The Role of Nitric Oxide in Renal Warm Ischaemia Reperfusion Injury

By

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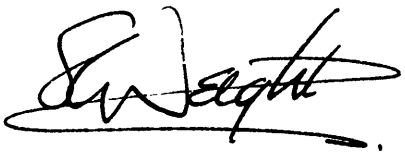

SC Weight

June 1998

To Alison & Sam

Finished despite the best of attentions!

If your experiment needs statistics, you ought to have done a better experiment.

Lord Rutherford

Publications Arising From This Work

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SC Weight, PN Furness, PRF Bell, ML Nicholson. A new model of renal warm ischaemia reperfusion injury. *Transpl Proc* 1997; 29: 3002-3003.

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SC Weight, PN Furness, ML Nicholson. Nitric oxide generation is elevated in renal warm ischaemia reperfusion injury. *Br J Surg* 1998; 85: 1663-1668.

SC Weight, ML Nicholson. Nitric oxide and renal reperfusion injury: a review. *Eur J Vasc Endovasc Surg* 1998; 16: 98-103.

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SYNOPSIS

The purpose of the work described in this thesis was twofold. Firstly, to determine what role nitric oxide (NO) played in renal warm ischaemia reperfusion injury and secondly, once the role was clarified, to assess if this injury could be ameliorated by the pharmacological manipulation of renal NO. The introduction to this thesis comprises two parts. Chapter 1 reviews the pathophysiology of renal ischaemia reperfusion and outlines some of the treatment modalities that have been used in response. Chapter 2 focuses on the physiology of NO and the pathophysiological role it plays in renal reperfusion injury and contrasts some apparent differences between the kidneys and other organs.

The three main experimental Chapters are then presented. The first of these describes the development and verification of a NO assay for use with renal tissue homogenate. The next Chapter describes the development of a new model of renal warm ischaemia reperfusion injury in a rodent model which allows the collation of renal NO levels with comparative renal functional, pathological and pathophysiological data. The final experimental Chapter details both the effect of warm ischaemia reperfusion injury on renal NO generation and the subsequent effect of pharmacologically manipulating NO on the degree of renal injury sustained. In the final Chapter the experimental results are reviewed and possible avenues of further investigation are discussed.

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LIST OF ABBREVIATIONS

$\delta A_{3\ min}$	Rate of change of absorbance over three minutes
µl	Microlitre
µM/µmol	Micromolar / micromoles
β-NADPH	Reduced nicotinamide adenine phosphate dinucleotide
^{99m}Tc DTPA	Technetium-DTPA
ADMA	Asymmetric dimethyl arginine
ADO	Adenosine
AG	Aminoguanidine
ANF	Atrial natriuretic factor
ATP	Adenosine triphosphate
ATP-MgCl	ATP-magnesium chloride
C	Control group
CGRP	Calcitonin gene-related peptide
CI	Confidence interval
cNOS	Constitutive nitric oxide synthase
COX	Cyclooxygenase
cpm	Counts per minute
DFO	Deferoxamine
DNA	Deoxyribonucleic acid
DSO	Desferrioxamine
D-to-O	(xanthine) dehydrogenase to oxidase
DTPA	Diethylene triamine penta-acetic acid
EDRF	Endothelium-derived relaxing factor
ET	Endothelin
f.c.	Final concentration
FAD	Flavin adenine dinucleotide
Fc	Fraction crystalline (antibody)
g	Gram
GFR	Glomerular filtration rate
H_2O_2	Hydrogen peroxide
HC	Hydrocortisone
H&E	Haematoxylin and eosin stain
HOCl	Hypochlorous acid
HPLC	High pressure liquid chromatography
hr	Hour
IRI	Ischaemia reperfusion injury
i.p.	Intraperitoneal
iNOS	Inducible nitric oxide synthase
J_{45}	via Jablonski method at 45min ischaemia

L-ARG	L-arginine
L-CITR	L-citrulline
L-NAME	N^G-nitro-L-arginine methyl ester
LOONO	Alkyl peroxynitrite
M/mol	Molar / moles
Mab	Monoclonal antibody
MBq	Millibecquerel
MD	Macula densa
min	Minute
ml	Millilitre
mM/mmol	MilliMolar / millimoles
MW	Molecular weight
N	Normal group
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NHBD	Non-heart beating donor
nM/nmol	nanoMolar / nanomoles
NO	Nitric oxide
NOS	Nitric oxide synthase
NRase	Nitrate reductase
O_2-	Superoxide anion
OFR	Oxygen free radicals
$OH^{\bullet}$	Hydroxyl radical
ONOO-	Peroxynitrite
PAF	Platelet activating factor
PGI_2	Prostacyclin
PLA2	Phospholipase A2
PMN	Polymorphonuclear (leucocyte)
RBF	Renal blood flow
S	Sham group
SMC	Smooth muscle cell
SNAP	Sodium nitroprusside
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TNF	Tumour necrosis factor
TxA_2	Thromboxane
w/v	Weight/volume ratio
WI	Warm ischaemia
WIRI	Warm ischaemia reperfusion injury
XD	Xanthine dehydrogenase
XO	Xanthine oxidase

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Chapter 1

A REVIEW OF THE PATHOPHYSIOLOGY OF RENAL ISCHAEMIA REPERFUSION INJURY

1.1 INTRODUCTION

Free radicals are ubiquitous toxic species. The over-production of these molecules during ischaemia-reperfusion is one of the factors responsible for the consequent cell injury and death. This introductory Chapter reviews the pathophysiology of such injury, contrasts post-ischaemic damage in the kidney with that seen in other tissues and surveys the current options available to ameliorate such injury.

1.2 CHEMISTRY OF FREE RADICALS

A free radical is any species containing one or more unpaired electrons which is able to exist independently. A normal covalent bond is comprised of a pair of electrons and it is the presence of an odd number of electrons in the outer orbital that renders free radicals chemically more active. If two radicals react then the unpaired electrons are extinguished and both radicals are eliminated. If however a radical and non-radical species react then a further free radical is necessarily produced and this capability to generate further molecules allow free radicals to initiate and propagate chain reactions (Dickstein, 1990).

Foremost within biological systems are the oxygen free radicals (OFR), with molecular oxygen (O_2) itself the most prevalent. Fortunately the reactivity of O_2 is limited by the particular configuration of the two unpaired electrons in its outer shell. Much more reactive is the superoxide radical (O_2-) and greater still is the hydroxyl radical ($OH^{\bullet}$). The nitrogen-centred free radical nitric oxide (NO) is now recognised as being of central importance (Radi et al, 1991), both in its own right and as peroxynitrite (ONOO-). This is formed in a reaction between NO and O_2- and is thought to decompose to the highly reactive $OH^{\bullet}$ and nitrogen dioxide (Beckman, 1990). Zweier et al (1987) have conclusively demonstrated using electron paramagnetic resonance spectroscopy that both oxygen and nitrogen-centred radicals are generated during tissue ischaemia-reperfusion. Of the many other radicals the transition metal ions are important, due to the ease with which they facilitate electron transfer; as is the iron-centred perferryl radical (Fe^{3+}-O_2-Fe^{2+}) (Bucher et al, 1983)., Hydrogen peroxide (H_2O_2) is formed *in vivo* by the dismutation of O_2- and, although reactive, possesses no unpaired electrons and thus is not a free radical. The reactivity

of H_2O_2 is low but homolytic fission of the oxygen-oxygen bond leads to the formation of $OH^{\bullet}$ (Biemond et al, 1986; Halliwell, 1987; Grace, 1994).

1.3 CELLULAR PRODUCTION OF FREE RADICALS

Production of free radicals can be ascribed to three main areas:

(1) Exogenously derived production.

Electromagnetic radiation and heat can induce the formation of intracellular radicals, hence the term ionising radiation (Reilly et al, 1991).

(2) Endogenous physiological production.

During oxidative phosphorylation a proportion of the oxygen 'leaks' out of the mitochondria. This is then available to undergo an autocatalytic reduction (Turrens & Boveris, 1980) in the presence of reduced nicotinamide adenine dinucleotide (NADH) with the consequent generation of O_2-. This low-grade production presumably accounts, at least in part, for the prevalence of the O_2- scavenger superoxide dismutase (SOD) within both the cytosol (McCord & Fridovich, 1969) and the mitochondria (Freeman & Crapo, 1982).

(3) Endogenous pathophysiological production.

1.3a Xanthine oxidase system

During metabolism, the purine substrate hypoxanthine is oxidised to xanthine and then to uric acid. This reaction is normally catalysed by the enzyme xanthine dehydrogenase (XD), with the concomitant reduction of nicotinamide adenine dinucleotide (NAD) to NADH (Stripe & Della Corte, 1968; McCord, 1985). Xanthine dehydrogenase also exists as the xanthine oxidase (XO) isoform and catalyses the reaction via the transfer of an electron to molecular oxygen with the generation of O_2- (McCord & Fridovich, 1968). Conversion of XD to XO (D-to-O) is effected by a calcium dependent protease which is triggered by ischaemia (Parks et al, 1985), tumour necrosis factor (TNF), interleukin 1 and 3, neutrophil elastase, the fifth component of the complement system (C5) and the chemotactic peptide N-formyl-Met-Leu-Phe (fMLP) (Friedl et al, 1989. Bulkley, 1993).

Although the exact mechanism has yet to be determined, it appears that ischaemia, in leading to a depletion in adenosine triphosphate (ATP) (Green et al, 1989a) has two sequelae: a rise in hypoxanthine (Mandel et al, 1988) (which is a breakdown product of ATP metabolism) and the dysfunction of ATP-dependent membrane ion pumps. This latter effect leads to a selective rise in both mitochondrial (Wilson et al, 1984) and intracellular calcium (Snowdowne et al, 1985; Fuller et al, 1988) and thus D-to-O conversion. Whilst the ischaemic conditions persist hypoxanthine cannot be oxidised and therefore accumulates. Upon reperfusion, a relative abundance of oxygen is available to oxidise the accumulated substrate with consequent XO-dependent production of O_2-. Fluctuation in the oxygen delivery to the reperfused tissue may accentuate the damage as this has been shown to upregulate XO (Hallett et al, 1985).

The duration of ischaemia required for D-to-O conversion has been demonstrated to vary from organ to organ. The gastrointestinal (GI) tract is apparently the most susceptible with D-to-O conversion taking 10 seconds in a rat model, whilst it takes 30 minutes in the kidney (McCord, 1985). More recently however, Parks et al (1987) have shown that D-to-O conversion in the GI tract of the cat was less than 70% complete after 3 hours of ischaemia. Such a wide disparity suggests more than just a species-specific difference.

1.3b The Fenton Reaction.

With the production of the superoxide radical via the XO pathway the generation of $OH^{\bullet}$ through the Fenton reaction can occur. Superoxide first reduces ferric iron to the ferrous state which then donates the electron to H_2O_2 with subsequent formation $OH^{\bullet}$ (Halliwell & Gutteridge, 1984; Reilly et al, 1991). This reaction occurs at a rate sufficient to exceed the quenching capacity of the free radical scavengers (Freeman & Crapo, 1982). In fact the rate limiting step is the production of H_2O_2 (Baker & Gebicki, 1986) and not the availability of iron because both XO and O_2- are able to mobilise iron from the intracellular storage protein ferritin (Mazur & Carleton, 1965; Biemond et al, 1984). Angel et al (1986) have suggested that haemoglobin can act as a source of iron for the Fenton reaction *in vivo* although *in vitro* experiments have had conflicting results (Sadrzadeh et al, 1984; Baker & Gebicki, 1986). Certainly

haemoglobin is not essential as a source of iron. In a renal model an increase in free iron was seen during reperfusion with an oxygenated asanguinous solution (Green et al, 1989a).

1.3c The Plasma Membrane

In addition to being one of the sites of free radical induced cell injury the cell membrane can act as a source of OFR generation. Following ischaemia-reperfusion the increase in intracellular calcium is thought to activate phospholipase A2 (PLA2) which initiates the arachidonic acid cascade (Welbourn et al, 1991; Koike et al, 1992). The generation of the metabolites of this cascade, i.e., thromboxane, prostaglandins and leukotrienes (Lelcuk et al, 1985a; Klausner et al, 1988a; 1989a; 1989b) is associated with the concomitant production of free radicals (Freeman & Crapo, 1982). Furthermore, H_2O_2 has been shown to activate cyclooxygenase (Ullrich et al, 1989) and this presumably potentiates the cascade.

1.4 CELLULAR TARGETS FOR FREE RADICAL INJURY

The highly reactive nature of free radicals dictates that their site of action is very close to their site of formation. It has been suggested therefore that the lipid membrane is a central target in OFR-mediated cellular damage. This process of lipid peroxidation, which may be initiated by iron (Halliwell & Gutteridge, 1984) or free radicals (Radi et al, 1991) and can become autocatalytic, produces malondialdehyde as one of the metabolites. Many of the *in vivo* measurements of lipid peroxidation are based on the reaction of malondialdehyde with thiobarbituric acid (the TBA test) as described by Ohkawa et al (1979). Doubts have been cast on the validity of this test however (Fuld & Urbaitis, 1986) because of the possibility of contamination of the sample with blood, which demonstrates a similar absorption peak as malondialdehyde. Utilising the more specific high performance liquid chromatography (HPLC) McCoy et al (1988) found that there was little lipid peroxidation after ischaemia-reperfusion in the rat kidney. Furthermore Ceconi et al (1991) compared HPLC directly with the TBA test in rabbit hearts exposed to ischaemia-reperfusion and demonstrated no correlation between the two tests and concluded that TBA overestimated the extent of lipid peroxidation.

Also at risk of oxidative damage are proteins, both free and membrane bound, structural and enzymatic, which are denatured by free radicals. The other major cellular target is deoxyribonucleic acid (DNA), with one of the major oxidative products being 8-oxydeoxyguanosine (Aruoma et al, 1989). The consequent hydroxylation, cross-linking and scission of DNA can result in cell death (Reilly et al, 1991).

1.5 THE ROLE OF POLYMORPHONUCLEAR LEUCOCYTES

1.5a Attraction, Activation and Diapedesis

The polymorphonuclear leucocyte (PMN) is thought to play a central role in the evolution of post-ischaemic injury. Like the endothelial cell (Ratych et al, 1987) the PMN is intrinsically capable of producing free radicals in ischaemia-reperfusion; using the same mechanism as during phagocytic destruction (Babior, 1978; 1984). The primary event is the chemoattraction of PMN, followed by their margination and diapedesis.

Initial PMN chemoattraction is mediated by the endothelial cells. Xanthine oxidase, whether directly or via the secondary production of OFR, has been demonstrated to have a chemoattractive effect (Hernandez et al, 1987), as has platelet activating factor (PAF) which is released from hydrogen peroxide stimulated endothelial cells (Lewis et al, 1988). Both thromboxane and leukotrienes are also implicated in this white cell sequestration (Klausner et al, 1989c; Paterson et al, 1989c; Goldman et al, 1991). Rubin et al (1990) have demonstrated that after ischaemia-reperfusion injury in canine muscle leucosequestration lasts more than 48 hours.

Margination is a complex sequence involving an interaction between adhesion molecules on the endothelial cells (ICAM & VCAM and the E & P selectins) and on the granulocytes (L selectin, CD14 and the β integrins, especially the CD11/CD18 complex). These molecules mediate adhesion, activation and migration (Beekhuizen & van Furth, 1993; Windsor et al, 1993). Adhesion is essential (Carden et al, 1990a) and takes place in two phases, firstly through the rolling of PMN on the endothelial selectin CD62, a process that is reversible and calcium dependent (Lawrence & Springer, 1991). Then CD18 expression is upregulated possibly secondarily to the

action of a protease enzyme released from the granulocyte (Von Andrian et al, 1991). The adhesion of PMN through the CD11/18 complex is a prerequisite for the cytokine-induced respiratory burst (Nathan et al, 1989). During ischaemia, endothelial cell transcription and secretion of endothelin (ET) is amplified (Kourembanas et al, 1991) leading to both an upregulation of CD11/CD18 expression (López-Farré et al, 1993) and to the activation of the alternative pathway of the complement system (Beekhuizen & van Furth, 1993), with the subsequent generation of further chemoattractant species.

