplate reader set at 540 nm.

Nitrate Reduction Assay Procedure

The reconstituted NADH and Nitrate Reductase should be kept on ice throughout the duration of the assay. Bring all other reagents to room temperature before use. It is recommended that all samples and standards be assayed in duplicate.

This assay procedure measures total nitrite by converting nitrate to nitrite. To determine the nitrate concentration in the sample, the endogenous nitrite concentration measured from the Nitrite Assay Procedure must be subtracted from the converted nitrite concentration measured in this assay procedure.

1. Prepare all reagents, working standards, and samples.

2. Remove excess microplate strips from the plate frame, return them to the storage bag.

3. Add 200 µl of Reaction Buffer (1X) to the blank wells.

4. Add 50 µl of Reaction Buffer (1X) to the zero standard wells.

5. Add 50 µl of Nitrate Standard or sample to the remaining wells.

6. Add 25 µl of NADH into all standard and sample wells.

7. Add 25 µl of Nitrate Reductase into all standard and sample wells. Mix well and cover with the adhesive strip provided.

8. Incubate for 30 minutes at 37° C.

9. Add 50 µl of Griess Reagent I to all wells except Blank wells.

10. Add 50 µl of Griess Reagent II to all wells except Blank wells. Mix well by tapping the side of the plate gently.

11. Incubate for 10 minutes at room temperature.

12. Determine the optical density (OD) of each well using a microplate reader set at 540 nm.

Major Adjustment to Our Methodology

We have concentrated on measuring serum nitrate as an end product of nitric oxide for the following reason:

1. We found that the measurement of nitrite (NO^-2) in serum and urine were **negligible.** In the first 15 recipients where we measured nitrite in serum and urine, the serum nitrite was <1 micromole and urine nitrite <2 micromole, and this value did not alter with the repeated assay. The measurement of serum and urine nitrate (NO^-3) was more significant and correlates well with the clinical pictures.
2. Other research groups have concluded that within one hour of collecting the blood sample 95% of nitrite is converted to nitrate (Moshage 1995). This conclusion is confirmed by our laboratory results and thus confirms point (1).
3. Nitrite entering the circulating blood reacts rapidly with oxyhaemoglobin to yield nitrate. Therefore, the synthesized nitric oxide is detected in the plasma as nitrate (Tanaka 1995).

Technical Notes

The following precautions and notes were taken into account while conducting the measurement of nitrate and nitrite, and should be considered when conducting future measurements:

1. Do not use the kit beyond the expiration date on the kit label.
2. Do not mix reagents from different lots.
3. Allow reagents to warm to room temperature before use.

4. The reconstituted NADH and Nitrate Reductase must be kept on ice during the assay.

5. When mixing or reconstituting protein solutions, always avoid foaming.

6. Pre-rinse the pipette tips when pipetting standards.

7. To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.

8. Pipette standards and samples to the bottom of the wells.

9. Add all other reagents to the side of the wells to avoid contamination.

10. If samples generate higher values than the highest standard, further dilute the samples and repeat the assay.

11. Any variation in operator, pipetting technique, washing technique, incubation time or temperature, as well as kit age, can cause variation in optical density.

12. Precautions should be taken to avoid the contamination of samples or buffers with outside sources of nitrates or nitrites. Possible sources of contamination are skin, saliva, food, drink, and water.

13. Deionized or distilled water should be used to avoid the contamination of reagents with nitrate or nitrite.

14. Care should be taken in the selection of gloves and disposable pipette tips as these products maybe a source of nitrite or nitrate contamination.

Statistical Analysis

SPSS software (version 18) was used for the data analysis. Data is presented as a median value ± semi-interquartile (semi-IQ) range. A non-parametric test, the Mann-Whitney test, was used to test for differences between groups. A P value ≤ 0.05 was considered to indicate statistical significance. An ROC curve was used to find the sensitivity and specificity for various levels of serum nitrate in diagnosing acute rejection. Age was presented as mean value ± SD.

Renal dysfunction caused by post transplant complications (acute rejection, UTI, raised serum creatinine, PD peritonitis) can occur in the same recipient at the same or different points in time. Similarly, post transplant complications in the 44 recipients could occur at the same or different points in time. This can introduce bias in our statistical analysis: to avoid this, the most appropriate method to analyse the data is to analyse the first adverse event that occurred in each of the 44 recipients.

Chapter 6

Results

Serum Nitrate in Healthy Volunteers

The healthy volunteers had median serum nitrate of 47.5 ± 10.88 μmol/l.

Serum Nitrate in End Stage Renal Failure

We measured serum nitrate in the pre-transplant blood samples of the 47 recipients (day 0). The median serum nitrate was 55 ± 30.5 μmol/l., which represented the serum nitrate levels in ESRF.

Serum Nitrate in Recipients with Previous Renal Transplants

The median nitrate measured in recipient Nora 21 (CK) was 115 ± 40.25 *μmol/l*, recipient Nora 26 (JF) was 55 ± 27.5 *μmol/l* and recipient Nora 27 (AL) was 107 ± 16.5 *μmol/l*. None of these recipients had an episode of acute rejection, but they underwent 4 biopsies, none of which showed acute rejection. Figure 6.1, 6.2 and 6.3 show that levels of serum nitrate remained high despite falling serum creatinine.

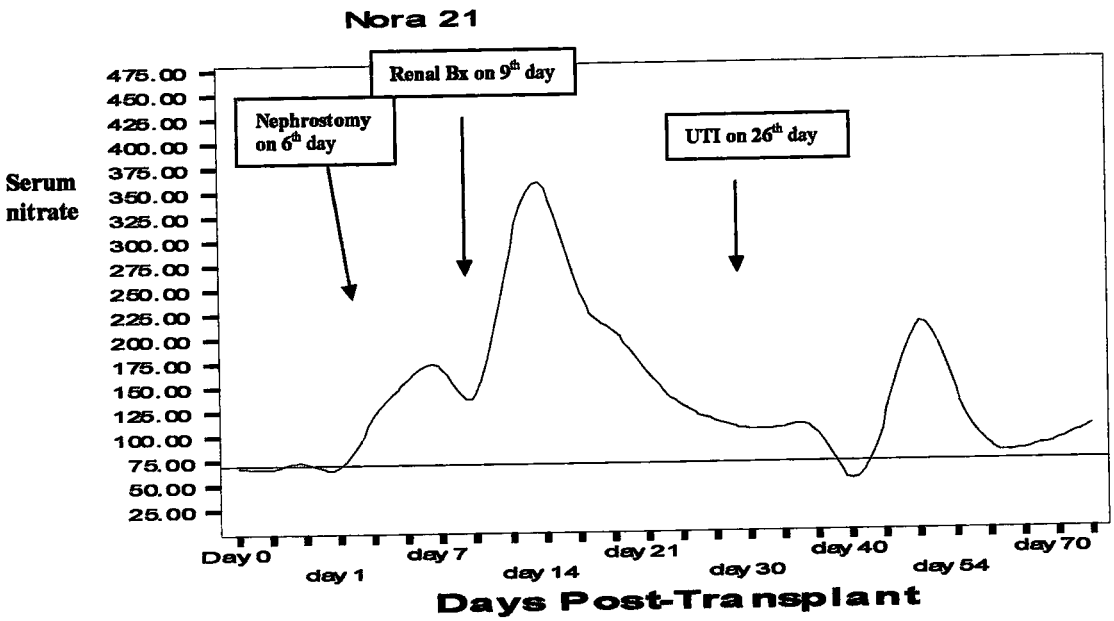


Figure 6.1: Recipients N21 had a previous failed renal transplant. Over a 3 month period, he had serum nitrate > 70 *μmol/l*. On the 6th day, nephrostomy was carried out for ureteric obstruction. On day 9, renal biopsy (Bx) was carried out, showing Acute Tubular Necrosis (ATN). On the 26th day, the recipient had UTI. Reference line at 70 *μmol/l*.

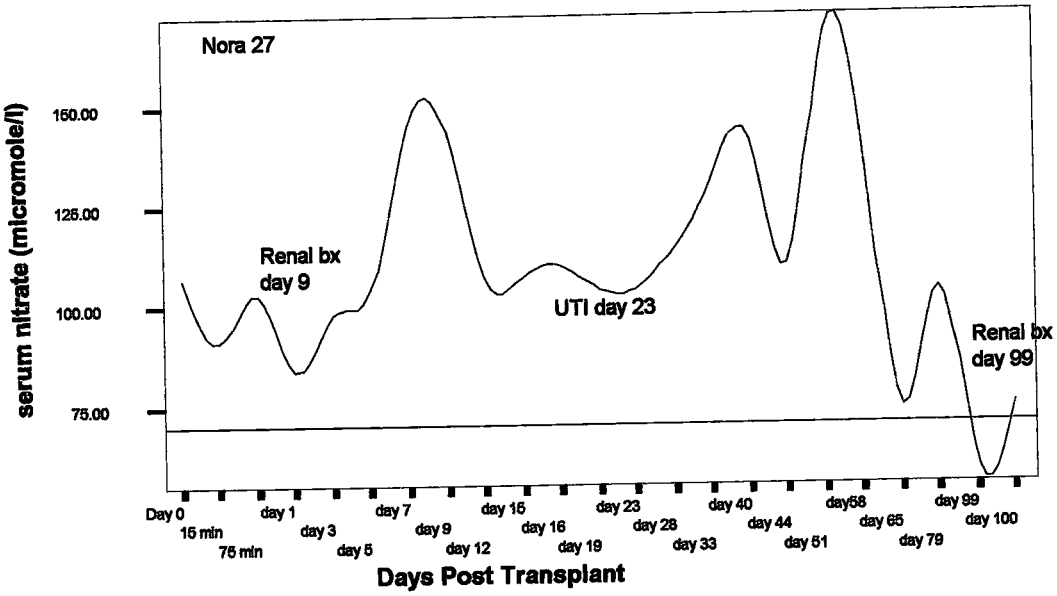


Figure 6.2: Serum nitrate of Recipient N27 (2 previous failed renal transplants) over 3 months period ws >70 *μmol/l*. On the 7th and 99th day, renal bx were carried out, both showing Acute Tubular Necrosis (ATN). UTI was diagnosed on the 23rd day. Reference line at 70 *μmol/l*.

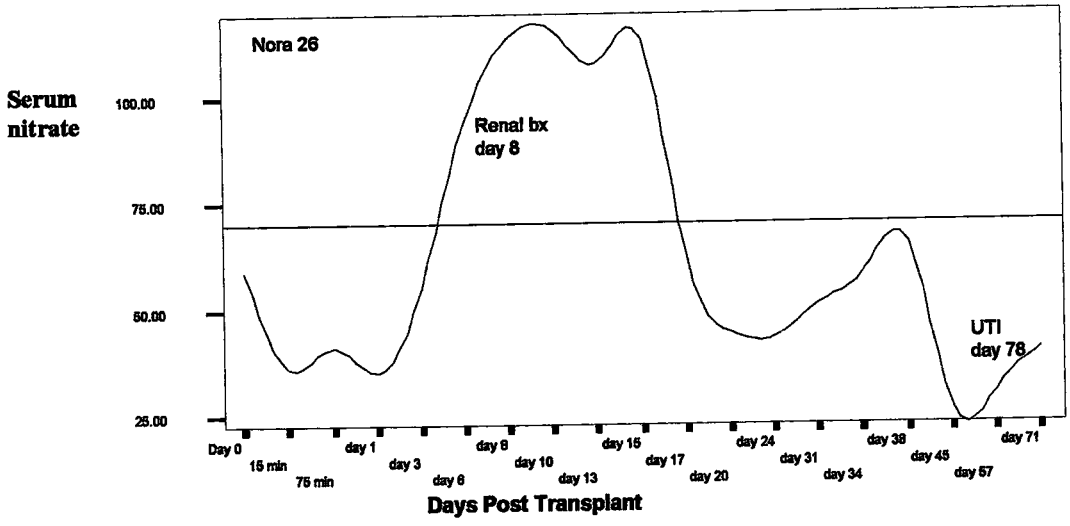


Figure 6.3: Serum nitrate of Recipient N26 over 3 months period ws >70 *μmol/l* from the 3rd day to the 21 day post renal transplant. On the 8th day, renal bx were carried out, both showing ATN. UTI was disgnosed on the 78th day. Reference line at 70 *μmol/l*.

Figures 6.4, 6.5 and 6.6 show falling serum creatinine despite sustained raised levels of serum nitrate.

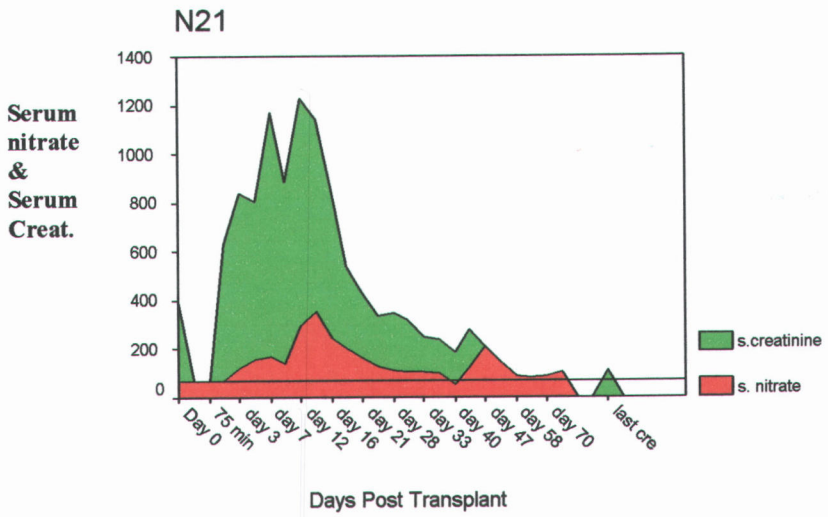


Figure 6.4: In N21, Serum nitrate and serum creatinine post renal transplant. S creatinine continues to fall towards a normal value while serum nitrate stays at high levels. Reference line at 70 μmol/l.

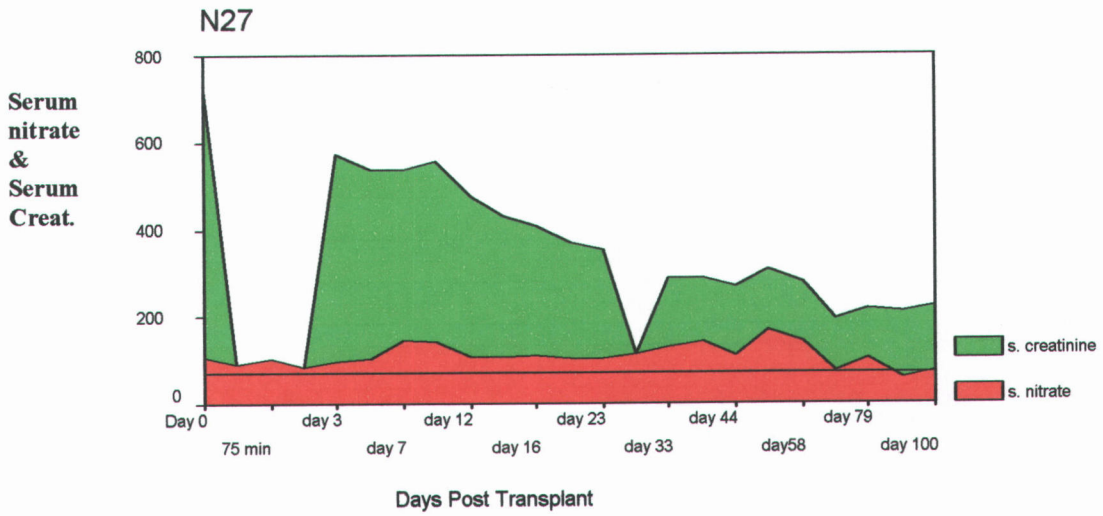


Figure 6.5: In N27, Serum nitrate and serum creatinine post renal transplant. S creatinine continues to fall towards a normal value while serum nitrate stays at high levels. Reference line at 70 μmol/l.

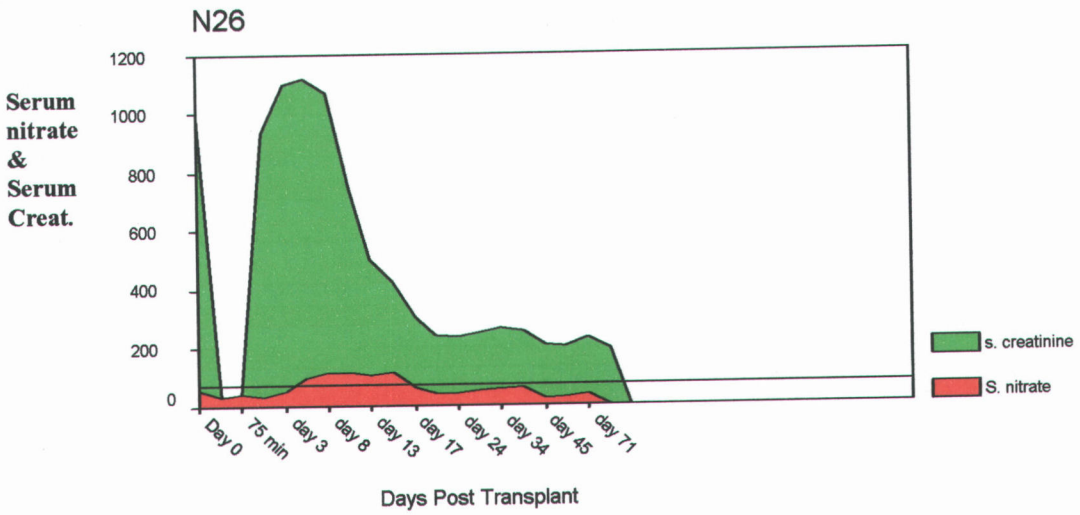


Figure 6.6: IN N26, Serum nitrate and serum creatinine post renal transplant. S creatinine continues to fall towards a normal value while serum nitrate stays at high levels. Reference line at 70 μmol/l.

Serum Nitrate in Recipients with Stable Graft Function (SGF)

Out of the 44 renal allograft recipients, 17 recipients (mean age 46, 8 males and 9 females) had SGF for the period of three months post renal transplantation. They had a median serum nitrate of 42 ± 14 μmol/l. The serum nitrate fell to low levels for the duration of the follow-up (3 months). The graphs in Figure 6.7, 6.8, 6.9, 6.10 and 6.11 demonstrate this.