Once adhesion has occurred the system is augmented by endothelial cell secretion of cytokines. The most important of which, in this context, are PAF and interleukin-8 (IL-8) (Baggiolini et al, 1989) with TNF also released, at least during hepatic ischaemia (Colletti et al, 1990). These recruit and activate further PMN (Gónzalez-López et al, 1985) and also upregulate the expression of E-selectin (Bevilacqua et al, 1989).

1.5b Leucocyte Mediated Injury

An activated PMN can cause tissue destruction through three pathways: (a) production of free radicals via the respiratory burst; (b) release of intrinsic enzymes, (c) physical obstruction of capillaries.

The site of production of OFR has been localised to a membrane bound NADPH oxidase. This generates O_2- (Babior & Peters, 1981) in a graded response to stimulation (Bass et al, 1983), enhanced by the local acidic microenviroment (Baker & Gebicki, 1986). The superoxide radical is then converted to the relatively stable oxidant hypochlorous acid (HOCl) in a reaction with H_2O_2, catalysed by the enzyme myeloperoxidase which is stored within PMN granules (Weiss, 1989).

Marletta et al (1988) demonstrated that activated PMN are also capable of producing the free radical NO and this has subsequently been confirmed by others (Albina et al, 1989. McCall et al, 1989). However, whether NO is responsible for significant post-ischaemic tissue damage is unclear as Bassenge (1992) has suggested that the release of NO is impaired in ischaemia-reperfusion. This will be discussed further in Chapter 2.

The second pathway is through the release of enzymes located within the PMN granules. Of the 20 or more enzymes thus present, elastase has been demonstrated to be especially destructive although proteinase, collagenase and gelatinase enzymes are also released (Smedly et al, 1986; Carden et al, 1990b). Here again the pivotal role of OFRs are once more evident. The body's natural defence against PMN elastase includes α1-antitrypsin, α2-macroglobulin and secretory leucoproteinase inhibitor and these are all oxidised and thus inactivated by HOCl. Furthermore, it has been demonstrated that HOCl activates both the collagenase and gelatinase enzymes (Weiss, 1989).

Thirdly, it has been shown by Bagge et al (1980), utilising vital microscopy in a feline model of hypovolaemic shock, that capillaries are physically plugged by PMN, with an inevitable impairment in the microcirculation. The concept of this "no-reflow" phenomenon in extending an ischaemic insult has been widened to encompass ischaemia-reperfusion injury (Engler, 1987; Carden et al, 1990a). The evidence indicates that it is ischaemia per se that leads to PMN plugging (Engler et al, 1983). Plugging may be due to an increase in PMN stiffness rather than through integrin/selectin adhesion, for it has been demonstrated that activation renders PMN less deformable (Windsor et al, 1993). Furthermore, the use of a monoclonal antibody (Mab) to block the CD11/CD18 complex has been shown not to limit PMN plugging (Worthen et al, 1989).

1.6 SYSTEMIC EFFECTS OF ISCHAEMIA-REPERFUSION INJURY

Damage is not limited to the post-ischaemic tissue alone but can be demonstrated to involve distant organs. Widely investigated is the link between ischaemia-reperfusion and pulmonary injury in the form of respiratory distress syndrome (RDS). Reperfusion following lower limb ischaemia in both animal and human models leads to a rise in the plasma levels of thromboxane (TxA2) and leukotriene (LTB4) with increased pulmonary microvascular permeability (Anner et al, 1988) and white cell sequestration (Paterson et al, 1989a, 1989b).

The lung contains a large number of PMN (about 50% of the total population) which are physically impeded within the capillaries due to their large size (Lien et al, 1987) although they remain in constant flux with the circulating population. Increased

leucocyte sequestration occurs following activation because they are rendered less deformable and thus less able to traverse the capillary bed (Worthen et al, 1989). These activated PMN then degranulate (Windsor et al, 1993) and almost certainly produce free radicals (Klausner et al, 1989a). Experiments using leucocyte-depleted blood, which ameliorates the pulmonary injury, have added credence to this model (Klausner et al, 1988b) although this cannot be the whole story as RDS can still develop in severely neutropenic patients (Ognibene et al, 1986).

Activated PMN have also been demonstrated to accumulate in the heart after renal ischaemia (López-Farré et al, 1993) and Bernier et al (1989) have shown that OFR generated within the myocardium increase the incidence of ventricular fibrillation. A further example of the potential for neutrophils to amplify ischaemic injury has been elegantly demonstrated by Linas et al (1992). They showed that mildly ischaemic rat kidneys suffer severe damage if perfused by primed PMN but not if non-primed neutrophils were used. Such priming can take place in the GI tract as outlined below.

The GI tract has long been thought of as pivotal. The original hypothesis was that gut ischaemia allowed transmural bacterial translocation with subsequent sepsis and the risk of multi-organ failure (MOF) (Rush et al, 1988; Wilmore et al, 1988). Recent evidence is at odds with this theory however for such bacterial translocation occurs infrequently (Moore et al, 1992) and endotoxaemia cannot be held responsible either (Koike et al, 1994). An alternative explanation is that ischaemia-reperfusion leads to activation of phospholipase A2 (PLA2), which is highly concentrated in the gut mucosa (Koike et al, 1992), with subsequent leucocyte activation. In support of this is a study by Vedder et al (1989) which indicated that MOF following hypotensive shock could be reduced by preventing PMN adhesion with a monoclonal antibody.

One further mechanism exists through which gut ischaemia-reperfusion can have systemic complications. Abdominal chemosensitive sensory nerve fibres are stimulated by $OH^{\bullet}$ and H_2O_2 and this reflexly excites the cardiovascular system (Stahl et al, 1992; 1993).

1.7 ENDOTHELIN AND NITRIC OXIDE

The endothelium produces these two vasoactive substances which are important in cardiovascular and renal microvascular homeostasis (Parrella et al, 1991; Lerman & Burnett, 1992). Within the arterial circulation nitric oxide (NO) tends to reverse the vasoconstrictive effects of endothelin (ET) whereas endothelin predominates over NO-derived vasodilation within veins (Lüscher et al, 1990). In ischaemia-reperfusion the physiological ratio of ET to NO is altered and this may be central to the resultant injury.

Endothelin, a 21-residue peptide, is the most potent vasoconstrictor yet identified (Yanagisawa et al, 1988) and the kidney is around 10 times more sensitive to this effect than other vascular beds (Vane et al, 1990). As mentioned previously, ischaemia leads to a prolonged increase in endothelial cell transcription and secretion of ET.

Palmer et al (1987) have shown that endothelial derived relaxing factor (EDRF) is identical to NO. Currently four isoforms of nitric oxide synthase (NOS) have been identified (Marsden et al, 1992; Lamas et al, 1992; Nishida et al, 1992; Lowenstein & Snyder, 1992). NOS I and NOS III are both constitutive enzymes (cNOS) that are activated by calcium and calmodulin. NOS II and NOS IV are inducible (iNOS) whereby expression is dependent upon transcription and once induced the activation is prolonged (Bachmann & Mundel, 1994). In the kidney, NOS I is predominantly expressed in the macula densa (Ujiie et al, 1994) where the NO produced may act in a paracrine fashion both to control arteriolar vascular tone and local renin release (Mundel et al, 1992; Johnson & Freeman, 1994). NOS III is expressed within the arteriolar endothelium whilst NOS II has been localised principally to the preglomerular portion of the afferent arteriole, as well as the mesangial cells (Bachmann & Mundel, 1994).

The role of NO in renal ischaemia reperfusion injury (IRI) will be discussed in depth in Chapter 2. Renal ET production is increased in IRI (Kosaka et al, 1989; Kon & Badr, 1991). This impairs renal function by decreasing both renal blood flow and glomerular filtration rate with an associated increase in plasma renin and atrial natriuretic factor (Firth et al, 1988; King et al, 1989; Miller et al, 1989). López-Farré

et al (1991a, 1991b) have suggested that the renal effects of ET are mediated through the release of PAF which is produced within the glomerulus (Pirotzky et al, 1984; Schlondorff et al, 1986). In a rabbit model, PAF has been demonstrated to cause vasodilation when present at low concentrations (Juncos et al, 1993), possibly via NO as cNOS has been shown to be upregulated by PAF (Radomski et al, 1990; Bachmann & Mundel, 1994). At higher concentrations however PAF causes vasoconstriction, which may be mediated via the products of the cyclooxygenase pathway (Juncos et al, 1993).

1.8 RENAL SPECIFIC FACTORS

As will be seen in the next section on therapeutic intervention, marked anomalies are apparent when comparing different organs. Several factors stand out to account for the pathophysiology encountered in renal ischaemia-reperfusion injury.

Morphological studies have demonstrated that ischaemia irreversibly damages the distal segment of the proximal tubule (S3) whilst the more proximal segments, i.e., S1 & S2, suffer reversible injury. The S3 cells undergo necrosis and are shed into the tubule lumen (Venkatachalam et al, 1978) and it is thought this causes a physical blockage accountable, at least in part, for the decrease in the glomerular filtration rate (GFR).

Mason et al (1984; 1985; 1987) have demonstrated that renal ischaemia leads to erythrocyte sludging in the outer medullary inner stripe capillaries which is not ameliorated by prior treatment with acetyl salicylic acid or heparin. However a reduction of the haematocrit to about 30% prevented this phenomenon and improved renal perfusion and post-ischaemic function.

Camazine et al (1976) have shown that a proportion of the mesangial glomerular cells express Fc and C3 receptors and are highly phagocytic. Therefore they are presumably capable of generating OFR and this may account in part for the equivocal role demonstrated by PMN in renal ischaemia-reperfusion injury (Thornton et al, 1989; Paller, 1989).

With such a complex, interrelated sequence of events (Bulkley, 1987), the difficulty encountered in effectively treating ischaemia-reperfusion injury is understandable. This problem is compounded because animal models are not necessarily comparable with the human situation. Southard et al (1987) have shown that human (and canine) kidneys have a SOD to XO ratio 40 times greater than that seen in the rat and thus may well be less susceptible to OFR damage. Further discrepancies have also been documented between rat and canine kidneys whereby post-ischaemic RBF is commonly maintained in the latter but not the former (Williams et al, 1981). In addition, Jablonski et al (1983) have demonstrated in a rodent renal model that the marked degree of morphological damage seen does not correlate with the relative preservation of function.

1.9 THERAPEUTIC INDICATIONS & INTERVENTIONS

Advances in the understanding of the pathogenesis of ischaemia-reperfusion injury have opened the way for therapeutic intervention. However, singly or in combination, such measures have afforded only partial protection *in vivo*; and the success varies from organ to organ.

1.9a Intervention within the Ischaemic Phase

Two main areas lend themselves to intervention. Firstly, the conversion of XD-to-XO and secondly the reduction in high energy phosphate compounds (Baud & Ardaillou, 1993).

The conversion of XD-to XO is protease dependent. The use of the protease inhibitor aprotinin to block this conversion has reportedly led to a significant improvement in the degree of post-ischaemic muscle oedema and thus by implication a decrease in microvascular damage (Korthius et al, 1985). Other explanations for this benefit are possible however. Hallett et al (1985) have demonstrated that aprotinin inhibits the PMN respiratory burst. In addition, protease inhibitors may limit the expression of CD18 and thus PMN adhesion (von Andrian et al, 1991). One final role for aprotinin is possible. Calcitonin gene-related peptide (CGRP) has been shown to be the most potent vasodilator yet discovered (Brain et al, 1985; O'Halloran & Bloom, 1991) and a physiological role for CGRP has been postulated. It acts by opening an ATP

sensitive potassium channel (Nelson et al, 1990) and is broken down by the action of protease enzymes (Brain & Williams, 1988). Thus it may be that by inhibiting this proteolysis aprotinin increases the level of CGRP in post-ischaemic tissues.

Several studies have demonstrated the role of calcium channel blockers in improving primary graft function following renal transplantation (Wagner et al, 1987; Frei et al, 1987; Neumayer et al, 1989). Green et al (1989a) have suggested this effect is due to the prevention of calcium influx during the ischaemic phase. The fact that D-to-O conversion is calcium dependent (McCord, 1985) may be one reason that calcium channel blockers ameliorate injury. In addition, calcium influx into the cytosol activates phospholipases which potentiate membrane damage, arachidonic acid metabolism and mitochondrial injury (Finn, 1990). Other possible modes of action for calcium channel blockers exist. By decreasing calcium influx into erythrocytes during ischaemia red blood cell deformability is maintained which aids microvascular perfusion (Van Neuten & Vanhoutte, 1980). More importantly perhaps, endothelin induced vasoconstriction is reliant on calcium influx and this has been shown to be antagonised by nicardipine (Yanagisawa et al, 1988).

Although ATP levels fall quickly during ischaemia, tissue viability is not affected in such a precipitant manner. It appears that the ability to resynthesise ATP after ischaemia is more crucial than the diminution per se (Neumayer et al, 1989). *In vitro* studies using rabbit renal proximal tubules have demonstrated that the administration of extracellular ATP-$MgCl_2$ significantly improves cellular respiratory function during the recovery from anoxia (Takano et al, 1985). Another report suggested this treatment restored membrane permeability towards normal (Chaudry et al, 1981) which was confirmed by Mandel et al (1988) who also found complete recovery of cellular ATP levels with the extracellular administration of ATP-$MgCl_2$, ADP-$MgCl_2$ (adenosine diphosphate) or AMP-$MgCl_2$ (adenosine monophosphate).

The results obtained with the extracellular administration of adenosine (ADO) are less clear-cut. Although *in vitro* studies (Mandel et al, 1988) suggest that the regeneration of intracellular ATP is dependent on extracellular ADO, the addition of ADO-$MgCl_2$ to the post-ischaemic rat kidney demonstrated little benefit on renal function (Sumpio et al, 1987). One reason for the discrepancy between *in vitro* and *in*

vivo results may be because ADO exerts a negative influence on *in vivo* renal function by causing afferent arteriolar constriction (Homer-Vanniasinkam et al, 1994). Interestingly it has been shown that sufficient endogenous adenosine is produced during renal ischaemia to cause such vasoconstriction (Miller et al, 1978). Adenosine not only increases cellular ATP, but has also been demonstrated to inhibit O_2- production in activated PMN by binding to neutrophil A_2 receptors (Granger et al, 1987; McGarrity et al, 1989; Hsu et al, 1994).

1.9b Manipulation of Free Radical Production

Superoxide dismutase (SOD) is an OFR scavenger whilst allopurinol is a xanthine oxidase inhibitor (Green et al, 1989b). In most models of renal ischaemia the administration of intravenous SOD or allopurinol prior to reperfusion has demonstrated a protective effect (Paller et al, 1984; Baker et al, 1985; Green et al, 1989b; Paller et al, 1991). Gamelin and Zager (1988) however failed to demonstrate any such benefit and indeed question whether OFRs are consistent mediators in post-ischaemic renal failure.

Catalase, a H_2O_2 scavenger, has not demonstrated protection in the post-ischaemic kidney, possibly because it's large size limits glomerular filtration and thus its availability at the site of injury (Paller et al, 1984). Alternatively it may be because SOD, but not catalase which specifically scavenges O_2-, prevents both the quenching of NO (Gryglewski et al, 1986; Rubanyi & Vanhoutte, 1986; Lüscher, 1992) and subsequent formation of peroxynitrite (Beckman et al, 1990). It also inhibits the superoxide dependent Fenton reaction, with the subsequent initiation of lipid peroxidation (Thomas et al, 1985). However catalase as well as SOD and allopurinol has been shown to diminish the injury in post-ischaemic heart and skeletal musculature (Korthius et al, 1985; Aoki et al, 1988; Bernier et al, 1989). In the gut, Droy-Lefaix et al (1991) demonstrated that the administration of SOD prior to ischaemia improved the histological outcome.