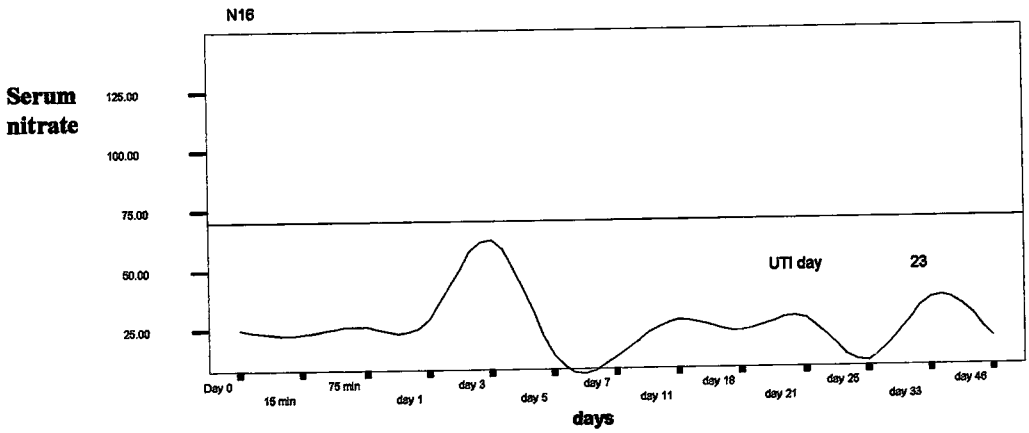


Figure 6.7: Serum nitrate in Nora 16 during the 3 month period post renal transplant. Over 3 months period the mediam serum nitrate was 25 µmol/l . Reference line is 70 µmol/l

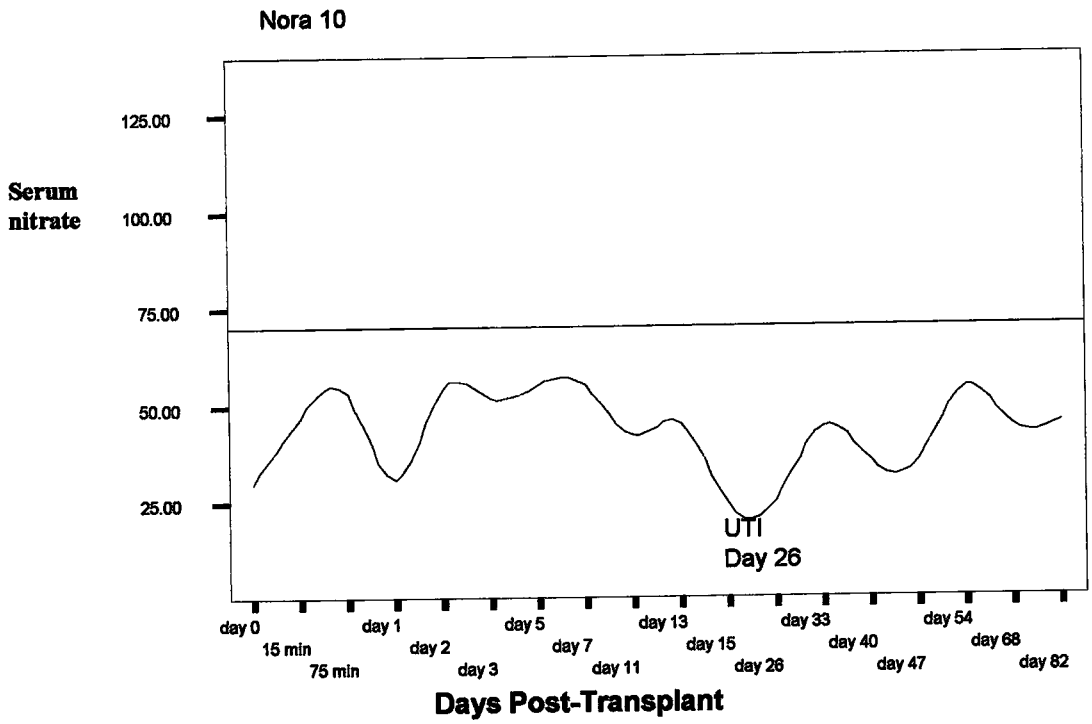


Figure 6.8: The median serum nitrate in Nora 10 during the 3 month period post renal transplant was 44.5 µmol/l. One episode of UTI did not cause a rise in serum nitrate. Reference line is 70 µmol/l

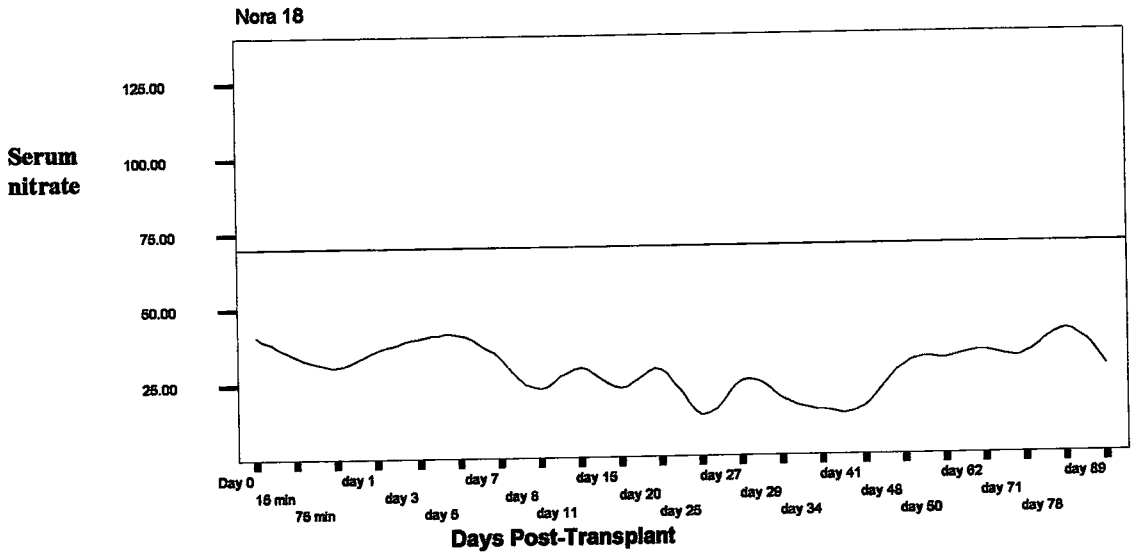


Figure 6.9: The median serum nitrate in Nora 18 during the 3 month period post renal transplant was 30.5 μmol/l.

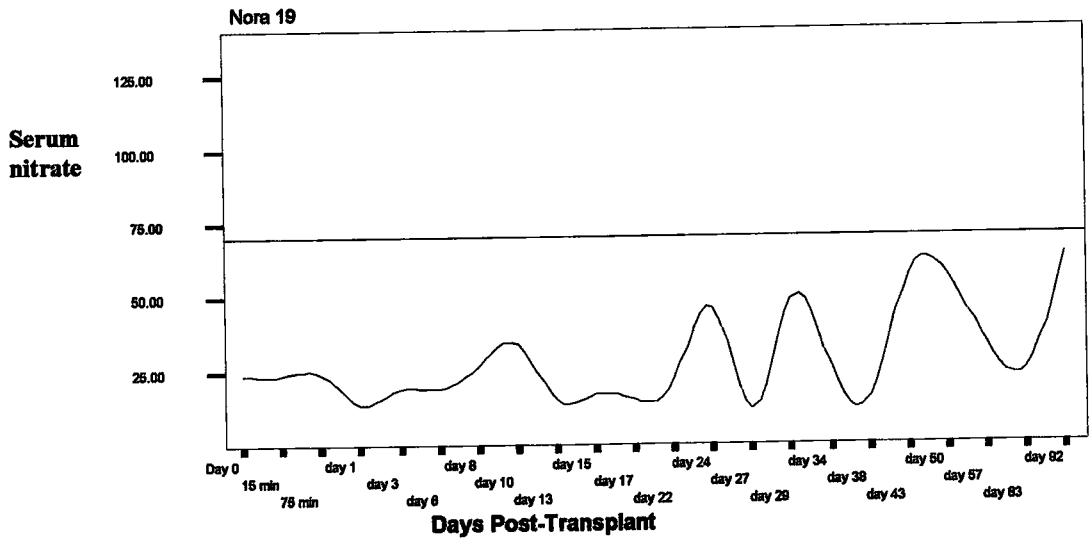


Figure 6.10: The median serum nitrate in Nora 19 during the 3 month period post renal transplant was 24μmol/l .

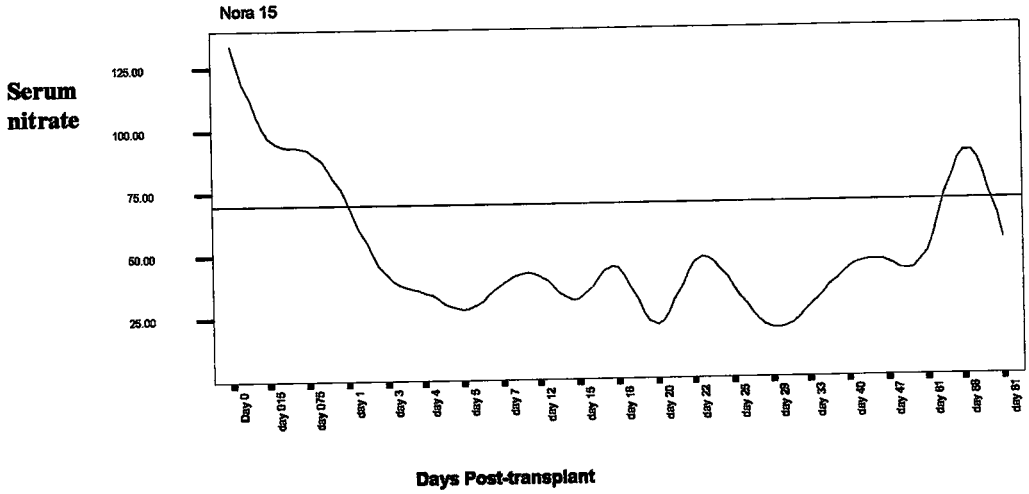


Figure 6.11: The median serum nitrate in Nora 15 during the 3 months period post renal transplant was 43 µmol/l.

Urinary tract infection did not cause a rise in serum nitrate in recipients with SGF, as shown in Figures 6.7 and 6.8, and Figures 6.12, 6.13, 6.14 and 6.15.

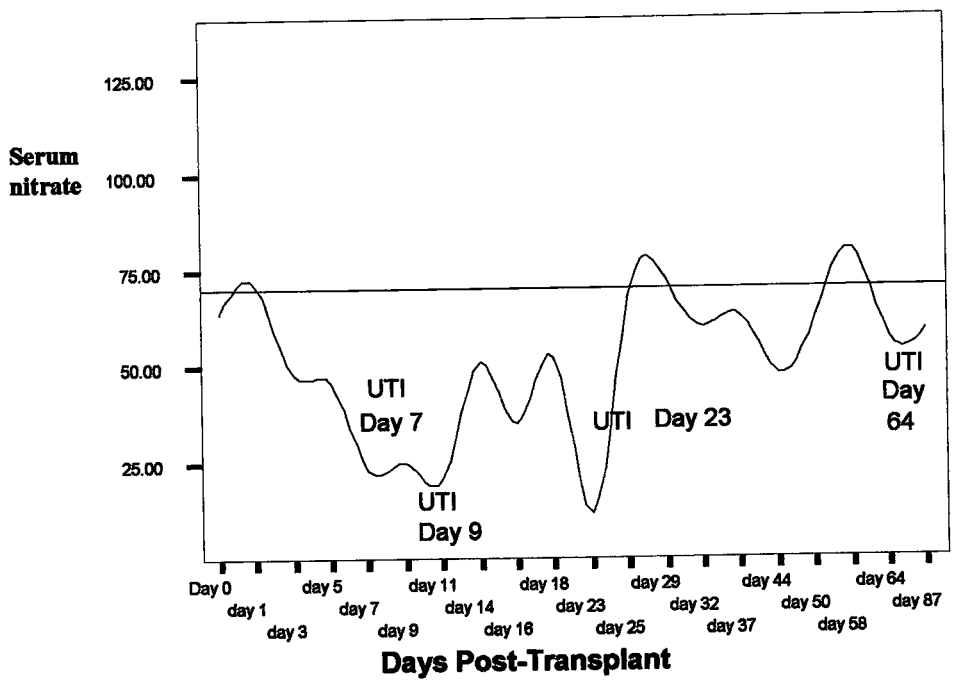


Figure 6.12: In Nora 1, UTI does not cause a significant rise in serum nitrate. the median serum nitrate was 54.50 µmol/l.

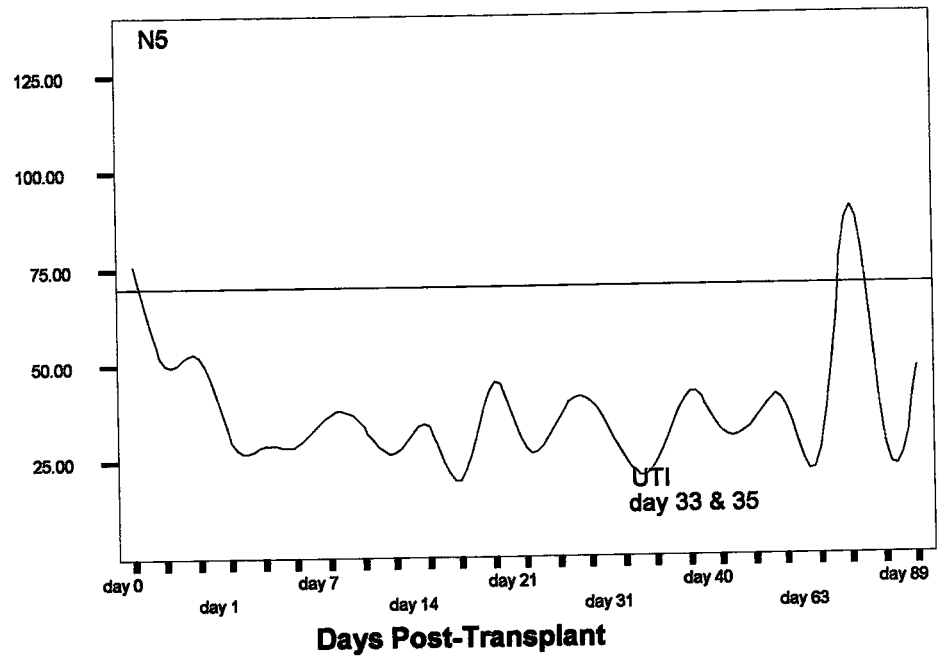


Figure 6.13: In Nora 5, UTI does not cause a significant rise in serum nitrate. The median serum nitrate was 34 μmol/l.

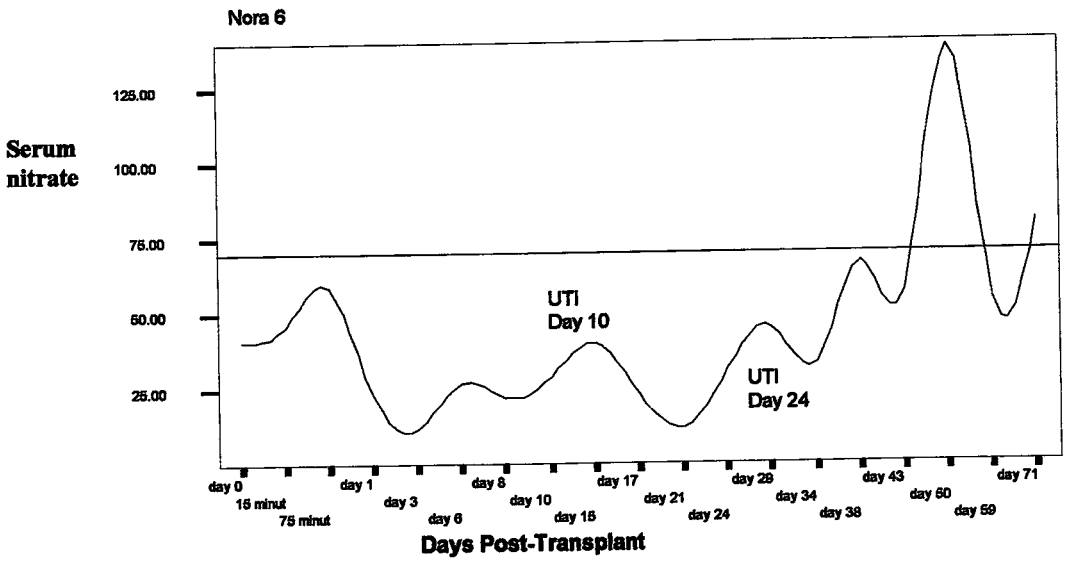


Figure 6.14: In Nora 6, UTI does not cause a significant rise in serum nitrate. The median serum nitrate was 40 μmol/l.

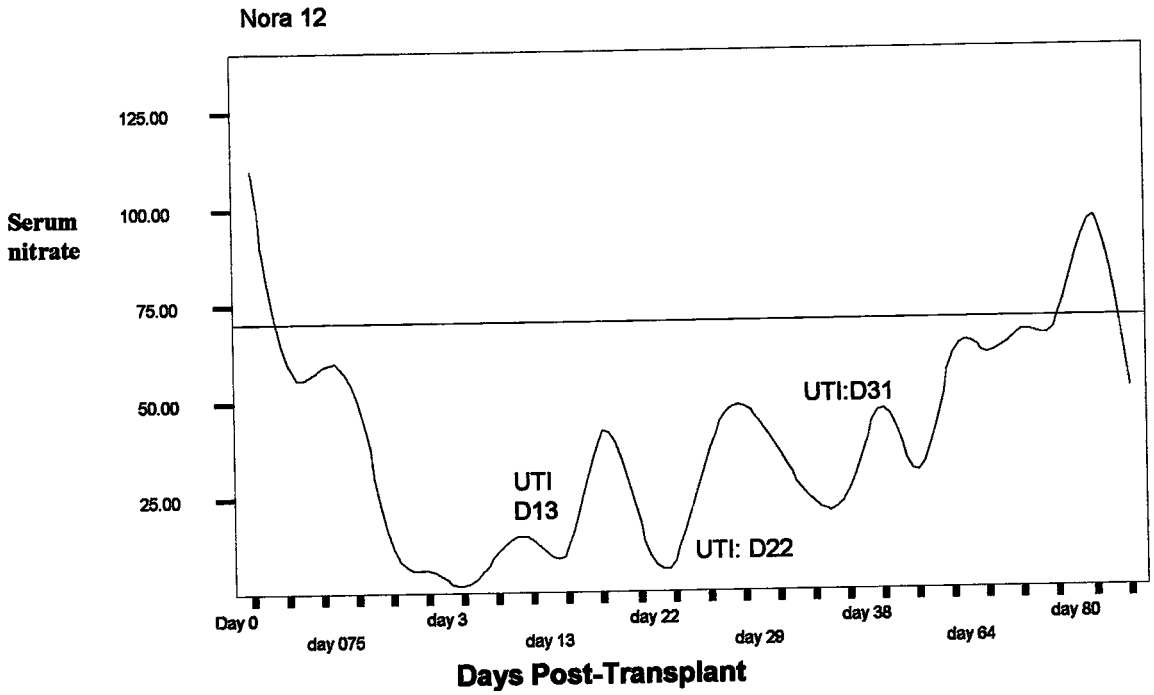


Figure 6.15: In Nora 12, UTI does not cause a significant rise in serum nitrate. The median serum nitrate was 39.5 μmol/l.