Several other compounds have shown promise as OFR inhibitors. Histidine, which is another O_2- scavenger, offers myocardial protection during reperfusion (Kukreja & Hess, 1992), whilst *in vitro*, Ebselen (Cotgreave et al, 1989) and Nafazatrom (Bednar

et al, 1985) have been demonstrated to inhibit superoxide production in the leucocyte and also migration in the latter case.

Further along the pathway, the use of iron chelators to minimise the production of $OH^{\bullet}$ via the Fenton reaction has shown partial success. Desferrioxamine (DSO) and deferoxamine (DFO) have been the agents commonly used, both *in vitro (*Paller et al, 1984; Stahl et al, 1992*)* and *in vivo.* Menasché et al (1988; 1990) have demonstrated that DFO limits OFR production and lipid peroxidation associated with human cardiopulmonary bypass. Similar results have been obtained in post-ischaemic skeletal muscle (Fantini & Yoshioka, 1993). In a series of experiments in rabbits, Green et al 1986a, 1986b, 1989a) have demonstrated the protective effect of DSO administration on reperfused kidneys after exposure to either cold or warm ischaemia.

In an endeavour to avoid the toxicity and short half-life of these chelators others have been sought by binding DFO to biocompatible polymers (Hallaway et al, 1989). However care is needed because some chelators allow lipid peroxidation to continue after the iron has been chelated (Mostert et al, 1987). Also dextran conjugated DFO does not undergo renal filtration and thus in the rat provided no protection for the post-ischaemic kidney (Paller & Hedlund, 1988).

1.9c Membrane Stabilisers

In a model of cardiac ischaemia chlorpromazine has been shown to protect the cell membrane from damage, with a temporally associated inhibition of calcium uptake (Chien et al, 1979). Similarly, Pavlock et al (1981) demonstrated that two hours of warm ischaemia-reperfusion in the rabbit kidney led to severe mitochondrial damage that was completely ameliorated by pretreatment with chlorpromazine. Such protection was not seen if the warm ischaemia was extended to 3 hours.

A further group of compounds recently described are the 21-aminosteroids, or lazaroids, which can inhibit both iron-catalysed lipid peroxidation and arachidonic acid metabolism (Reilly et al, 1991. Kirschner & Fantini, 1994). The use of lazaroids in rat skeletal muscle subjected to ischaemia-reperfusion has demonstrated significant preservation of myocyte viability (Homer-Vanniasinkam et al, 1993).

1.9d Manipulation of Arachidonic Acid Metabolism

Intervention at most stages during the conversion of arachidonic acid to its active metabolites has been attempted. Early inhibition of the pathway by use of the cyclooxygenase inhibitor ibuprofen prevents the rise in TxA_2 in post-ischaemic kidneys. However it also prevents an increase in prostacyclin (PGI_2) which has been shown to ameliorate the injury (Klausner et al, 1989b). The use of a specific thromboxane synthetase inhibitor circumvents this allowing an increase in PGI_2 and, in the renal model, prevents histological damage (Lelcuk et al, 1985b). Others have demonstrated similar benefit in skeletal muscle injury using both thromboxane synthetase inhibitors and thromboxane receptor blockers (Paterson et al, 1989c. Homer-Vanniasinkam et al, 1994).

In addition, there is a further mechanism to account for the efficacy of thromboxane receptor blockers within the kidney. It is apparent that in renal ischaemia-reperfusion a vasoconstrictive prostaglandin (8-epi-prostaglandin $F_{2\alpha}$) is active. This is produced via lipid peroxidation through a non-cyclooxygenase pathway and exerts its effects through TxA_2 receptors (Morrow et al, 1990. Takahashi et al, 1992). This alternative pathway suggests a further reason for the poor efficacy of cyclooxygenase inhibitors.

An alternative strategy to increase the PGI_2:TxA_2 ratio is to administer the stable, semisynthetic prostacyclin analogue iloprost. Neumayer et al (1986) demonstrated that a seven day intra-aortic infusion of iloprost following renal ischaemia-reperfusion injury in dogs led to early maintenance of the renal blood flow (RBF) and a significantly improved GFR by the third day when compared to the controls. Such invasive administration may not be necessary however because when iloprost is added to the perfusate in cadaveric renal transplants a similar improvement in function is seen as well as a decrease in the incidence of primary non-function of the graft (Frei et al, 1987). Using a different PGI_2 analogue, Suzuki et al (1991) have shown that pretreatment prior to hepatic ischaemia in the rat results in improved cell survival as measured by both morphological and biochemical criteria.

Mannitol has also been demonstrated to offer protection in renal ischaemia-reperfusion injury (Pavlock et al, 1981; Green et al, 1986a) and to diminish TxA_2

production (Paterson et al, 1989a). This is probably secondary to its property as a free radical scavenger rather than any direct effect on TxA_2 production.

1.9e Cytokines & Chemoattractants

Complement depletion has been shown to diminish the size of infarcted myocardium in experimental models of cardiac ischaemia (Pinckard et al, 1980; Weisman et al, 1990). Such protection is not seen however in the pulmonary injury which follows lower limb ischaemia (Klausner et al, 1989a) although anti-tumour necrosis factor (TNF) antiserum given prior to hepatic ischaemia-reperfusion has been shown to prevent the secondary pulmonary microvascular injury (Colletti et al, 1990). Direct ischaemic lung injury can be ameliorated by a monoclonal antibody (Mab) to interleukin-8 (IL-8) (Sekido et al, 1993). The use of NOS inhibitors have been shown to reduce post-ischaemic myocardial and skeletal muscle injury (Sternbergh et al, 1992; Matheis et al, 1992) respectively.

The infusion of a PAF antagonist prior to inducing ischaemia demonstrates a protective effect in both renal and gut models (Droy-Lefaix et al, 1991; Torras et al, 1993). As PAF is thought to mediate the effects of endothelin within the ischaemic kidney it is not surprising that use of an anti-endothelin antibody also improves post-ischaemic renal function (Kon et al, 1989).

1.9f Manipulation of Leucocyte Function and Involvement

The use of a Mab to the leucocyte CD11/18 complex has been shown to prevent both adhesion and migration, although endothelial rolling is not affected (Afors et al, 1987). In skeletal muscle such a Mab has been demonstrated to prevent post-ischaemic microvascular damage (Carden et al, 1990a). A similar Mab also decreased the histological size of experimentally induced myocardial infarction by almost 50% (Simpson et al, 1988). Protection was not evident, however, in a renal model of ischaemia-reperfusion (Thornton et al, 1989).

The use of leucocyte-depleted blood for the reperfusion of ischaemic tissue is a further strategy. The relative ease with which this can be achieved during cardiopulmonary bypass has seen it used in clinical practice. During cardiac

transplantation, Pearl et al (1992a; 1992b) have demonstrated a decrease in cardiac biochemical dysfunction and histological damage, although without any apparent functional benefit. Several other studies concur with these results (Pillai et al, 1990; Bando et al, 1991). Breda et al (1989) found that PMN depletion almost completely preserved subsequent myocardial function in isolated neonatal piglet hearts after 12 hours of cold ischaemia (CI). Reperfusion with PMN depleted blood, achieved using anti-neutrophil serum, does not demonstrate such clear results on post-ischaemic renal function. Although Klausner et al (1989d) found significant benefit others have found no protective effect, either functionally (Paller, 1989) or morphologically (Thornton et al, 1989).

1.9g Other Strategies

Mandel et al (1990) have used extracellular glutathione, a OFR scavenger, to protect renal proximal tubules from anoxia *in vitro*, with an improvement in function during reoxygenation. This cytoprotective effect appears related to the intracellular production of glycine from glutathione rather than a direct effect.

Exogenous thyroid hormone affords protection in kidneys subjected to ischaemia-reperfusion (Neumayer et al, 1989). This may be due to the stimulation of mitochondrial respiration.

One further treatment merits comment. Atrial natriuretic factor (ANF) is a polypeptide hormone that is stored within atrial granules and also non-cardiac sites most notably the kidney. Functionally ANF is natriuretic, hypotensive and inhibits renin and aldosterone secretion (De Bold, 1985). Shaw et al (1987) demonstrated functional and morphological protection in renal ischaemia-reperfusion when ANF was administered after the ischaemic period. The ANF appeared to increase the GFR by causing efferent vasoconstriction and thus increasing glomerular capillary pressure (Schafferhans et al, 1986). However the marked systemic hypotensive effect of ANF has precluded clinical trials. Conger et al (1989, 1991) circumvented this by infusing dopamine with the ANF. They found that the post-ischaemic renal GFR was maintained at control levels and the RBF was significantly elevated. This was as a result of an increase in both glomerular plasma flow rate and glomerular capillary pressure, leading to a doubling in the single nephron glomerular filtration rate.

1.10 RENAL TRANSPLANTATION

In many clinical situations the ischaemic damage is a fait accompli whilst the clinician does have a degree of control over the injury sustained during reperfusion of the tissue. One area where the ischaemic injury is amenable to greater intervention is solid organ transplantation.

The differential between the number of renal transplants performed and those on dialysis awaiting a graft is widening. Thus the expansion of the renal transplantation program is essential but the traditional source of kidneys, from cadaveric donors via the intensive care unit, is diminishing (Varty et al, 1994a). A further potential source is through non-heart beating (NHBD) donation (Koostra et al, 1991; Kozaki et al, 1991) and this has refocused attention on the issue of ischaemic damage. NHBD grafts are exposed to significant warm ischaemia (WI) and consequently have a higher incidence of delayed primary function and acute tubular necrosis (ATN) than cadaveric grafts (Rigotti et al, 1991).

Less than 1 hour of human renal WI is not usually associated with anything more than temporary dysfunction, whilst 3 hours or more results in irreversible damage (Calne et al, 1963). Within these bounds of WI lies a therapeutic window. A similar opportunity exists during CI preservation (where the duration of CI lies between 18 and 48 hours) when treatment with SOD or allopurinol is efficacious. In a canine model, Satoh et al (1993) have demonstrated that 30 minutes of WI followed by cold storage preservation leads to an endothelium dependent rise in renal vascular resistance that may be a result of impaired NO secretion. Beyond this therapeutic window however, the renal damage is a consequence of the ischaemia *per se* (Koyama et al, 1985; Hoshino et al, 1988; Schneeberger et al, 1990) and treatment is without benefit. Indeed, it has been demonstrated that free radical production within post-ischaemic tissue actually diminishes with prolonged ischaemia (Henry et al, 1993).

The duration of ischaemia not only impacts on initial graft function however, for several studies have linked prolonged CI to the development of chronic vascular rejection. The somatastatin analogue angiopeptin has been shown to prevent or delay

this and is currently being evaluated clinically (Yilmaz et al, 1992; Wanders et al, 1993).

Renal preservation prior to transplantation can be achieved in one of two ways: on a continuous perfusion machine or by cold storage. Simple cooling allows preservation for 12 hours (Calne et al, 1963) by slowing down cellular reactions (Levy, 1959) whilst this can be extended to 72 hours by flushing the kidney with UW (University of Wisconsin) solution (Belzer & Southard, 1988) prior to cold storage. This solution contains glutathione and adenosine to aid the regeneration of ATP, lactobionate, phosphate and raffinose to prevent cellular swelling and several other components (Southard et al, 1990) including allopurinol. Although this solution is buffered to pH 7.4 a recent report has shown that systemic alkalosis helped to preserve post-ischaemic renal function in rats (Lennon et al, 1993) and this warrants further investigation.

1.11 CONCLUSION

Free radical species can be seen to be responsible for a wide spectrum of damage at a cellular level. This is not only true in reperfusion injury for OFR have also been linked to the development of cancer, rheumatoid arthritis (Halliwell & Gutteridge, 1984) and systemic lupus erythematosus (SLE) (Blount et al, 1991) as well as immune-mediated renal damage (Oberle et al, 1992).

In the majority of models discussed ischaemia primes the tissue for the injury incurred during reperfusion, primarily through XD-to-XO conversion and the secretion of chemoattractants. In the kidney however the situation is somewhat more complex, for in addition to priming the tissue, ischaemia also causes irreversible proximal tubule damage.

In summary, the effective treatment of ischaemia-reperfusion injury remains elusive. It may well transpire that combination strategies aimed at multiple sites in the pathway of OFR generation and damage offers the best hope. However, the evident species and organ specific differences will continue to hamper a quick resolution. Although the place of OFR in general during IRI has been well established the specific role of NO has not been determined. The next Chapter summarises current

knowledge regarding both the physiology of NO in normal renal homeostasis and the pathophysiology of NO in renal IRI, which remains contentious.

Chapter 2

NITRIC OXIDE: A REVIEW OF THE BIOLOGY, PHYSIOLOGY AND PATHOPHYSIOLOGY IN ISCHAEMIA REPERFUSION INJURY

2.1 CLINICAL BIOLOGY

Research into the clinical role of nitric oxide has expanded dramatically over the past decade, leading to Science declaring it the molecule of the year in 1992 (Koshland, 1992). In 1980 Furchgott and Zawadski demonstrated in an arterial bioassay that acetylcholine-dependent vasodilation (i.e. receptor-dependent) was effected by a non-prostanoid, endothelium-dependent factor which was termed endothelium derived relaxing factor (EDRF). Work on identifying this substance culminated seven years later when Palmer et al (1987) showed that EDRF was NO derived from L-arginine (Palmer et al, 1988). Concurrent with these seminal papers was work in a different field looking at the cytotoxic activity of neutrophils. This activity was found to be dependent on the metabolism of L-arginine and could be mimicked by nitric oxide (Marletta et al, 1988)

Beyond the role of NO as EDRF it is also implicated in the modulation of platelet function, neurotransmission (Egdell et al, 1994), inhibition of neutrophil chemotaxis, adherence and activation (Ma et al, 1993) and immunomodulation following an allograft (Ioannidis et al, 1995).

2.1a Biochemistry and Biosynthesis

Nitric oxide is formed during the conversion of L-arginine (L-ARG) to L-citrulline (L-CITR). Oxidation of the terminal guanidino nitrogen of L-ARG (Nathan et al, 1992) occurs under the control of the enzyme nitric oxide synthase with the subsequent elimination of equimolar amounts of NO and superoxide (O_2-) and generation of L-CITR (Yacoob et al, 1996b). In the absence of L-arginine, however, NOS can continue to generate the superoxide anion (Pou et al, 1992). Nitric oxide is a free radical (Radi et al, 1991) with a short half-life in biological solutions, primarily due to: (i) oxidation to nitrate and nitrite (Ignarro, 1990), (ii) quenching with the superoxide radical (Beckman et al, 1990) and (iii) binding to haem-containing proteins with Fe-S sites such as oxyhaemoglobin (Kuo & Schroeder, 1995).

Four main isoforms of NOS have been identified (Table 2.1). Two of these are constitutive (cNOS) and two inducible (iNOS) enzymes. The constitutive form of the enzyme is responsible for a continual, basal release of NO although this production can be upregulated. It is membrane bound and possibly linked to the cytoskeleton which accounts in part for the regulatory effect on endothelial NOS of vascular shear

stress and flow (Davies et al, 1995). The constitutive isoforms are dependent on calcium and calmodulin for their activation whereas calmodulin is already bound to activated iNOS, which therefore remains independent of the free calcium concentration. Inducible NOS is a free cytosolic enzyme which is basally quiescent but once induced is permanently active for the life of the enzyme (up to 24 hours) (Morris & Billiar, 1994) and produces NO in amounts 1000-fold greater than cNOS (nanomolar rather than picomolar concentrations) (Forstermann et al, 1994).

FORM	ISOFORM	PRINCIPAL SOURCE	? BASAL NO PRODUCTION	ENDOGENOUS INDUCERS	ENDOGENOUS INHIBITORS
CONSTITUTIVE	Ia	Brain	Yes	ACh ATP ADP thrombin SS AA	ADMA
	Ib	EC	Yes		
	Ic	PMN	Yes		
	III	EC	Yes		
INDUCIBLE	II IV	Macrophage	No	cytokines endotoxin	IL-4 IL-10 GC

Abbreviations: EC = endothelial cells. PMN = polymorphonuclear leucocytes. ACh = acetylcholine. ATP = adenosine 5'- triphosphate. ADP = adenosine 5'-diphosphate. SS = shear stress. AA = excitatory amino acids. ADMA = asymmetric dimethyl arginine. IL-4 =interleukin-4. IL-10 =interleukin -10. GC =glucocorticoids.

Table 2.1 Classification and properties of nitric oxide synthase (modified from Davies et al, 1995)

2.1b Interaction between Nitric Oxide and the Vascular Endothelium

The vascular endothelium is one of the prime effector sites for NO with endothelial cells also able to generate NO via both cNOS and iNOS (Radomski et al, 1990). Furthermore the underlying smooth muscle cells (SMC) can also synthesise iNOS under conditions of large-scale cytokine release such as sepsis or the systemic inflammatory response syndrome (Davies et al, 1995). Vasodilation is mediated through soluble guanylyl cyclase (sGC) which is activated by haem group nitrosylation and produces cyclic guanylate monophosphate (cGMP). Kinases dependent on this secondary messenger

phosphorylate SMC proteins leading to relaxation (Kiechle & Malinski, 1993). Nitrosylation of other iron-centred enzymes in both the Krebs cycle and electron transport chains in target cells are believed to account for NO-dependent macrophage cytotoxicity (Nathan, 1992).

The activity of NO outlined above, where it acts via protein nitrosylation, summarises the situation under physiological conditions. Following ischaemia reperfusion the pathophysiology of NO is considerably more complex.

2.2 NITRIC OXIDE AND ISCHAEMIA REPERFUSION INJURY

The injury incurred as a consequence of ischaemia and reperfusion is mainly a result of oxygen derived free radical (OFR) production during the reperfusion rather than the ischaemic phase. The dynamic equilibrium between endogenous NO and these OFR may be critical in determining the degree of post-ischaemic damage for nitric oxide may either quench these free radicals or alternatively act as a substrate for further OFR production. This balance is further highlighted by evidence that NOS can generate OFR (the superoxide anion) when there is insufficient L-ARG for NO production (Pou et al, 1992).

Probably the most important interaction is between NO and O_2- (Gryglewski et al, 1986) which react rapidly (rate constant 10^9/msec) (Blough & Zafiriou, 1985) with the ultimate elimination of both radicals and the generation of peroxynitrite ($ONOO^{\bullet}$). This short-lived oxidant species (half-life of less than one second) (Beckman & Crow, 1993) decomposes to form HOONO which behaves similarly to the hydroxyl radical (Beckman et al, 1990) and thus is highly toxic at the cellular level. One particular target is the lipid membrane, which is peroxidised leading to membrane pump inactivation and ultimately cell death. This lipid peroxidation is propagated by the formation of peroxyl radicals and thus their inactivation would help to maintain cellular integrity. Such inactivation is seen in the NO mediated nitrosylation of these peroxyl radicals to alkyl peroxynitrate (LOONO) which is therefore cytoprotective especially if the subsequent metabolism of LOONO does not generate further free radicals (Darley-Usmar et al, 1995).

Thus several possibilities emerge for the role of NO during reperfusion, without considering the vasoactive function in maintaining microvascular perfusion. It is undoubtedly too simplistic to suggest NO is either solely cytotoxic through the

generation of $ONOO^-$ or cytoprotective via LOONO. However the balance of these free radicals during reperfusion may have a central role.

There is a plethora of *in vivo* experimental work evaluating the effect of manipulating the post-ischaemic levels of both O_2- and NO. In the case of the former, superoxide dismutase (SOD) is commonly employed to reduce superoxide to water, whilst a variety of agents are available to manipulate NO levels. In particular there are NO donors such as sodium nitroprusside (SNAP) and S-nitrosothiols, which by directly providing NO are thus endothelium and receptor independent, and L-ARG which acts as a promoter of NO generation.

Nitric oxide production can be blocked using structural analogues of L-ARG. Compounds typically used include N^G-nitro-L-arginine methyl ester (L-NAME) and N-methyl-L-arginine (L-NMMA) which are both iNOS and cNOS inhibitors, although L-NAME may be predominately a cNOS inhibitor (Gross et al, 1990; Stuehr & Griffiths, 1992; Bryant et al, 1995). Aminoguanidine (AG) is a selective iNOS inhibitor (Corbett et al, 1992) whilst glucocorticoids such as hydrocortisone (HC) are non-specific (i.e. their effects are not specific to NOS alone) iNOS inhibitors (Radomski et al, 1990).

Caution must be exercised however when interpreting the results of experiments utilising these compounds when the effect of the pharmacological manipulator is presumed rather than proven. Furthermore, the structural analogues of L-arginine can also interfere with iron-centred enzymes (Peterson et al, 1992) and even stimulate NO generation in endothelial cells (Archer & Hampl, 1992). Therefore unless the NO levels are measured directly such studies remain inconclusive.

2.2a Experimental Measurement of Nitric Oxide

The measurement of NO in biological samples is difficult primarily because of the small quantities of NO present (typically nanomolar) and also the lability of NO in the presence of O_2. Measurement of NO can be either indirect or direct.

Indirect measurement of NO relies on the detection of the metabolic end products of metabolism or the secondary messengers of cellular interaction. Nitrite can be measured fairly simply in a diazotisation assay with Griess reagent and the resultant purple azo dye quantified spectrophotometrically (Gomez-Jimenez et al, 1995). Nitrite appears to be a

reasonable marker of NO for *in vitro* cell studies but *in vivo* nitrate is the more sensitive marker as nitrite is oxidised rapidly to nitrate in biological solutions (Zeballos et al, 1995). There are two methods of determining nitrate in common use, both of which rely on reduction to nitrite; one is an end-point assay whilst the other is kinetic. The end-point assay employs a cadmium column through which the sample is passed to reduce the nitrate and the resultant nitrite can be measured (Green et al, 1982). The kinetic assay utilises the enzyme nitrate reductase, which can be extracted from Aspergillus (Gilliam et al, 1993). The reduction of nitrate by this enzyme is driven by nicotinamide adenine dinucleotide phosphate (NADPH) which is oxidised to NADP at an equimolar rate to the reduction of nitrate. The oxidation of NADPH can be monitored spectrophotometrically. Several other somewhat more indirect assays of NO have also been described. The measurement of L-CITR (Cristol et al, 1993) and the detection of NOS by either immunohistochemistry (Bachmann et al, 1995) or reverse transcriptase-polymerase chain reaction (RT-PCR) (Ujiie et al, 1994) are quantitative and semi-quantitative measures respectively of NOS activity. Cyclic GMP can be measured via a radioimmunoassay (Ignarro et al, 1984) and is an indication of activated guanylate cyclase, although this is not activated specifically by NO (Rapoport, 1986).

The direct measurement of nitric oxide is more complex and costly, relying on sophisticated detection equipment. The various strategies include: chemiluminescence where NO reacts with ozone and generates light (Chung & Fung, 1990); electron paramagnetic resonance in which NO is trapped by nitroso compounds to form more stable compounds which give a signature spectrum when they return to a low energy state from the higher one. Similarly NO can be trapped by haemoglobin and the resultant shift in absorbence from 433nm to 406nm as methaemoglobin is produced can be measured (Ignarro et al, 1987). Another NO trap is nitrosothioproline, which has been used to quantify NO in a mass spectrometer (Gustafsson et al, 1991). One final method is worthy of note. Malinski & Taha (1992) have described a porphyrinic-based microsensor with a detection threshold of 10^{-20} mols (in comparison to the methods outlined above with thresholds of approximately picomols of NO) allowing the quantification of NO released from single cells. Furthermore this sensor allows real-time, direct measurement of NO and can be used to monitor the NO generation within organs from the effluent blood (personal communication, Malinski).

2.2b Modulation of Post-ischaemic Oxygen Free Radical Levels

The scavenging of oxygen free radicals in the post-ischaemic period has been demonstrated to ameliorate injury in a variety of organs. In particular protection has been shown in the heart (Aoki et al, 1988; Bernier et al, 1989), the small intestine (Droy-Lefaix et al, 1991), skeletal muscle (Korthius et al, 1985) and the kidney.

Much of the work on renal ischaemia reperfusion injury has stemmed from the field of transplantation where the use of SOD and/or allopurinol (a xanthine oxidase inhibitor) prior to reperfusion has been shown to confer a significant functional benefit (Hoshino et al, 1988; Koyama et al, 1985). Similar benefits can be gained by quenching the hydroxyl radical, as outlined by Paller (1992). Experimental models of renal reperfusion injury, without the potential confounding effect of transplantation, mirror these results with the use of OFR scavengers leading to a partial preservation of renal functional and morphological parameters (Baker et al, 1985; Paller et al, 1984).

2.2c Modulation of Post-ischaemic Nitric Oxide Levels

In marked contrast to the work outlined above there appears less consensus as to the effect of ischaemia reperfusion on endogenous NO levels or the effect of manipulation of these levels on subsequent outcome.

It is apparent from the literature that there is a lack of unanimity regarding levels of post-ischaemic NO production, not just between different organs but also in studies on the same organ. In the former, for example, a diminution in the endogenous NO level is reported in skeletal muscle (Sternbergh et al, 1992) and the coronary endothelium (Ma et al, 1993) whilst an increase is seen in the lung (Ischiropoulos et al, 1995) and small bowel (Mueller et al, 1994). One example of intra-organ variance is the coronary endothelium where as mentioned previously Ma et al conclude that NO production is diminished following reperfusion and this finding is supported by others (Maulik et al, 1995; Fukuda et al, 1995). Depré et al (1995) however reach the opposite view point and suggest that NOS inhibitors are cardioprotective following ischaemia reperfusion injury.

There exist several possible reasons for such disparate results. Perhaps the most pertinent of these lies in the mechanism by which the experimental conclusions are reached. In essence either there has been a direct assay of NO (or it's stable metabolic end products nitrate and nitrite) or conclusions are reached empirically without

quantification. The former must be the most reliable and includes those papers by Mueller (small bowel), Ischiropoulos (lung), Maulik and Fukuda (heart). Other potential confounding factors include the temporal effect of different durations of ischaemia and reperfusion on endogenous NO production and the different animal models and age groups (Sabbatini et al, 1994). However such differences do not appear to affect the outcome of the studies investigating OFR production. Furthermore, the use of modulators of NO production with their divergent haemodynamic effects may skew the results; especially if the conclusions of such experiments are based on functional rather than quantifiable measures of post-ischaemic NO levels.

2.3 NITRIC OXIDE AND RENAL PHYSIOLOGY

A major role for NO in normal renal physiology has been established with both main forms of NOS expressed. Their relative distribution varies within the kidney, with the constitutive form of NOS predominately localised in the preglomerular vasculature, macula densa (MD) and collecting duct whilst iNOS has a more general distribution with the exception of the vessels and MD (Gabbai et al, 1995) (Table 2.3).

Utilising NOS blockers and micropuncture techniques it has proved possible to determine the role of NO in normal renal homeostasis. Correlating closely with the distribution of the constitutive isoform of the enzyme, NO antagonises the vasoconstrictive effect of angiotensin II on the afferent arteriole (Ito et al, 1991) and helps to maintain the GFR, RBF (Lahera et al, 1991; Granger et al, 1992) and sodium regulation (Javier-Salazar et al, 1995). The interaction of NO and renin remains unclear. One study, using (intravenously) anaesthetised rats, suggested NO may act in a paracrine fashion within the glomerulus to stimulate the release of renin (Johnson & Freeman, 1994) whilst another study found NOS blockade increased plasma renin activity (PRA) in conscious dogs (Javier-Salazar et al, 1995). The effect of certain anaesthetic agents on NOS may be one cause for such discrepancies (Kumagai et al, 1994) although inhalational anaesthesia has been demonstrated not to affect NOS activity (Johns et al, 1995).

Site	cNOS	iNOS
Preglomerular vessels	+	-
Glomerulus	+	++
Macula densa	++++	-
Proximal tubule	-	++
Loop of Henle	+	++
Distal tubule	-	++
Cortical collecting duct	+	++
Medullary collecting duct	++++	++

Table 2.3 Renal distribution and semi quantification (via reverse transcriptase-polymerase chain reaction) of cNOS and iNOS.
After Gabbai et al (1995)

2.4 NITRIC OXIDE AND RENAL ISCHAEMIA REPERFUSION INJURY

2.4a Background & Theory

The classical model of ischaemia reperfusion injury whereby ischaemia 'primes' the tissue for OFR generation during the reperfusion phase is now clearly not the whole story. Studies on isolated proximal tubules have demonstrated that early in the ischaemic phase NOS activity is increased with NO generation apparently maximal by 10 minutes (Yacoob et al, 1996a). The dynamic equilibrium between NO and the OFRs generated during reoxygenation may thus be critical in determining the degree of post-ischaemic damage. A reaction between these species may result in either quenching of both or alternatively act as the substrate for further OFR production. One of the major products is $ONOO^{\bullet}$, which rapidly decays generating $OH^{\bullet}$. The temporal separation of NO and O_2- during ischaemia and reperfusion cannot be discounted either. Whether or not the interaction of these two radicals is ultimately toxic or protective it appears that NO alone is generated during ischaemia. Throughout this unopposed phase NO may well damage the actin cytoskeleton (Salzman, 1995) through ATP depletion in addition to directly altering DNA

(Nguyen et al, 1992) and inhibiting the subsequent repair (Laval & Wink, 1994). Renal epithelial cell adhesion may also be impaired by actin degradation affecting integrin-ligand binding (Kellerman & Bogusky, 1992; Kroshian et al, 1994).

It would thus be easy to conclude that post-ischaemic NO is cytotoxic, except that in certain circumstances NO-mediated nitrosylation has been shown to have a cytoprotective effect. Firstly, as mentioned previously, through the nitrosylation of peroxyl radicals. Further evidence is seen in the interaction of NO with leucocytes. A number of post-ischaemic mediators including xanthine oxidase, platelet activating factor, thromboxane and leukotrienes are involved in leucosequestration. These neutrophils then adhere, activate and migrate under the control of adhesion molecules, most notably the B-integrins. Both these (the CD11/CD18 complex in particular) and the matrix proteins are integrally involved in the subsequent respiratory burst (Nathan et al, 1990). The involvement of nitric oxide in the proteolysis of β-integrin has been demonstrated (Giancotti et al, 1992) as has the effect of NO on the extracellular matrix (Stamler et al, 1992). Thus by interfering with the neutrophil respiratory burst NO should help ameliorate post-ischaemic renal damage and this has been shown experimentally (Kukrose et al, 1994; Linas et al, 1996).

Apparently opposing roles for NO during ischaemia and reperfusion are thus evident. Whilst the results of *in vitro* studies suggest the former *in vivo* work demonstrates the protective effect of NO on overall post-ischaemic renal function. Part of this discrepancy may be attributable to the vasoactive function of NO in maintaining microvascular perfusion (Kukrose et al, 1994) in addition to the complex interaction of cytokines and leucocytes absent from the *in vitro* models. Furthermore the balance of free radicals, especially NO and O_2-, during reperfusion may be critical in determining the degree of eventual damage with an excess of NO over O_2- in the post-ischaemic tissue having an overall cytoprotective effect to counteract $ONOO^{\bullet}$ toxicity (Rubbo et al, 1994). Such a balance can be achieved experimentally by manipulating the post-ischaemic levels of O_2- and NO.

2.4b Experimental Evidence

One of the first investigations was undertaken by Lieberthal et al (1989) on isolated rat kidneys and they concluded that NO activity was impaired following IRI. In contrast Rivas-Cabañero et al (1995) cultured glomeruli from ischaemic rat kidneys and measured nitrite production. This demonstrated a significant rise in NO production after one hour of ischaemia followed by 24 hours reperfusion (prior to glomerular harvest) when compared to either one hour ischaemia / one hour reperfusion or the control (no ischaemic insult). This is at odds with the work of Yacoob et al (1996b) who found NO actual increased during renal proximal tubular ischaemia before falling towards normal levels upon reoxygenation.