Serum Nitrate in Biopsy-proven Acute Rejection

The median serum nitrate in the recipients with biopsy-proven acute rejection (65.5 ± 40.75 μmol/l) was significantly higher when compared with serum $NO3^-$ in recipients without acute rejection (41.5 ± 13.25 μmol/l) (Table 6.1).

Post Transplant Complication - Acute Rejection	Recipient No.	Median of S nitrate (µmol/l)	Semi-IQ Range	Mann-Whitney Exact Sig. (2-tailed)
S nitrate in recipients with AR	8	65.5	53	
S nitrate in recipients without AR	36	41.5	23	0.001

Table 6.1: The median serum nitrate was significantly higher in recipients with biopsy-proven acute rejection compared to recipients without acute rejection.

The Sensitivity and Specificity of Serum Nitrate in Diagnosing Acute Rejection

Using an ROC and setting the threshold at 70 µmol/l (between 68 and 80 µmol/l) (see Table 6.2) we get a sensitivity of 50% and specificity of 90%.

Therefore, using the coordinates of the curve and moving the threshold will alter the sensitivity and specificity of serum nitrate during acute rejection (Table 6.2).

Serum Nitrate	Sensitivity	1-Specificity
13	1	1.
16.5	1	0.944
20	1	0.917
22	1	0.889
24	1	0.778

26	1	0.750
28	0.875	0.750
30	0.875	0.722
32	0.875	0.694
34	0.875	0.639
35	0.875	0.583
37	0.875	0.556
38	0.750	0.500
39	0.750	0.472
42	0.750	0.417
44	0.750	0.361
45	0.625	0.306
46	0.5	0.306
49	0.5	0.278
51	0.5	0.250
52	0.5	0.222
57	0.5	0.194
61	0.5	0.139
68	**0.5**	**0.111**
80	**0.5**	**0.083**
87	0.375	0.083
92	0.375	0.056
102	0.375	0.028
115	0.25	0.028
122	0.250	0.00
152	0.125	0.00
180	0.00	0.00

Table 6.2: Coordinates of ROC.

Behaviour of Serum Nitrate in Acute Rejection

During acute rejection, serum nitrate is expected to rise and drop with successful treatment of acute rejection. If acute rejection persisted or failed to respond to treatment, we expected the levels of serum nitrate to remain high.

Recipient Nora 23 (Figure 6.16) developed a single episode of acute rejection that was treated successfully with methyl predinsolone. Acute rejection was associated with a rise in serum nitrate to 125 μmol/l and when treated successfully serum nitrate fell to 17 μmol/l.

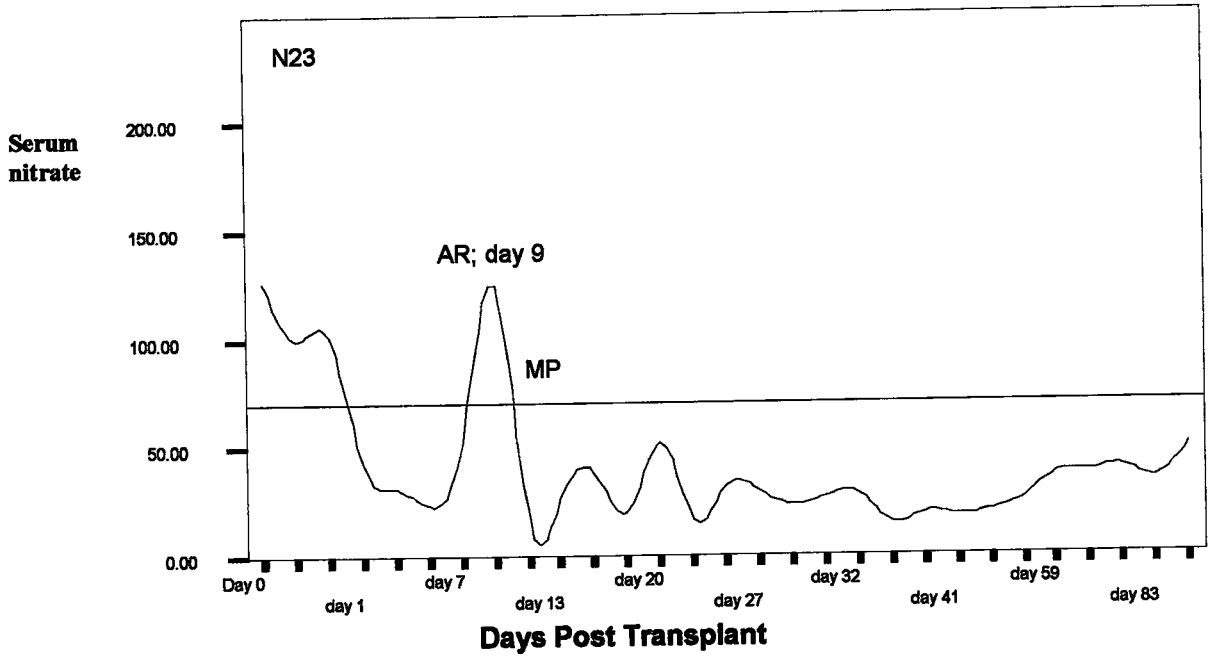


Figure 6.16: Serum nitrate rises during acute rejection and falls to levels < 70 μmol/l when treated successfully.

Recipient Nora 11 had two episodes of acute rejection; both were associated with a rise in serum nitrate to 72 μmol/l and 70 μmol/l and following successful treatment serum nitrate was 53 μmol/l and 42 μmol/l respectively. This recipient also had one negative biopsy during which serum nitrate was 19 μmol/l (Figure 6.17).

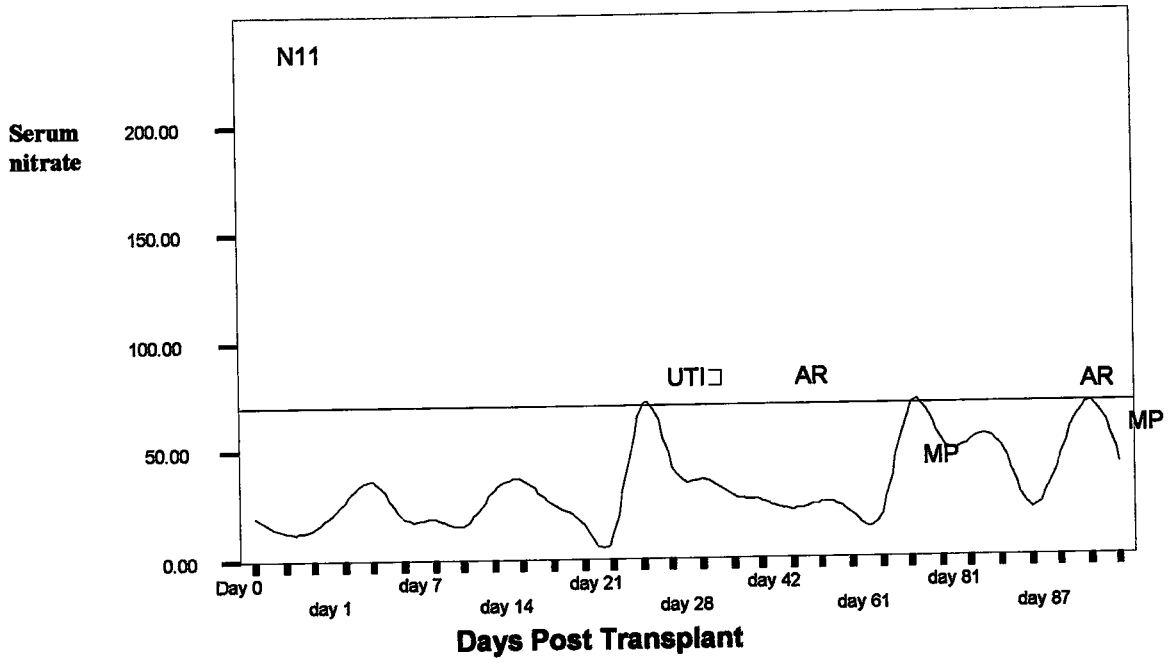


Figure 6.17: Nora 11 had two episodes of acute rejection. In both episodes it was associated with a rise in serum nitrate > 70 μmol/l and when treated successfully this level falls to < 70 μmo/l

Recipient Nora 22 had a rise in serum nitrate to 80 μmo/l with biopsy-proven acute rejection. Following treatment with methyl prednisolone the level of serum nitrate was 38 μmol/l. Interestingly, serum creatinine remained high (250 mmol/l), which lead to a second renal biopsy which showed recovering episodes of acute rejection. Serum nitrate continued to be low (30 μmol/l), and renal function normalised without any further treatment (Figure 6.18).