Both Chintala et al (1993) and Kin et al (1995) concluded that endogenous NO production is important in maintaining post-ischaemic renal function during the first two and twenty four hours respectively of reperfusion injury. A similar picture emerges from the work of Conger et al (1995) and Cristol et al (1993) in assessing the role of endogenous and augmented (by L-ARG infusion) NO respectively, with an additional insight into NOS activity. Conger et al used a renal artery infusion of norepinephrine to induce acute renal failure (ARF) and then measured renal blood flow (as the index of NO activity) following the infusion of L-NAME, AG and SNAP. They concluded that one week after inducing ARF the activity of renal NOS was already maximal and therefore exogenous attempts to stimulate NO production were futile. Furthermore, when correlating the effects of iNOS blockade with immunohistochemical studies it appeared that iNOS expression was not upregulated.

The model of Cristol et al used a rather more direct method of inducing renal ischaemia (renal artery occlusion) and demonstrated a similar decline in renal blood flow (RBF) in response to L-NAME. They also used a more sophisticated process to elucidate which form of NOS was responsible for post-ischaemic NO generation (although they did not determine if this was upregulated over baseline). Briefly, by using tritiated arginine as the substrate for NO production the consequent [^{3}H]-citrulline generated could be quantified by scintillation counting; and by either adding or chelating calcium the relative roles of cNOS (calcium-dependent) and iNOS (calcium-independent) was assessed. They concluded that the constitutive but not inducible form was responsible for post-ischaemic NO generation. In contrast to

this Noiri et al (1996) used antisense oligonucleotides to selectively knock-down iNOS. When directed at renal specific iNOS the post-ischaemic injury was ameliorated whilst knock-down of vascular SMC iNOS exacerbated the deterioration in renal function

There is however no more unanimity on the role of NO in renal ischaemia reperfusion injury than in other organ models and López-Neblina et al (1995) conclude the antithesis of this. Using a rat model of renal warm ischaemia they found no role for endogenous NO in maintaining renal function but exogenous NO (via SNAP) not only improved function but also the morphological picture and survival.

Attempts to maximise NO production have tended to focus on the provision of L-ARG as a precursor and hence enhancer of NO generation rather than through the use of nitric oxide donors such as SNAP. Once again however the evidence for an associated increase in NO is often assumed rather than affirmed and whilst L-ARG can under certain circumstances enhance NO production this has not been shown in the post-ischaemic kidney. For example Dagher et al (1995) demonstrated a beneficial effect on glomerular filtration and tubular function from a L-ARG infusion prior to the onset of ischaemia whereas Conger et al (1995) found that infusion during ischaemia conferred no benefit. The conclusion that the former is the result of augmented NO generation is unproven, as no measure of NO was made. Certainly the reaction kinetics of L-ARG -to-NO do not suggest substrate availability would be rate limiting (Forsterman et al, 1994) and this has been demonstrated clinically by MacAllister et al (1995), albeit not under pathophysiological conditions. Furthermore they showed that L-ARG, in the massively supraphysiological doses (×20 normal plasma concentration) commonly achieved in such studies, caused elevated levels of hormones including insulin, glucagon and prolactin. These may be responsible for some of the vascular effects attributed to the L-ARG : NO axis.

2.5 NITRIC OXIDE, PROSTANOIDS AND THE KIDNEY

One of the enzymes responsible for the production of prostanoids exists in two isoforms: cyclooxygenase I (COX 1) and cyclooxygenase 2 (COX 2). COX 1 represents the constitutive form and COX 2 the inducible, which is expressed principally on monocytes (Fu et al, 1990) and endothelial cells (Maier et al, 1990). Vasodilator prostanoids such as

prostacyclin (PGI_2) are important in maintaining RBF in the post-ischaemic kidney (Cristol et al, 1993) and recent evidence points to a linkage between NO and these prostanoids.

Using a model of renal inflammation Salvemini et al (1994) have shown that both endogenous and exogenous NO can induce COX 2, i.e. COX acts a NO receptor, and this acts to support the inflammatory process. However, measuring 6-keto-prostaglandin$_{1\alpha}$ (the stable metabolite of PGI_2) and nitrite in a model involving both *in vitro* work on a macrophage cell line and also *in vivo* renal experiments, Swierkosz et al (1995) came to very different conclusions. Whilst concurring that iNOS and COX 2 can be co-induced and also that COX would appear to act as a receptor for NO they concluded that both endogenous and exogenous NO inhibits the expression of COX 2 *in vitro*. It appears that low levels of NO potentiate the activity of COX 2 and high levels inhibit the activity. Whilst no renal NO - COX interaction was apparent *in vivo* this was not an ischaemia reperfusion model which may be important for it has recently been demonstrated (Zou and Ullrich, 1996) that peroxynitrite rapidly and irreversibly blocks PGI_2 biosynthesis whilst NO (or the superoxide radical) alone has no effect.

2.6 CONCLUSION

Since EDRF was first identified as nitric oxide in 1987 there has been a plethora of research delineating the biosynthesis and physiology involved in NO biology. In summary nitric oxide can be seen to be a simple molecule involved in a complex sequence of events both physiologically and pathophysiologically during ischaemia and reperfusion. What is not so clear is the precise effect of NO in post-ischaemic organs and whether it has a predominately protective or injurious role. Clarity on this will remain elusive until the experimental measurement of NO becomes the norm.

Chapter 3

THE DEVELOPMENT AND VALIDATION OF A NITRIC OXIDE ASSAY FOR USE WITH RENAL TISSUE HOMOGENATE

3.1 GENERAL INTRODUCTION

Chapter 2 outlined some of the experimental methods available for quantifying NO in biological samples. These varied from the costly and complex direct measurement via chemiluminescence (Chung & Fung, 1990), methaemoglobin (Ignarro et al, 1987) or mass spectrophotometrically (Gustafsson et al, 1991) to the more simple indirect measurement of NO through its metabolites nitrite and nitrate.

The latter route was chosen for the development of a simple, robust assay utilising equipment that was easily available in any basic laboratory. This was of particular importance, as a major criticism of much of the NO-centred work published is the absence of any objective measurement of NO. There is a growing trend whereby the effects of pharmacological manipulation of NOS are directly (but possibly erroneously) extrapolated to changes in NO levels. Thus one of the aims of the work of this thesis was the establishment of a universally available assay of NO in tissue homogenates. Since the completion of this work a commercially available nitrate/nitrite assay kit has become available (Cayman Chemical, Ann Arbor, USA) which facilitates this process. This kit is based around the enzymatic reduction of nitrate to nitrite and subsequent detection of this using Griess reagent and as such is similar to the technique described in this work.

The quantification of nitrite in biological solutions is relatively straightforward. The basic principles have not changed since Griess first described the process in 1879 of the diazotisation (formation of a chemical group containing two nitrogen atoms bonded to one carbon atom) of an aryl amine by nitrite and the coupling of this product to form an azochromophore. The (Griess) reagent used consists of 1 part 0.1% naphthylethylenediamine dihydrochloride in distilled water and 1 part 1% sulphanilamide in 5% concentrated phosphoric acid (H_3PO_4), the two parts being mixed immediately prior to use and discarded after 12 hours. The combination of Griess reagent and nitrite results in the formation of a purple azo dye with a linear relationship between nitrite concentration and the spectrophotometric absorbance of the dye measured at 546nm (Green et al, 1982).

There is no simple assay for the measurement of nitrate and accordingly it is generally quantified following reduction to nitrite. The standard method to achieve

this reduction is via a cadmium wire (Stainton, 1974). Although this appears to work well for nitrite in water, when more complex biological solutions are investigated there appear to be endogenous inhibition of cadmium reduction of nitrate to nitrite (Radomski & Palmiri, 1978; Witter & Gatley, 1979). One way around this is to increase the amount of cadmium present (Davison & Woof, 1979) and this was refined by Green et al (1982). They utilised the principles of HPLC, in particular a large sample dilution and a high surface-to-volume ratio of column pathway to sample. Using a high-pressure column packed with fine particles of copper-plated cadmium they achieved nitrate reduction efficiency of 99 ± 5%. By splitting the sample and passing half through the cadmium column prior to reaction with Griess reagent and bypassing the column with the other aliquot the relative proportions of nitrite and nitrate can be quantified.

The second method for establishing nitrate reduction is via the medium of nitrate reductase. The reaction can be characterised thus:

$$NO_3^- + NADPH + H^+ \xrightarrow{\text{Nitrate Reductase}} NO_2^- + NADP + H_2O$$

Nitrate reductase (NRase) has been isolated from a number of plant and fungal species, with soybean leaves (Jolly et al, 1976) and rice seedlings (Shen, 1972) among the most plentiful sources. The enzyme used in the following experiments was derived from Aspergillus.

Nitrate reductase is capable of using the reduced form of pyridine nucleotides, flavins, or benzyl viologen as electron donors for reduction of nitrate to nitrite (Hageman & Reed, 1980) but as NADH or NADPH are the usual donors *in vivo* these tend to be used experimentally. The enzyme is composed of a pyridine nucleotide-cytochrome *c* reductase bonded non-covalently to a molybdenum-containing subunit with a total molecular weight ranging from 160000 to 500000 (Relimpio, 1971). Flavin adenine dinucleotide (FAD) is a constituent component of the NRase enzyme (Betts & Hewitt, 1966) where it acts as an electron acceptor in the reduction of nitrate:

$$NADPH \rightarrow FAD \rightarrow \text{Cyt } b \rightarrow \text{Mo protein subunit} \rightarrow (NO_3 \rightarrow NO_2)$$

Two distinct enzymes have been identified: that utilising NADPH (EC 1.6.6.2) and the other NADH (EC 1.6.6.1). The Michaelis-Menten constant (K_m) for substrate affinity of nitrate reductase shows interspecies variation, being 0.11mM and 4.5mM for soybean NADH and NADPH enzymes respectively (Jolly et al, 1976; Campbell, 1976) and 0.2mM for Aspergillus NADPH-dependent NRase (Gilliam et al, 1993).

The nitrate assay used in the following experiments is based on that developed by Gilliam et al (1993), which in turn is a modification of the Sigma enzymatic procedure for determining nitrate reductase activity. It is a kinetic, spectrophotometric method that measures the change in absorbance (at 340nm) as NADPH is oxidised during the electron transfer to nitrate by the NRase.

This Chapter details the development of both the nitrite and nitrate assays as well as the method used to prepare the kidney for analysis.

3.2 DEVELOPMENT OF THE NITRITE ASSAY

3.2a. Production of Nitrite Standards

The previously described assays for nitrite (Green et al, 1982) and nitrate (Gilliam et al, 1993) were accurate within the range of 1μM to 300μM and 1.25μM to 40μM respectively. As there was no available experimental data for the range of nitroxides in the post-ischaemic kidney, calibration curves were constructed to lie within these parameters.

A one molar solution of sodium nitrite (Sigma, Poole, UK. S2252) was prepared by dissolving 0.69g of sodium nitrite (MW 69) in distilled, deionised water to a final volume of 10ml. This was vortexed to ensure optimal mixing and then serial dilutions were performed (1 part nitrite solution to 9 parts distilled, deionised water) to achieve a 0.1M, 0.01M, 0.001M and a 0.0001M (i.e. 100μM) nitrite solution. The 100μM nitrite solution was then used to generate a series of nitrite standards ranging from 100μM to 2.5μM (Table 3.2i) which were used in the subsequent experiments. Standards were stored in the dark at room temperature in sealed universal containers and discarded after 7 days.

3.2b Determination of the Point of Maximal Spectrophotometric Absorbance through the Nitrite-Griess Azo Dye Complex

Griess reagent (BDH, Poole, UK. 19062/ 19063) is supplied as two separate components which need to be mixed (1:1) prior to use and discarded after 12 hours. For this experiment a 50μM standard was chosen as it lay in the middle of the expected experimental range of nitrite. The spectrophotometer (Philips PU8620, Philips, Netherlands) was switched on and allowed to warm up for 30min prior to use in all experiments. The point (i.e. wavelength of light) of maximal absorbance would correspond to the point of greatest sensitivity of the assay, and would thus be used in subsequent assays.

A 1ml aliquot of nitrite standard and a 1ml aliquot of Griess reagent were mixed by vortexing in a test tube (Stardest, Leicester, UK) and placed in water bath at 60°C for 15min to allow diazotisation to proceed. A 1ml sample was then pipetted into a semi-micro spectrophotometer cuvette (Sigma. C5416), mixed by inversion and the absorbance read having first zeroed the spectrophotometer using a control of 1 part distilled, deionised water to 1 part Griess reagent. In this way, using a fresh sample for each reading, the absorbance was noted at increasing wavelengths of light between 500-600nm at 10nm increments (Figure 3.2i). This demonstrated the maximal absorbance lay between 520-530nm and therefore the process was repeated within this range, using duplicate samples at 2nm increments, to show that the optimal wavelength of light was 523nm (Figure 3.2ii). This value was therefore used in all subsequent nitrite analyses.

3.2c Determination of the Optimal Ratio of Griess Reagent to Nitrite Solution

This experiment was designed to assess if a 1:1 ratio of Griess reagent to nitrite solution was optimal or whether increasing the proportion of reagent would encourage the diazotisation and thus the development of a deeper (i.e. more absorbent) dye colour. This might be expected to increase the accuracy of the assay especially at low nitrite concentrations.

To a 1ml aliquot of nitrite standard (in the range of 25-150μM) was added either 1ml, 2ml or 4ml of reagent. Samples were analysed in duplicate and were incubated for 15min as described in (3.2b) before the absorbance was read at 523nm. The relevant

spectrophotometric controls were distilled, deionised water and Griess reagent in ratios of 1:1, 1:2 or 1:4 (Figure 3.2iii). A ratio of 1:1 was found to be optimal.

3.2d Assessment of the Accuracy of the Spectrophotometer Autocell Sampler.

The Philips PU8620 spectrophotometer used throughout these experiments was fitted with an autocell sampler. This consisted of an internal cuvette which could be filled (and emptied) via a length of fine bore tubing and a simple pump. This obviated the need for individual cuvettes for each sample and thus seemed attractive. Before using this sampler however the accuracy of the device was tested.

Nitrite standards ranging between 2.5-100μM were used with Griess reagent added in a 1:1 ratio and the procedure as described in (3.2b) followed. Distilled, deionised water: Griess (1:1) was used as the control. Using the sampler programmed to draw up a volume of 1ml the spectrophotometer was first zeroed on the control and then five consecutive readings were taken at each concentration of nitrite. The spectrophotometer was re-zeroed in between each nitrite standard (i.e. after every five readings). The discrepancy between the first reading and the mean of the subsequent four could then be calculated (Figure 3.2iv). At low nitrite concentrations there proved to be considerable cross-contamination with the subsequent sample. Therefore individual cuvettes were used thereafter for all spectrophotometric readings.

3.2e Construction of the Nitrite Calibration Curve

Nitrite standards ranging between 5-100μM were used with Griess reagent added in a 1:1 ratio and the procedure as described in (3.2b) followed. Four separate samples were analysed at each concentration of nitrite and the calibration curve constructed (Figure 3.2v) with a coefficient of variation of <2%. This process was repeated on a subsequent day using freshly prepared nitrite standards (Figure 3.2vi) which demonstrated an almost identical calibration curve. The minor variations were likely to stem from inaccuracies during diluting and preparing the standards.

A further calibration curve (Figure 3.2vii) was then constructed using a spiked solution of renal homogenate, as this would be the experimental medium for nitrite

analysis. Nitrite standards were prepared as described in (3.2a) with the exception that the nitrite was dissolved in renal homogenate (for preparation see 3.3c) that had been diluted (in total) ×50 in distilled, deionised water to prevent endogenous nitrite interfering with the final nitrite concentration (Green et al, 1982). The calibration curve was then constructed in an identical manner to that detailed earlier in this section. The curve thus plotted was almost identical to the standard curve indicating that renal homogenate did not affect the accuracy of this system.

3.3 PREPARATION OF THE RENAL HOMOGENATE

In order to maximise the sensitivity of the spectrophotometric assay and avoid non-standardised errors it was essential to measure the change in absorbance (i.e. 1/ transmission) of the sample in an essentially transparent carrier medium. This was particularly pertinent as homogenised kidney has a distinct red colour, similar in fact to the colour of the azo dye to be detected. Prior clarification was thus a necessity.

3.3a Clarification with 10% Sulfosalicylic Acid

Kidney sections that had been harvested and frozen as described in section (4.2a) were used; although for the development of these assays the kidneys were obtained from animals culled by stunning and neck dislocation without any prior inclusion in the experimental protocol. Otherwise the protocol was identical, with care taken to avoid warming the renal tissue to prevent possible metabolism of nitroxides.

Kidney sections were thawed on ice before weighing (Maettler AE200, Germany) and then quickly macerated with a scalpel and placed in a glass homogeniser. Chilled (4°C) distilled, deionised water was added to achieve a concentration of 20% w/v and the kidney was homogenised on ice with 20 strokes of the plunger. A 20% w/v ratio was chosen as preliminary trials had shown that this was the minimum dilution that would allow effective homogenisation without the sample congealing. The homogenate was then transferred to a test-tube and mixed 3:1 with iced 10% sulfosalicylic acid (Sigma. S2130) pre-prepared by dissolving 10g of sulfosalicylic acid to 100ml in distilled, deionised water. This was vortexed for 5sec every 5min for 30min whilst maintained throughout on ice (except for vortexing) and then

centrifuged at 1000g for 15min at 4°C. The clear supernatant was then decanted and snap frozen in liquid nitrogen prior to storage at -70°C.

This proved an extremely effective mode of clarification and was ideal for the nitrite assay with initial calibration curves constructed using supernatants derived thus. However it became apparent during preliminary trials with the nitrate assay that a method of clarification not based upon acidification was needed (section 3.3b). This was developed (section 3.3c) and the nitrite calibration curves recalculated using supernatant derived from this method (Figure 3.2vii).

3.3b Determination of the Equivalence Point for 10% Sulfosalicylic Acid

The enzyme nitrate reductase is pH dependent with one unit reducing 1μM of nitrate in the presence of β-NADPH at pH 7.50 and 25°C. Deviation from this pH leads to a rapid diminution of enzyme efficiency (section 3.4b). Therefore although not relevant to the nitrite assay, it was necessary to buffer the sulfosalicylic acid for the nitrate assay. Two molar (2M) sodium hydroxide was used as excessive dilution of the sample was theoretically undesirable with the actual levels of nitroxides in the homogenate unknown.

Two molar sodium hydroxide (Sigma, S5881) was prepared by dissolving 8g NaOH to 100ml in distilled, deionised water. Aliquots were then used to titrate a 5ml sample of 10% sulfosalicylic acid in a universal container through the equivalence point (Figure 3.3i). This was found by vortexing the container after each aliquot of NaOH and measuring the resultant pH using an electronic meter (Whatman PHA 230). Despite repeated measurements it proved extremely difficult to reliably buffer the solution to pH 7.5. Although the use of more dilute NaOH would make this problem less pronounced it would have the corollary of further diluting the sample. Therefore the decision was taken to develop an alternative, pH neutral method of clarifying the renal homogenate.

3.3c Clarification of Homogenate Through Freeze-Thaw Cycling

Renal tissue was homogenised as described in section (3.3a) with the exception that iced 0.14M potassium phosphate buffer was used in place of distilled, deionised

water. This buffer, at pH 7.5, is the recommended medium for the nitrate assay and was therefore used throughout these experiments and kept as a stock solution, stored in the dark at 4°C (for preparation see section 3.4a). Initial attempts to clarify the homogenate were based around either simple centrifugation, centrifugal-driven ultrafiltration through a synthetic membrane, or a combination of both. Simple centrifugation, to a maximum of 15000g for up to one hour, proved an unacceptable method of clarification (as assessed visually). Ultrafiltration across a microporous membrane of pore size 0.45μm or 0.65μm at 5000g using commercial filter units (Sigma, M9660 and M9785), whether or not preceded by prior sample centrifugation, achieved excellent clarification. Unfortunately however the membrane tended to block after filtering only 100-200μl of homogenate and this method of clarification was therefore not pursued.

The method finally chosen was based on that described by O'Donovan et al (1995) whereby cycling the sample through alternate freezing and thawing leads to cell membrane fracture and then centrifugation is all that is required for clarification. Samples were homogenised in phosphate buffer as described earlier in this section, placed in 2ml Eppendorfs and then snap frozen in liquid nitrogen to-196°C for 30s. Each Eppendorf was then transferred to the 4°C refrigerator for one hour to allow thawing and then this cycle was repeated two further times. Samples were then centrifuged at 15000g for 15min at 4°C and the supernatant decanted into fresh Eppendorfs, snap frozen and stored in the -70°C freezer until analysis. Prior to this analysis the supernatant was thawed in the 4°C refrigerator for one hour and then used undiluted for the nitrite assay or diluted 1:3 with phosphate buffer for the nitrate assay. Preliminary work had demonstrated this brought the sample nitrate within the measurable range of the assay (see section 3.5b).

3.4 DEVELOPMENT OF THE NITRATE ASSAY

Evaluation of an End-Point Nitrate Assay

Following the verification of the nitrite assay outlined in (3.2e) the nitrate assay was developed. The basic choice lay between nitrate reduction using the reductase enzyme or reduction over a cadmium column with the former chosen as it fulfilled the criteria of utilising equipment that was easily available in any basic laboratory.

Once this route was elected upon there remained the question of whether an end-point assay (Jacob et al, 1993. Ioannidis et al, 1995) or a kinetic assay (Gilliam et al, 1993) would be the most accurate and reliable and both techniques were thus evaluated.

Whilst the methodology of both the kinetic nitrate reductase assay and the cadmium column end-point assay had been described and verified in a leading biochemistry journal (Green et al, 1982; in Analytical Biochemistry) I was unable to find a similar peer reviewed article assessing the end-point assay using nitrate reductase. The use of the end-point assay however was appealing as it tied in with the already established nitrite assay and would allow the 'one-shot' measurement of nitroxides (i.e. nitrate and nitrite) rather than their separate measurements. Therefore the initial experiments were directed towards assessing the viability of an end-point nitrate assay.

This assay was developed from the Sigma quality control test procedure used to determine the activity (i.e. quality) of nitrate reductase. However if instead of using a known concentration of nitrate and an undetermined enzyme activity one commences with a known enzyme activity it becomes possible to calculate the experimental, unknown nitrate concentration. The reagents used were initially those outlined in the Sigma test procedure and modified if necessary as described.

3.4a Production of Nitrate Standards & Assay Reagents

A one molar solution of sodium nitrate (Sigma. S5506) was prepared by dissolving 0.85g of sodium nitrite (MW 85) in distilled, deionised water to a final volume of 10ml. This was vortexed to ensure thorough mixing and then serial dilutions were performed (1 part nitrate solution to 9 parts distilled, deionised water) to achieve a 0.1M, 0.01M, 0.001M (1mM) and a 0.0001M (i.e. 100μM) nitrate solution. Both the 1mM and 100μM nitrate solution were then used to generate a series of nitrate standards ranging from 2.5μM to 500μM (in an identical manner to that used to generate nitrite standards, see table 3.2i) which were used in the subsequent experiments. Standards were stored in the dark at room temperature in sealed universal containers and discarded after 7 days.

A 0.14M potassium phosphate buffer was used both to dissolve the lyophilised NRase and as the reaction medium. It was prepared from two stock solutions, dibasic potassium phosphate and monobasic potassium phosphate. A 0.14M dibasic potassium phosphate (Sigma. P5504) solution was prepared by dissolving 3.195g of $K_2HPO_4.3H_2O$ (MW 228.2) to 100ml using distilled, deionised water and similarly a 0.14M monobasic (Sigma. P5379) solution by dissolving 1.905g of KH_2PO_4 (MW 136.1). Both these solutions were placed in a water bath at 25°C and left 30min to equilibrate. The final buffer was then generated by titrating approximately 3parts dibasic solution with 1part monobasic solution to a final measured pH of 7.5. Both the buffer and stock solutions were stored in the dark at room temperature in sealed containers and discarded after seven days.

The remaining reagents were prepared freshly on each day the assay was performed. A 0.1mM solution of flavin adenine dinucleotide (Sigma. F6625) was prepared by dissolving 8.3mg of FAD (MW 829.5) in 10ml of distilled, deionised water and then diluting this 1 in 10 with distilled, deionised water. It was kept on ice for the duration of the experiment and protected from the light by encasing the universal container with aluminium foil. A 12mM solution of reduced nicotinamide dinucleotide phosphate (Sigma. 201-205) was prepared by dissolving the contents of a 10mg pre-weighed vial of β-NADPH (MW 833.4) in 1ml of distilled, deionised water and kept on ice for the duration of the experiment. A 3.3 unit/ml solution of nitrate reductase (Sigma. N7265) was prepared by dissolving the contents of a 24mg vial (10U) in 3ml of potassium phosphate buffer. This was allowed to reactivate for 30min and then placed on ice for the duration of the experiment.

3.4b Determination of the Optimum pH for Nitrate Reductase Efficiency

By varying the relative proportions of the two stock solutions of potassium phosphate it was possible to prepare a number of buffers ranging in pH from 7.00 to 8.10. The following reagents were pipetted into a test tube: 400µl of buffer, 300µl of distilled, deionised water, 250µl of 1mM nitrate solution (final concentration: 250µM), 25µl of 0.1mM FAD (fc: 2.5µM), 8µl of 12mM β-NADPH (fc: 96µM) and 17µl of 3.3 units/ml NRase (=0.056 U). Thorough mixing was ensured by inversion, the pH was checked and the mixture incubated at 25°C for 10min. A one ml aliquot of Griess

reagent was then added and the test tube transferred to a 60°C water bath for 15min. A one ml sample was then pipetted into a semi-micro cuvette and the absorbance read against a control consisting of 1 part the same reagents (except for substitution of distilled, deionised water for the nitrate solution) to 1 part Griess (Figure 3.4i)

3.4c Comparison of Two Buffers on Enzyme Efficiency

Following the demonstration of the importance of pH to enzyme efficiency two readily available buffers were compared. Both the phosphate buffer described in (3.4a) and HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]), a biological buffer (Sigma. H9897), have a pKa of 7.50 at 25°C and might be expected to perform equally well in this system. If this were demonstrated then HEPES, being a stock buffer within the laboratory, would be used preferentially in subsequent experiments.

The basic protocol described in 3.4b was followed. The experiment was carried out in duplicate test tubes, one using 0.14M phosphate buffer and the other 0.1M HEPES buffer. Four nitrate standards were chosen between 40-160μM (fc) and the remaining reagents were the same: FAD 2.5μM (fc), β-NADPH 96μM (fc) and NRase 0.056 U (fc). The reaction time was 10min at 25°C (Figure 3.4ii). Following this the pH was checked and found to lie within the range 7.46-7.52, prior to the addition of the Griess reagent. Conversion of nitrate to nitrate was found to be more efficient using phosphate buffer, which was accordingly used in all subsequent experiments.

3.4d The Optimal Concentration of Nitrate Reductase and the Co-Factors

The Sigma test procedure aims to determine the activity of NRase and as a consequence may not provide the optimal concentration of reagents to measure nitrate. Therefore a preliminary trial was undertaken to determine the optimal concentrations of enzyme, β-NADPH and FAD.

The basic protocol described in 3.4b was followed. The experiment was carried out using 0.14M phosphate buffer with nitrate standards between 10-160μM (fc). The remaining reagents were the same: FAD 2.5μM (fc) and β-NADPH 96μM (fc). Four concentrations of NRase were prepared by dissolving the contents of a 24mg vial

(10U) in 1.5ml of phosphate buffer to give a 6.7U/ml reagent (0.112U fc). An aliquot of this then underwent serial 1:1 dilution with phosphate buffer to generate: 0.056U, 0.028U and 0.014U (all fc) enzyme solutions. The reaction time was 10min at 25°C followed by Griess reagent and measurement of the absorbance in the spectrophotometer (Figure 3.4iii). The control consisted of 1 part of the same reagents (except for substitution of distilled, deionised water for the nitrate solution) to 1 part Griess. There appeared to be little difference in efficacy between 0.112U and 0.056U for the nitrate range used and therefore the latter concentration was used in subsequent experiments.

In the second series of experiments the basic protocol in 3.4b was followed using 0.14M phosphate buffer with a nitrate standard of 100μM (fc) and a NRase of 0.056U (fc). A 0.2mM FAD solution (5μM fc) was prepared by dissolving 16.6mg of FAD in 10ml of distilled, deionised water and then diluting this 1:10. Thereafter serial dilution 1:1 with distilled, deionised water gave: 2.5μM, 1.25μM and 0.625μM (all fc) FAD solutions. A series of β-NADPH solutions were generated in an identical manner, commencing with dissolving the contents of a 10mg vial in 0.5ml distilled, deionised water to give a 24mM solution (192μM fc) and diluting this to give: 96μM, 48μM and 24MμM (all fc) β-NADPH solutions. For all the FAD readings β-NADPH was kept constant at 96μM and similarly FAD was held constant at 2.5μM for the β-NADPH readings.

A spot measurement could then made for each individual FAD and β-NADPH concentration on the nitrate to nitrite conversion efficiency. The reaction time was 10min at 25°C followed by Griess reagent and measurement of the absorbance spectrophotometrically. The control consisted of 1 part of the same reagents (except for substitution of distilled, deionised water for the nitrate solution) to 1 part Griess. As Figure 3.4iv shows decreasing the concentration of co-factors impedes the enzymatic conversion of nitrate to nitrite. It is possible that increasing the concentration of co-factors further would increase the efficiency of the reduction of nitrate but the graph does demonstrate that there is in fact only a slight improvement in conversion in doubling the co-factor concentration from the middle value used to the maximum.

3.4e The Optimal Duration for Enzymatic Nitrate to Nitrite Conversion

The Sigma test procedure and the paper by Gilliam (1993) suggest a reaction time of five and eight minutes respectively for the kinetic assay. However the duration of enzymatic action in the end-point assay for optimum nitrate to nitrite conversion was undetermined.

The basic protocol in 3.4b was followed. The experiment was carried out using 0.14M phosphate buffer with a nitrate standard 100μM (fc). The remaining reagents were: FAD 2.5μM, β-NADPH 96μM and NRase of 0.056U (all fc). The reaction times were: 10min, 30min, 60min, 6hr, 12hr and 24hr at 25°C. This was followed by diazotisation and measurement of the absorbance. The control consisted of 1 part of the same reagents (except for substitution of distilled, deionised water for the nitrate solution) to 1 part Griess. It can be seen from Figure 3.4v that there is little to be gained in terms of nitrate reduction by continuing the incubation beyond 10min.

3.4f Assessment of End-Point Assay Variability

Following the experiments detailed above, which determined the optimum parameters for the enzymatic end-point conversion of nitrate to nitrite, the reliability of the assay needed to be defined.

The basic protocol in 3.4b was followed. The experiment was carried out in triplicate using 0.14M phosphate buffer with nitrate standards ranging from 2.5 to 100μM (fc). The remaining reagents were: FAD 2.5μM, β-NADPH 96μM and NRase of 0.056U (all fc). The reaction time was 10min at 25°C. This was followed by nitrite diazotisation and measurement of the absorbance spectrophotometrically. The control consisted of 1 part of the same reagents (except for substitution of distilled, deionised water for the nitrate solution) to 1 part Griess. To measure the intra-day assay variability the triplicate samples were processed together and consisted of aliquots from the same batch of reagents on the same day (Figure 3.4vi). It was evident that not only was the conversion efficiency about 40% (Figure 3.4vii) but more importantly the coefficient of variation of this assay (from 5-50%) was such that it could not be relied upon to provide accurate data. Therefore the end-point assay was abandoned and a kinetic assay to determine nitrate concentration developed.