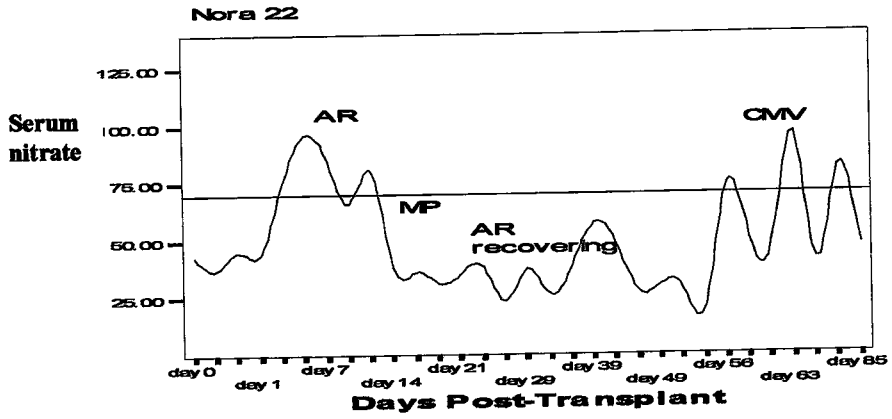


Figure 6.18: In Nora 22 serum nitrate rises during acute rejection and falls once successfully treated. The second biopsy was taking because of high serum creatinine while serum nitrate was within normal limits.

Serum Nitrate in Persistent Acute Rejection

Two Recipients (N9 and N3) had persistent rejection and required OKT3 therapy. Failure of treatment with methyl prednisolone of acute rejection episodes was associated with high levels of serum nitrate. Only successful treatment of acute rejection with OKT3 was associated with fall of serum nitrate (Figure 6.19, Figure 6.20).

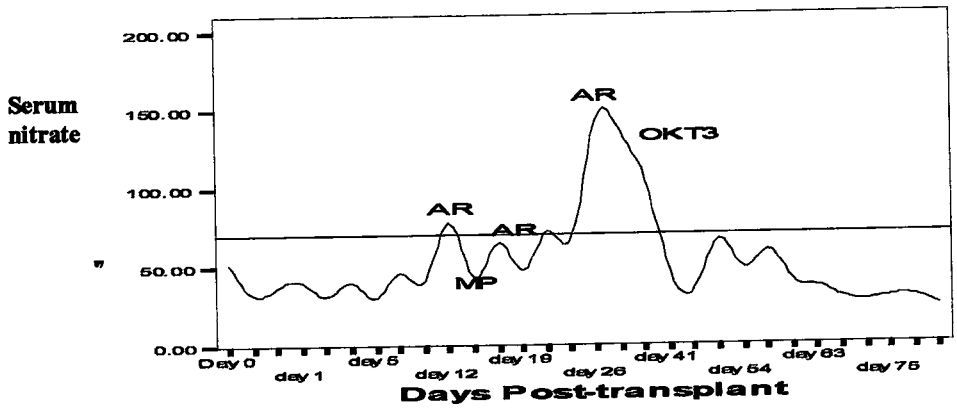


Figure 6.19: In N9, 3 episodes of AR with rise in serum nitrate (78 µmol/l, 72 µmol/l, 143 µmol/l respectively) which fell to levels < 70 µmol/l (45 µmol/l) only when acute rejection was resolved by successful treatment with OKT3.

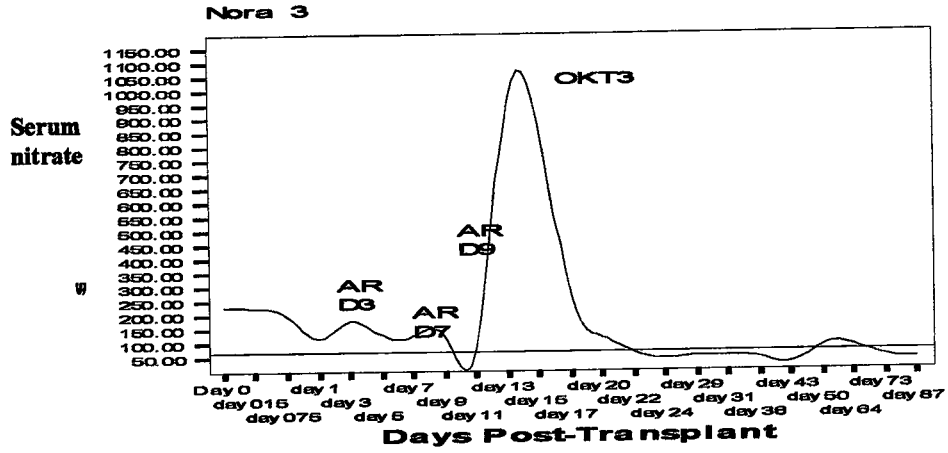


Figure 6.20: In N3, serum nitrate rises with acute rejection (179 µmol/l, 125 µmol/l, 120 µmol/l respectively) but does not fall to levels < 70 µmol/l until treated successfully with OKT3 to serum nitrate 43 µmol/l.

One recipient (N13) (Figure 6.21) had persistent rejection and did not respond to methyl prednisolone therapy or to treatment with OKT3; his serum nitrate levels were persistently high throughout. The recipient lost the kidney transplant.

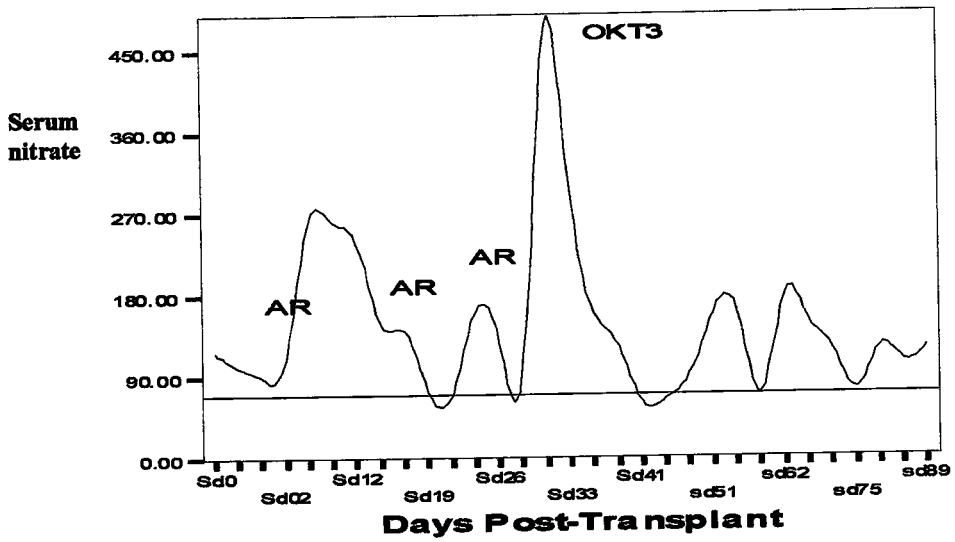


Figure 6.21: In this recipient persistent rejection was associated, as expected, with a persistent rise in serum nitrate. In this situation the graft was lost.

Serum Nitrate in Other Post Renal Transplant Complications

This would include the following complications:

1. Urinary tract infection
2. Tacrolimus toxicity
3. Raised serum creatinine >10%
4. Peritoneal catheter peritonitis

Serum Nitrate in UTI

There were 71 episodes of UTI in 37 recipients (aged 47, 26 males and 11 females).

The cultures showed 10 different types of organism (Table 6.3): the commonest isolated organisms were enterococcus (32.3%) and E.coli (17.7%). All episodes were treated successfully with oral antibiotic according to sensitivity, irrespective of the number of colonies or the white cell count in HPF.

Type of Organism	Frequency	Percentage
Enterococcus	42	32.3
E.Coli	23	17.7
Candida	17	13.1
Streptococcus	17	13.1
Coliform	15	11.5
Vancomycin Resistence Enterococcus (VRE)	8	6.2
Staphylococcus	4	3.1
Proteus	1	0.8
Pseudomonas	1	0.8
V. Faecalis	1	0.8
E.coli & Candida	1	0.8

Table 6.3: Different types of organism in recipients.

There was no significant difference between the median serum nitrate during episodes of UTI (21.83μmol/l) when compared with the median serum nitrate (22.61 μmol/l) during normal urinary cultures (Table 6.4).

Post Transplant Complication - Acute Rejection	Recipient No.	Median of S nitrate (μmol/l)	Semi-interquantile Ranges	Mann-Whitney Exact Sig. (2-tailed)
S nitrate in recipients with UTI	6	36.5	38.75	
S nitrate in recipients with normal urinary culture	38	42	12.25	0.891

Table 6.4: There is no significant difference in serum nitrate between recipients with UTI and those with normal urinary cultures.

Serum Nitrate in Tacrolimus Toxicity (TT)

Out of the 44 recipients, 40 recipients (mean age 46 years, 28 men and 12 women), were taking tacrolimus. There were 128 episodes where tacrolimus level was >15 ng/dl. Only 1 recipient (33 years old, male), on cyclosporine, had 7 episodes were CsA trough levels were >250 ng/dl.

The median serum nitrate during tacrolimus toxicity was not significantly higher when compared to median serum nitrate in recipients with normal tacrolimus trough levels Table 6.5)

Post Transplant Complication - TT	Recipient No.	Median of S nitrate (µmol/l)	Semi-interquantile Ranges	Mann-Whiteny Exact Sig. (2-tailed)
S nitrate in recipients with TT	22	35.5	14.88	
S nitrate in recipients with normal tacrolimus trough levels	22	45	15.12	0.205

Table 6.5: No significant difference in serum nitrate in recipients with Tacrolimus Toxicity (TT) and recipients with normal tacrolimus levels.

Serum Nitrate with Rise in Serum Creatinine >10%

Out of the 44 recipients, 40 recipients (mean age 46 ± 2.05, 31 males and 9 females), had at least one episode where there was a rise in serum creatinine >10%. In total, there were 100 instances of a rise in serum creatinine >10% in the 40 recipients. There was a no significant difference of median serum nitrate during the rise in s. creatinine >10% when compared to median serum nitrate during normal levels of serum creatinine (Table 6.6).