3.5 DEVELOPMENT OF THE NITRATE ASSAY

Evaluation of A Kinetic Nitrate Assay

The nitrate assay used in the following experiments is based on that developed by Gilliam et al (1993). It is a kinetic, spectrophotometric method that measures the change in absorbance (at 340nm) as NADPH is oxidised during the electron transfer to nitrate by the nitrate reductase.

The experiments detailed in sections 3.4b-3.4e for the end-point nitrate assay clearly delineated the optimum conditions for the maximal efficiency of nitrate reductase. These were in line with those documented in the validated kinetic assay and thus these experiments were not repeated. The kinetic assay was therefore developed and assessed for intra-day and inter-day variability.

3.5a Development of the Kinetic Assay

This series of experiments was designed to establish the kinetics of nitrate reduction and to determine the range of the sensitivity of the assay. The nitrate standards, buffer and reagents were prepared as described in (3.4a). The water bath was set to 25°C and separate flasks of buffer (pH 7.5) and distilled, deionised water were placed within to equilibrate to this temperature. The spectrophotometer was allowed to warm up and the wavelength programmed for 340nm and zeroed using a control of all the cuvette reagents with the exception of the reductase. All concentrations are the final reagent concentrations (fc) unless otherwise stipulated.

Nitrate standards from 2.5-40μM were used and the assay prepared in the following manner. Into each cuvette was measured 400μl of phosphate buffer, 300μl of distilled, deionised water and 250μl of nitrate standard. The cuvette was placed in the water bath for 2min to ensure the temperature equilibrated to 25°C (this was checked to be the case in a preliminary study). The cuvette was then quickly removed, dried and 25μl of FAD (2.5μM) and 8μl of NADPH (96μm) added with thorough mixing ensured by drawing the contents of the cuvette through a P1000 pipette twice. The cuvette was transferred to the spectrophotometer, 17μl of nitrate reductase added with thorough mixing again ensured and the absorbance noted at time 0 (T_0). The cuvette was then transferred back to the water bath for 50s before removing, drying

and noting the absorbance at one minute (T_1). This was repeated every minute for eight minutes (T_8). The temperature and pH in each cuvette were then measured to ensure these had not varied from the optimum (range: temperature 23.3-23.7 and pH 7.44-7.49). From this data it was possible to construct the reaction kinetics for nitrate reductase on a nitrate solution of concentration 2.5-40μM (Figure 3.5i) with the rate of change in absorbance for each consecutive minute plotted.

It was clear from this data that the reaction kinetics began to slow after 3-4min, presumably as a consequence of both reduced substrate availability and a slow decrease in reaction temperature. A further experiment was thus devised to standardise for this latter variable.

Preliminary studies had shown that 2min in the 25°C water bath was sufficient to ensure the reagent temperature in the 1ml cuvette was stable. In order to assure the maximum reaction time at 25°C before substrate availability became a potential confounding factor a T_2 reaction time was not evaluated but both T_3 and T_4 were. In each arm of this study the cuvettes (in duplicate) were set up in the manner described earlier in this section. An identical procedure was followed except that after adding nitrate reductase and measuring the absorbance at T_0 the cuvette was transferred to the water bath and incubated at 25°C for either three or four minutes prior to measurement of the final absorbance. This allowed two calibration curves to be constructed to assess whether a T_3 or T_4 time interval would be the more accurate (Figure 3.5ii). Although there was little difference the rate of change in absorbance over three minutes was greater than over four minutes therefore the former was chosen.

3.5b Construction of the Nitrate Standard Curve and Assessment of the Variability of the Kinetic Assay

The basic preparation was as described in 3.5a with nitrate standards from 2.5-70μM used with the assay prepared in the following manner. In to each cuvette was measured 400μl of phosphate buffer, 300μl of distilled, deionised water and 250μl of nitrate standard. The cuvette was placed in the water bath for 2min to ensure the temperature was stable at 25°C. The cuvette was then quickly removed, dried and 25μl of FAD (2.5μM) and 8μl of NADPH (96μm) added with thorough mixing

ensured by drawing the contents of the cuvette through a P1000 pipette twice. The cuvette was transferred to the spectrophotometer and used to zero the machine (i.e. each cuvette acted as an internal control). A 17µl aliquot of nitrate reductase was added (with the stopwatch immediately started), thoroughly mixed, and the cuvette transferred back to the water bath for two and one half minutes. At the end of this time the cuvette was dried and placed back in the spectrophotometer ready for measuring the change in absorbance over three minutes (δA_{3min}).

For each nitrate standard (2.5-70µM) this process was repeated in quintuplicate to construct the standard curve (Figure 3.5iii) and allow the coefficient of variation to be assessed. This was less than 5% for all the standards measured, which is an indication of the robustness of this assay. Furthermore, the entire procedure was repeated on a separate day using completely freshly prepared reagents to measure the inter-day variability of the assay. The slope for this second standard curve was almost identical to the first at y = x0.0021, indicating excellent reproducibility of the assay. One final monitor on the reliability of the assay was a spot check on a 40µM nitrate standard prior to analysing each batch of homogenate samples.

One further calibration curve was constructed to determine if any significant endogenous inhibitors of nitrate reductase (or the co-factors) were present within the renal homogenate to skew the results. Renal homogenates were prepared as described in 3.3c using kidneys obtained from freshly culled Wistar rats. A further dilution (×50) in phosphate buffer was then performed to ensure the endogenous nitrate was not sufficiently concentrated to be measurable within the range of the assay, although it must be noted that this would similarly dilute any endogenous inhibitors. Nitrate was dissolved in the homogenate (0.85g in 10ml to give a 1M solution, see 3.4a) which was serially diluted in distilled, deionised water to give a range of nitrate standards in homogenate from 70-2.5µM. These were then used to generate a standard curve in the manner described earlier in this section (Figure 3.5iv).

3.5c Assessment of any Potential Interference on the Assay from the use of Exogenous Manipulators of Nitric Oxide in the Animal Model

The compounds utilised in the therapeutic manipulation of post-ischaemic renal NO levels are outlined in 5.2c. It was obviously important however that whilst these

compounds affected endogenous NO they should not interfere with either the nitrate or nitrite assays. The following experiments were therefore undertaken to examine this proposition.

Working on the assumption that each kidney receives 10 percent of the cardiac output (i.e. the circulating volume) and $1/6^{th}$ of the individual kidney was used in each assay a quantity of compound equalling $1/60^{th}$ that given to the animals was utilised (Table 3.5i). This takes no account of any distribution beyond the intravascular compartment and thus may significantly overestimate the concentration of the agent providing a margin of safety in these experiments.

COMPOUND	BIO-QUANTITY	ASSAY QUANTITY
HC	10mg	167μg
L-ARG	150mg	2500μg
L-NAME	25mg	417μg
SNAP	1.25mg	21μg
ADMA	25mg	417μg
AG	25mg	416μg

Table 3.5i Quantity of each NO therapeutic manipulator used in the animal model (bio-quantity) and the consequent amount used in determining any confounding effect of these compounds on subsequent assay accuracy.

The final quantity of each agent was dissolved in 1.5ml of renal homogenate and placed in a water bath at 37°C for 85min to mirror the circumstances in the animal model (20min pre-ischaemic, 45min ischaemic and 20min reperfusion phases). This was diluted 1:5 prior to 'homogenisation', three cycles of freeze-thawing and centrifugation to mimic the processing of renal homogenates. A one millilitre aliquot (a) was separated for the nitrite assay and the remaining 0.5ml (b) was diluted 1:3 with phosphate buffer for the nitrate assay.

The nitrite assay was performed as described in 4.2e. The aliquot (a) was divided into separate 0.5ml samples and spiked with nitrite to achieve both a 50μM and 5μM test standard. Similarly for the nitrate assay 0.5ml aliquots of (b) were spiked with nitrate to achieve both a 50μM and 5μM test standard and the procedure described in 4.5b followed (Figure 3.5v). As can be seen the test standards are similar to the calibration standards for both assays, indicating that the NO donors and NOS inhibitors do not adversely influence these assays.

ALIQUOT OF 100μM NITRITE SOLUTION (ML)	ALIQUOT OF WATER (ML)	DILUTION FACTOR	FINAL NITRITE CONCENTRATION (μM)
1.0	0.11	1:1.11	90
1.0	0.25	1:1.25	80
1.0	0.43	1.1.43	70
1.0	0.67	1:1.67	60
1.0	1.0	1:2	50
1.0	1.5	1:2.5	40
1.0	2.33	1:3.33	30
1.0	4.0	1:5	20
0.5	4.5	1:10	10
0.5	9.5	1:20	5
0.5	19.5	1:40	2.5

Table 3.2i Dilution factors employed to produce nitrite standards

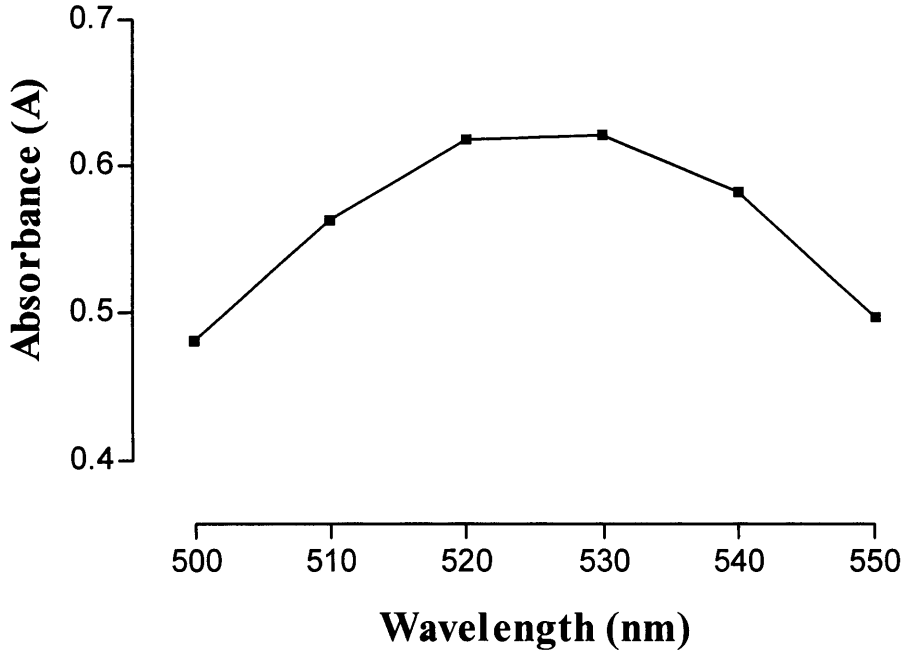


Figure 3.2i Crude determination of spectrophotometric wavelength at which light absorbance was maximal through a (50μM nitrite/Griess) azo dye solution

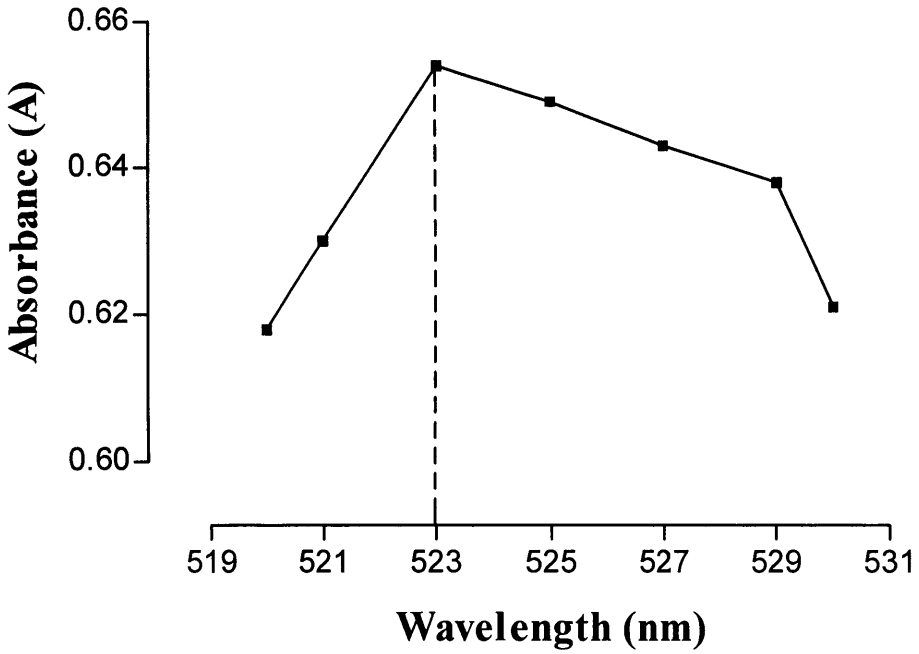


Figure 3.2ii Fine determination of spectrophotometric wavelength at which light absorbance was maximal through a (50μM nitrite/Griess) azo dye solution. Broken line indicates the wavelength at which absorbance was maximal.

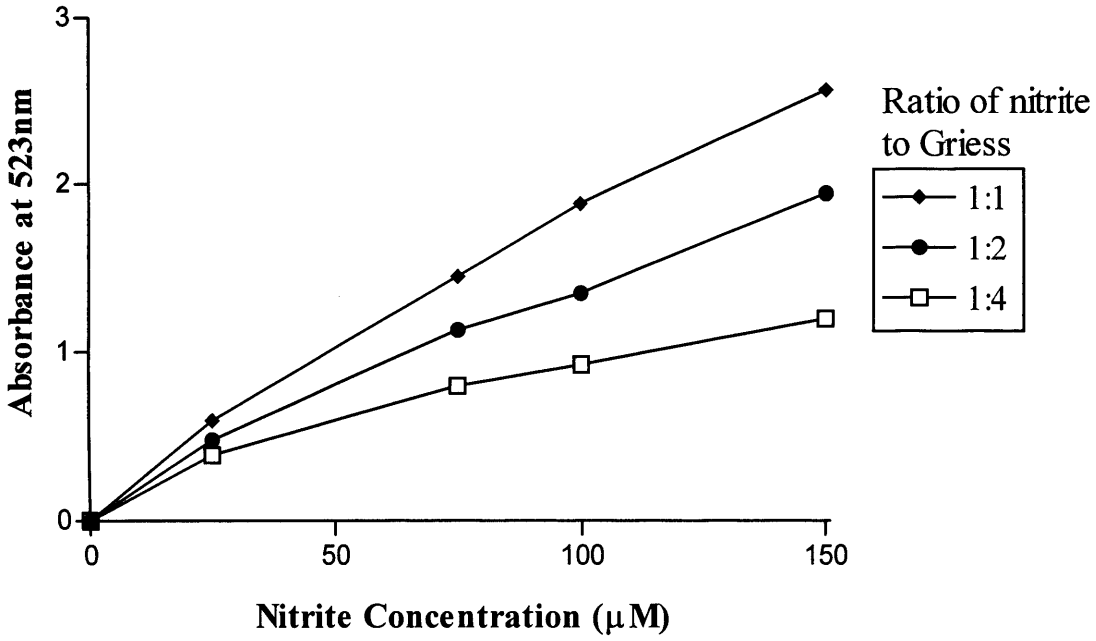
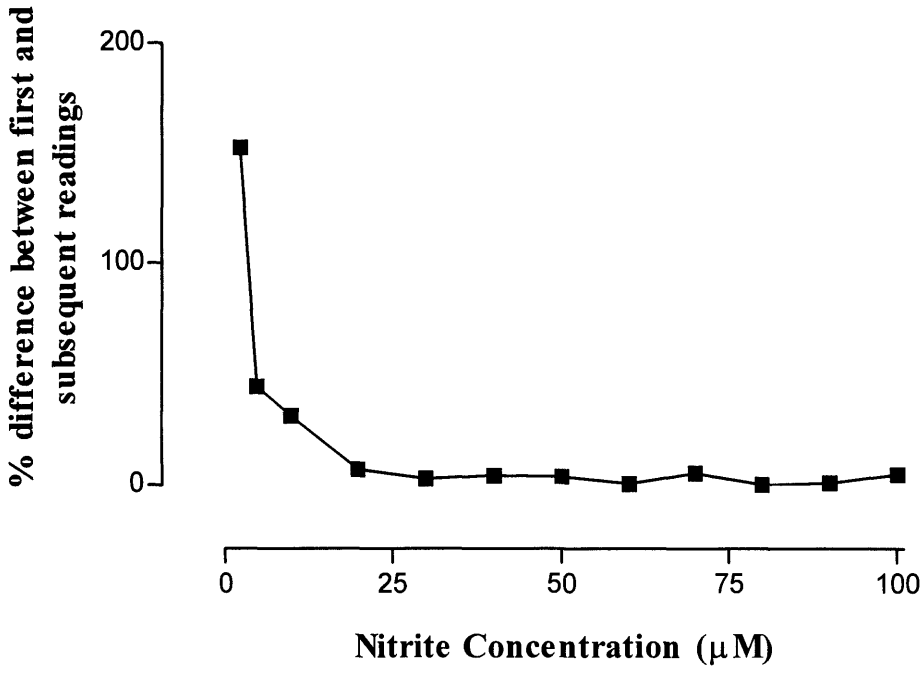


Figure 3.2iii Determination of the ratio of Griess reagent to nitrite solution for optimum assay sensitivity



3.2iv Evidence of contamination by the previous sample using the in-built spectrophotometer autocell at low nitrite concentrations. The results plotted are the difference (%) between the first reading and the mean of the four subsequent readings.