Post Transplant Complication – Rise in S Creatinine >10%	Recipient No.	Median of S nitrate (μmol/l)	Semi-interquantile Ranges	Mann-Whitney Exact Sig. (2-tailed)
S nitrate⁻ in recipients with rise in s.creatinine >10%	13	44	10.5	
S nitrate in recipients with normal serum creatinine	31	40	17.5	0.554

Table 6.6: No significant difference in serum nitrate in recipients with a rise in serum creatinine and those with normal serum creatinine.

Serum Nitrate in Peritoneal Dialysis Peritonitis

Five recipients (mean age 36 ± 3.98, 3 males and 2 females) out of the 4 recipients had PD peritonitis confirmed by culture and sensitivity and requiring surgical removal of the PD catheter. There are no significant differences in the median serum nitrate between recipients with or without PD peritonitis (Table 6.7).

Post Transplant Complication - PD Peritonitis	Recipient No.	Median of S nitrate (µmol/l)	Semi-interquantile Ranges	Mann-Whitney Exact Sig. (2-tailed)
S nitrate in recipients with PDP	2	37.5	23.5	
S nitrate in recipients with no PDP	42	40	12.25	0.640

Table 6.7: No significant difference in serum nitrate in recipients with or without PDP.

Chapter 7

Discussion and Conclusion

Finding an alternative test to diagnose acute rejection has always been an attractive prospect to researchers. NO is a molecule which plays a role in acute rejection. It contributes to organ function during physiological states and pathological conditions depending on the type of NOS activated (Davies 1995). Under physiological states, it is the cNOS which is up-regulated, generating small amounts of NO which is cytoptrotective and facilitates physiological functions (Radomski 1990). Up-regulation of iNOS generates large amounts of NO. This occurs during acute rejection and inflammatory conditions. The small amount of NO produced by cNOS forms nitrite and is difficult to measure, while iNOS-generated NO is produced in large quantities, forms nitrate and is detectable in the blood (Morris 1994, Weight 1998, Jefayri 2000, Goligorsky 2002). The production of NO in large amounts during acute rejection and the possibility of measuring it in the peripheral blood samples make NO measurement an attractive test.

ESRF and renal transplantation create a pool of complex and intriguing environments for NO. NO production in ESRF is affected by the pathology and by renal replacement therapy (HD or CAPD). On the other hand, NO in renal transplant recipients is influenced by the process of transplantation, organ retrieval, surgery, immunosuppression and post transplant complications, which include acute rejection and opportunistic infections.

ESRF patients have a high median serum nitrate level (55μmol/l) irrespective of the type of renal replacement therapy they were receiving. This could be attributed to the cause of ESRF and the role of NO. Once the patient undergoes a successful renal transplant, the serum nitrate drops to values comparable to those in healthy volunteers. This is because the transplanted kidney restores the normal NO production/secretion mechanism in addition to excretion of endogenous NOS inhibitors.

The vascularity and size of the transplanted organ, as well as the number of previously transplanted organs, may increase the total body NO production. Langrehr et al were the first to conclude that there is an increase in the total body NO production during the rejection of a vascularized organ (Langrehr 1992). Patients with previous multiple transplants may have high serum nitrate levels, which could be caused by the vascularity of the newly-transplanted kidney and the presence of previously transplanted organs which may not function, but may contribute to the total body NO production and haemodynamically active/renal NO production. Our results reflect the fact that although these recipients have a normally-functioning renal allograft transplant, they continue to have high serum nitrate levels which behave differently to the primary renal transplant recipients. This could be due to two possible reasons:

1. An increased total body NO production and haemodynamically active/renal NO production – more than can be handled by the transplanted kidney and/or
2. The failed or failing previous renal transplant which continues to contribute to NO production.

Despite the high serum nitrate levels in these recipients, serum creatinine falls to normal values. We have been the first to observe high levels of serum nitrate in multi-transplant recipients; as far as we know there are no previous studies reporting any such association. However, this study includes a small number of patients with previous failed transplants and may need further research. The possibility that this is only a random observation cannot be ruled out.

In the study group, there were 17 recipients with stable graft function. Their serum creatinine and serum nitrate dropped down to normal levels following transplantation. None of the recipients were subjected to dietary restriction except early morning fasting, until the blood sample was collected. The median serum nitrate in SGF was 42 ± 14 μmol/l (with a range of 15-60 μmol/l) despite their variable diets. The fall in serum nitrate levels towards normal in recipients with stable graft functions shows that the transplanted kidney achieves normal NO metabolism and shows the role of the renal system in clearing NO, as well as confirming the normal or expected range of NO end-products (serum nitrate) in humans.

Recipients with acute rejection show a significant rise in serum nitrate when compared to recipients with SGF and those with negative renal biopsies. Our results confirm the results of similar previous work. Langrehr, using a sponge matrix, showed a rise in **nitrite and nitrate** in the sponge fluids and a rise in serum **nitrite and nitrate** during acute rejection in solid organ transplantation in rats (Langrehr 1992). There was a rise in the level of serum **nitrate** during acute rejection in rat renal

transplants (Suzuki 2004, Lu 1999), rat pancreatic transplants (Tanaka 1995), rat liver transplants (Ohidan 1995) and rat lung transplants (Shirashi 1995). Most human studies involving renal transplantation have shown an increase in the levels of serum nitrate during acute rejection (Smith 1996, Takahashi 1998, Koyama 2000, Albrecht 2000). However, we measured only serum nitrate, recruited a larger number of recipients, followed-up for a longer period and imposed no dietary restriction except for fasting prior to harvesting of the blood sample. We found serum nitrate levels to be significantly higher in acute rejection, while serum nitrite level was low (< 1 micromol/l) at all times. This is supported by the published research that nitrate is the predominant blood oxidising product of NO and the indirect marker of the level of NO production. However, serum nitrite, in addition to serum nitrate, was used in diagnosing acute rejection (Ricart-Jané 2001, Viinikka 1996, Hibbs 1992, Moshage 1995, Wennmalm 1993, Langrehr 1991, Langrehr 1992, Langrehr 1993, Shiraishi 1995, Ohdan 1995, Dedeoglu 1996, Albrecht 2000, Tanaka 1995, Takahashi 1998, Lu 1999, Castillo 1996). This is because the methods make it possible to detect nitrate alone, nitrite alone or both. However, the recovery of nitrite from whole blood samples kept on ice or at room temperature for two-five hours was 0-7% while the recovery of nitrate was 91-95%. This is because of the red cells in the whole blood sample, which oxidise nitrite to nitrate (Moshage 1995, Wennmalm 2007). Furthermore, the normal nitrate level in human serum is 30-60 μmol/l, whereas the normal nitrite levels are 0.5-3 μmol/l; therefore the reported serum levels of the sum nitrite and nitrate in the serum represent the nitrate concentration. In addition, anticoagulants in blood sample bottles may interfere with the quantification of nitrite (Tsikas 2006). Therefore nitrate is the final end-product of NO.

We have shown a significant association between NO production and acute rejection. However, to make this test clinically applicable we need to identify a level at which acute rejection is diagnosed. There were 19 recipients who had a biopsy-proven acute rejection with a median serum nitrate of 66 ± 53 μmol/l, which was significantly higher than the median serum nitrate in the 13 recipients with negative biopsies (42 ± 6 μmol/l) and the recipients with SGF (42 ± 14 μmol/l). Furthermore, during acute rejection, serum nitrate rises to levels > 70 μmol/l and falls to 'normal' values (<66 micromol/l) with successful treatment – as diagrammatically displayed in Figures 6.16, 6.17, 6.18, 6.19 and 6.20. Similarly, recipients with persistent acute rejection had serum nitrate levels > 70 μmol/l, but these fell to levels of < 70 μmol/l only when acute rejection was successfully treated with OKT3. On the other hand, the reported normal levels of serum nitrate in humans is 30-60 micromol/l. In the volunteer group, the median serum nitrate level was 47.5 ± 10.88 μmol/l, while recipients with stable graft function had a median serum nitrate of 42 ± 14 μmol/l. Both groups fell within the reported range. Therefore, in acute rejection, serum nitrate rises to levels > 70 μmol/l, while in the absence of immune response and in physiological states, the serum nitrate falls to values < 70 μmol/l. Thus, serum nitrate at 70 micromol/l has a low sensitivity (50%) but high specificity (90%). However, as we showed using the ROC coordinate, the value of serum nitrate chosen can change and, accordingly, the sensitivity and specificity can change too. Choosing a lower serum nitrate level, therefore, will improve the sensitivity of the test.

There was a significant rise in the median serum nitrate level during acute rejection compared to the other post transplant complications. This further supports the association between serum NO production and acute rejection, and the usefulness of measuring serum nitrate as a diagnostic test.

UTI is an infectious process and the second-most common complication in renal transplant recipients, and studies have shown an associated rise in NO production (Kang 2004, Poljakovie 2003, Wheeler 1997). It causes renal impairment, manifesting as a rise in serum creatinine; it is also reported that serum NO rises during acute UTI, particularly serum nitrite. However, in our group of recipients, episodes of acute UTI were not associated with a rise in serum nitrate.

A second condition is peritoneal dialysis peritonitis, an inflammatory process which could be associated with up-regulation of iNOS and increased production of NO. However, there was no significant rise in serum nitrate in this condition either.

A rise in serum creatinine >10% was not associated with a rise in serum nitrate. Rise in serum creatinine >10% indicates renal dysfunction and the recipients may require a renal biopsy to rule out acute rejection. Thus, low levels of serum nitrate may avoid this invasive procedure.

Most of the immunosuppressive drugs used to control acute rejection, such as tacrolimus or cyclosporine (Watarai 2004, Strestíková 2001) and corticosteroids (Radomski 1990), are known to inhibit NOS and NO production. In immunosupressive toxicity there is renal dysfunction. Although high levels of tacrolimus >15 ng/dl or cyclosporine >250 ng/dl may clench the diagnosis, occasionally it is difficult to differentiate drug toxicity from acute rejection. However, serum nitrate dose not rise during immunosuppressive toxicity. Therefore, low serum nitrate levels and high drug trough levels will support toxicity rather than acute rejection.

Dietary restriction during nitrate analysis is controversial. In our study we did not impose dietary restriction with the exception of fasting before blood sample collection. In healthy volunteers the median serum nitrate was 47.5 ± 10.88 μmol/l

(range 24 - 77 μmol/l) without dietary restriction. This was comparable to reported values by other studies (30-60 μmol/l) without any dietary restriction and using different methods (Tsikas 2006, Green 1982, Suthanthiran, Blum 1998, Schmidt 1999, Viinikka 1996). Not all investigators imposed dietary restrictions on their study group (whether animal or human) (Langrehr 1992, Shiraishi 1995, Tanaka 1995, Dedeoglu 1996, Paul 1996, Takahashi 1998, Lu 1999, Smith 1996, Koyama 2000, Wennmalm 1993), however, and continued to use serum nitrate or NOx as a test to diagnose acute rejection in various organ transplants; they demonstrated a significant rise in serum nitrate or NOx during acute rejection. We do not believe that the absence of dietary restriction in our recipients (although our blood sampling was done while the patient was on an over-night fasting regime) affected the results, for the following reasons:

1. During an episode of acute rejection, there is up-regulation of iNOS leading to the production of NO in such large quantities that the serum nitrate would not be affected by the diet of the recipient or any other source.
2. Other research groups have demonstrated the usefulness of serum nitrate in acute rejection without dietary restriction.
3. All recipients with stable graft function had serum nitrate comparable to that of healthy volunteers despite not being subject to dietary restriction.

Wennmala et al conducted a study in healthy volunteers and in patients with congestive cardiac failure awaiting a heart transplant. The purpose of the study was to explore the NO metabolism and excretion in humans. No dietary restriction was imposed. NO was administered by inhalation in increasing doses to both groups. They reported a dose-related increase in serum nitrate, but serum nitrite did not change significantly (Wennmalm 1993). In fact, they demonstrated that nitrite is degraded to

nitrate when the whole blood sample is incubated in vitro for over two minutes. This clearly demonstrates that during conditions of increased NO production, particularly during pathological conditions where there was up-regulation of iNOS and production of large quantities of NO, there was an associated rise in serum nitrate to an extent that it is not affected by the dietary intake of nitrate (Wennmalm 1993, Wennmalm 1992).

Conclusion

Nitric oxide is a complex, unique molecule. Although we have a clear understanding of its formation, mechanism of action and degradation in vivo, its extensive role in pathophysiological conditions adds to its complex characteristics rather than shedding more light. Evidence supports NO's role in acute rejection; irrespective of its precise role, NO has been exploited as a marker or test to diagnose acute rejection, thereby avoiding renal biopsy.

We conclude from our study and results that serum nitrate is the final end-product of NO and that it is this molecule which should be measured in serum, and not nitrite.

Our results show that there is a significant rise of serum NO_3^- in acute rejection; therefore serum NO_3^- levels >70 μmol/l indicate high possibility of acute rejection. On the other hand, in the presence of other post renal transplant complications (UTI, drug toxicity, DGF, raised serum creatinine >10%) with low serum NO_3^- (<66 micromol/dl), the possibility of acute rejection is low and therefore we can avoid renal biopsy.

Clinically, post transplant complications may occur simultaneously; it becomes a clinical challenge to exclude or prove acute rejection and renal biopsy may be needed just to rule out acute rejection. However, our results demonstrate that serum nitrate

rises only with acute rejection. Therefore, where the cause of renal dysfunction is difficult to establish, measurement of serum nitrate becomes helpful. High levels of serum nitrate may indicate acute rejection and exclude other post transplant complications, and thus renal biopsy becomes justified. However, low levels of serum nitrate do not rule out the possibility of acute rejection, and in the presence of other possible post transplant complications such as UTI or tacrolimus toxicity, or a rise in serum creatinine, may avoid the renal biopsy.

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	days	snox	unox	s.creat	u.creat	unoucre	urincrea	fkl	uti	organis
1	Day 0	64	4	618	14.91	.27	.01	.	No UTI	.
2	day 1	71	71	538	11.30	6.28	.13	8.70	No UTI	.
3	day 3	48	149	629	5.15	28.93	.24	11.10	No UTI	.
4	day 5	46	106	316	9.06	11.70	.34	3.90	No UTI	.
5	day 7	23	435	176	8.71	49.94	2.47	3.90	UTI	E coli
6	day 9	25	920	138	9.20	100.0	6.67	3.90	UTI	E coli
7	day 11	21	1262	114	10.27	122.9	11.07	4.10	No UTI	.
8	day 14	51	835	98	5.53	151.0	8.52	3.80	No UTI	.
9	day 16	35	1095	99	7.39	148.2	11.06	6.70	No UTI	.
10	day 18	52	685	100	5.50	124.5	6.85	5.60	No UTI	.
11	day 23	12	1344	94	12.25	109.7	14.30	4.30	UTI	E coli
12	day 25	68	1388	103	17.94	77.37	13.48	9.20	No UTI	.
13	day 29	72	1554	106	7.60	204.5	14.66	9.60	No UTI	.
14	day 32	60	789	95	3.47	227.4	8.31	6.40	No UTI	.
15	day 37	63	1373	100	6.61	207.7	13.73	5.40	No UTI	.
16	day 44	48	1408	95	8.65	162.8	14.82	9.80	No UTI	.
17	day 50	62	1407	94	8.20	171.6	14.97	7.90	Contaml	.
18	day 58	80	1434	98	8.56	167.5	14.63	7.30	No UTI	.
19	day 64	57	1236	93	6.87	179.9	13.29	7.00	UTI	Coliform
20	day 87	59	1363	.	7.39	184.4	.	.	.	.

	days	average	unox	creatini	u.creat	unoucrea	urincrea	fkl	ar	uti
1	Sd0	117.50	7.00	711.00	10.22	.68	.01	.	.	.
2	d015	100.50	.00	711.00	.	.00	.00	.	.	.
3	d075	89.00	.00	711.00	.	.00	.00	.	.	.
4	Sd02	110.00	66.00	475.00	5.87	11.24	.14	9.80	acute rej	.
5	Sd07	261.00	248.00	781.00	9.23	26.87	.32	7.20	.	.
6	Sd09	261.00	357.00	694.00	6.44	55.43	.51	.	.	Cotamin
7	Sd12	240.00	287.00	482.00	.	.	.60	5.30	.	Yes
8	Sd14	148.00	357.00	326.00	4.40	81.14	1.10	4.70	.	Yes
9	Sd16	140.00	189.00	297.00	6.82	27.71	.64	9.80	acute rej	Yes
10	Sd19	73.00	249.00	230.00	5.12	48.63	1.08	8.70	.	Cotamin
11	Sd21	70.00	234.00	203.00	4.94	47.37	1.15	9.40	.	.
12	Sd23	162.00	359.00	193.00	7.77	46.20	1.86	12.00	.	.
13	Sd26	125.00	107.00	213.00	12.88	8.31	.50	9.90	acute rej	.
14	Sd28	102.00	135.00	239.00	6.82	19.79	.56	10.70	.	.
15	Sd30	479.00	381.00	291.00	5.52	69.02	1.31	2.70	.	.
16	Sd33	295.00	369.00	205.00	8.70	42.41	1.80	.	.	Yes
17	Sd35	159.00	351.00	174.00	5.32	65.98	2.02	.	.	Yes
18	Sd37	122.00	364.00	181.00	8.34	43.65	2.01	10.00	.	Yes
19	Sd41	60.00	621.00	184.00	7.12	87.22	3.38	10.30	.	.
20	sd44	64.00	547.00	191.00	9.66	56.63	2.86	15.80	.	.
21	sd49	91.00	663.00	187.00	8.34	79.50	3.55	.	.	.
22	sd51	160.00	933.00	210.00	12.00	77.75	4.44	12.00	.	.
23	sd53	159.00	936.00	198.00	.	.	4.73	.	.	.
24	sd56	69.00	671.00	198.00	8.73	76.86	3.39	18.50	.	.
25	sd62	180.00	980.00	194.00	8.16	120.10	5.05	13.70	.	.

	days	average	unox	creatini	u.creat	unoucrea	urincrea	fkl	ar	uti
26	sd65	151.00	726.00	183.00	4.43	163.88	3.97	13.40	.	.
27	sd72	119.00	698.00	205.00	12.83	54.40	3.40	10.80	.	.
28	sd75	74.00	374.00	178.00	12.56	29.78	2.10	9.50	.	Yes
29	sd79	121.00	624.00	173.00	4.35	143.45	3.61	18.10	.	.
30	sd86	105.00	459.00	201.00	13.10	35.04	2.28	8.30	no reject	Yes
31	sd89	120.00	225.00	205.00	14.85	15.15	1.10	13.70	.	Yes