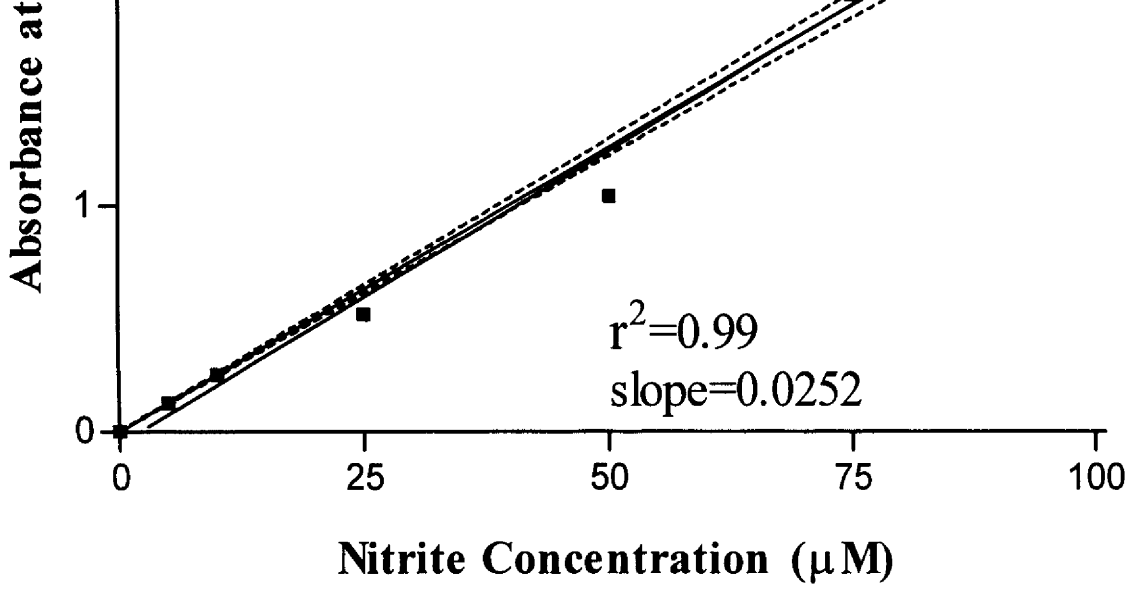


Figure 3.2v Calibration curve for nitrite in distilled, deionised water (mean w
95% CI). Slope is: y=*n*x.

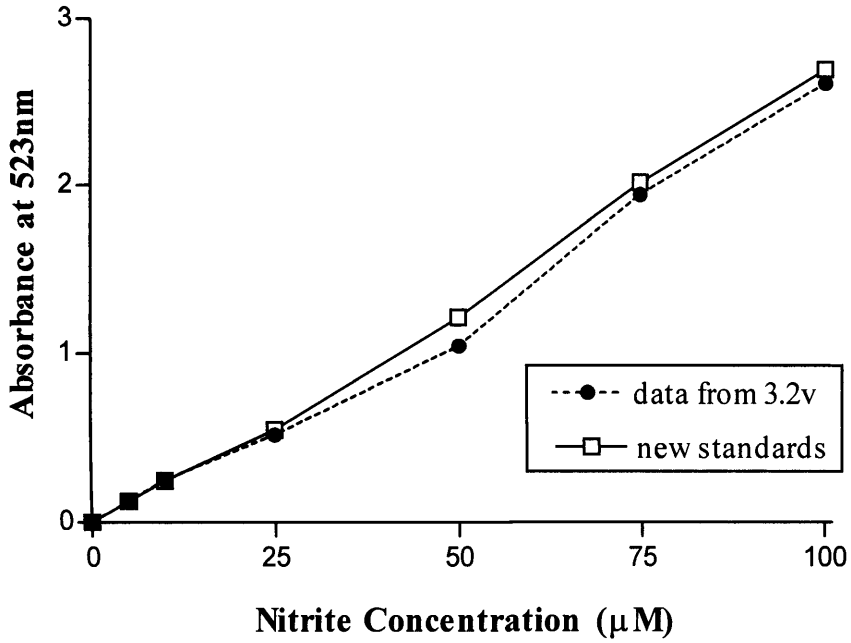


Figure 3.2vi Comparison between the original calibration curve (closed circles) and that obtained using freshly prepared nitrite standards on a subsequent day. The slope ($y = nx$) being 0.027 as compared to 0.025 for the original calibration curve (i.e. 3.2v).

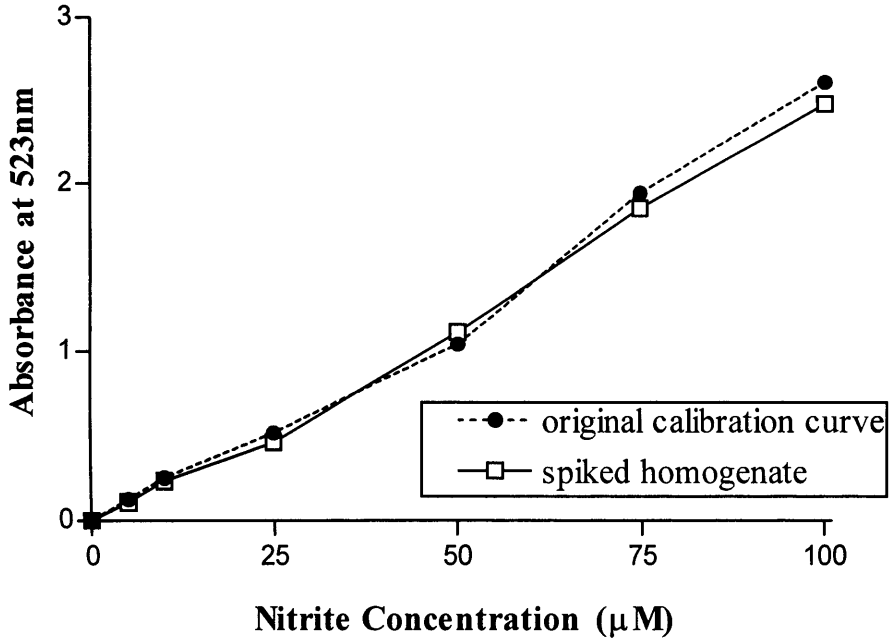


Figure 3.2vii Comparison between nitrite standard curve in distilled, deionised water (closed circle) and nitrite in renal homogenate.

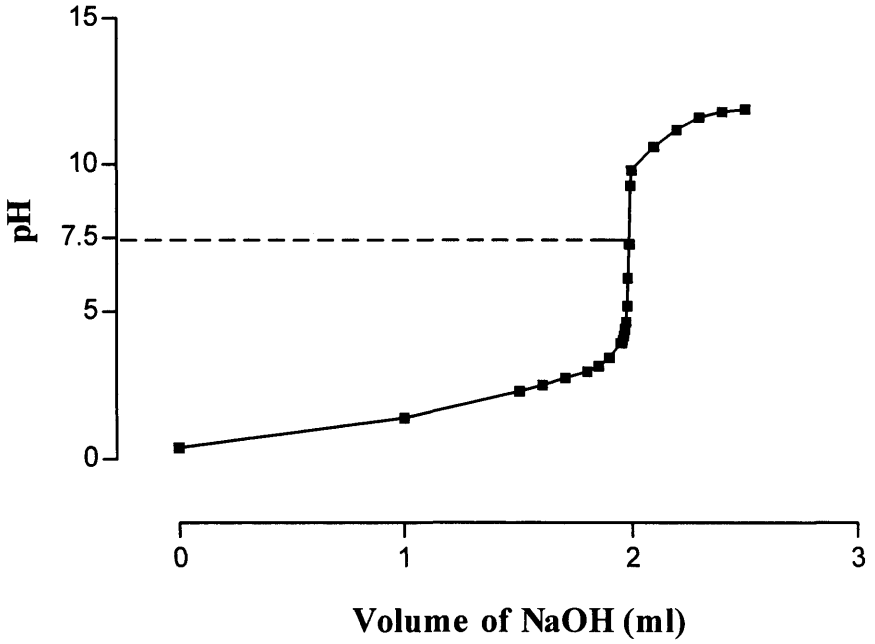


Figure 3.3i Determination of the equivalence point of 5ml 10% sulphosalicylic acid using 2M NaOH. The indicated pH of 7.5 is that needed for the optimum enzymatic conversion of nitrate to nitrite (by nitrate reductase) in subsequent assays. It proved impossible to reliably titrate the pH to 7.5.

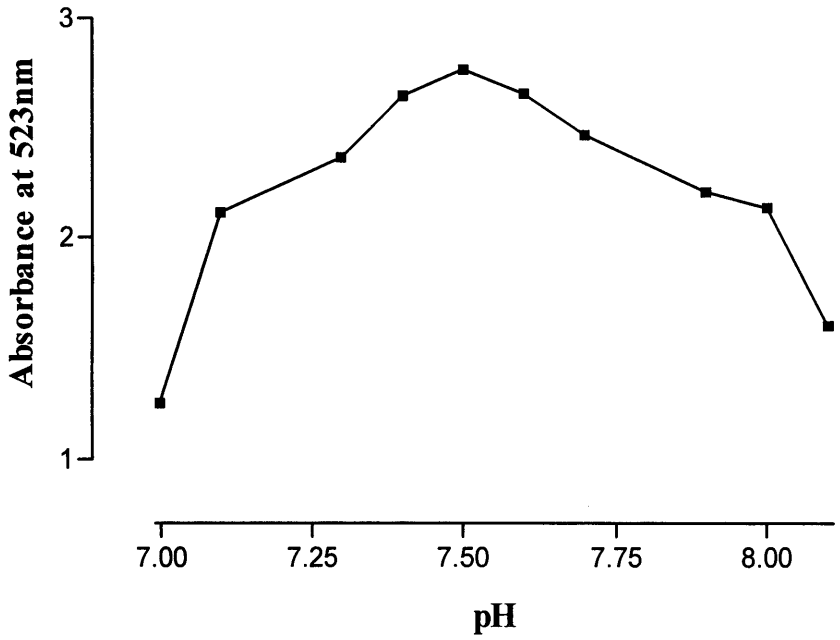


Figure 3.4i Determination of the optimum pH for conversion of nitrate to nitrite by nitrate reductase in an end-point assay

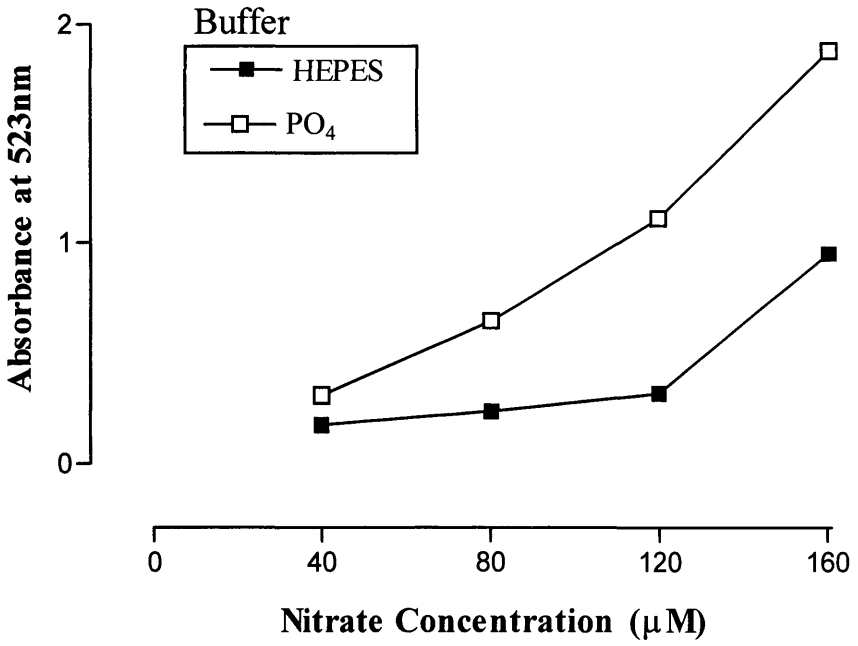


Figure 3.4ii Comparison between HEPES and phosphate buffers on the efficiency of conversion of nitrate to nitrite by NRase.

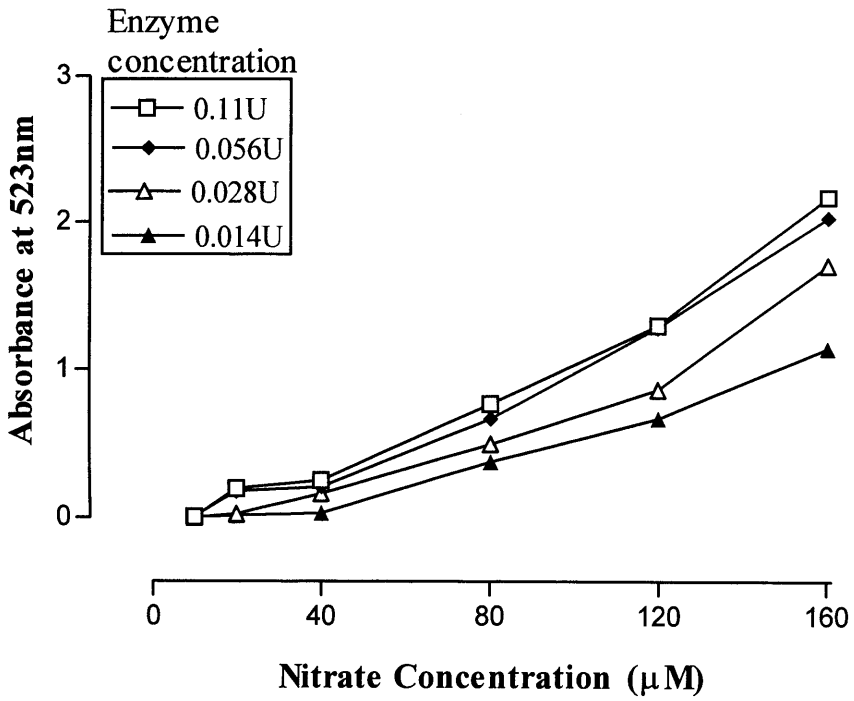


Figure 3.4iii Determination of the optimum (final) concentration of NRase to convert nitrate to nitrite in the range 10-160μM.

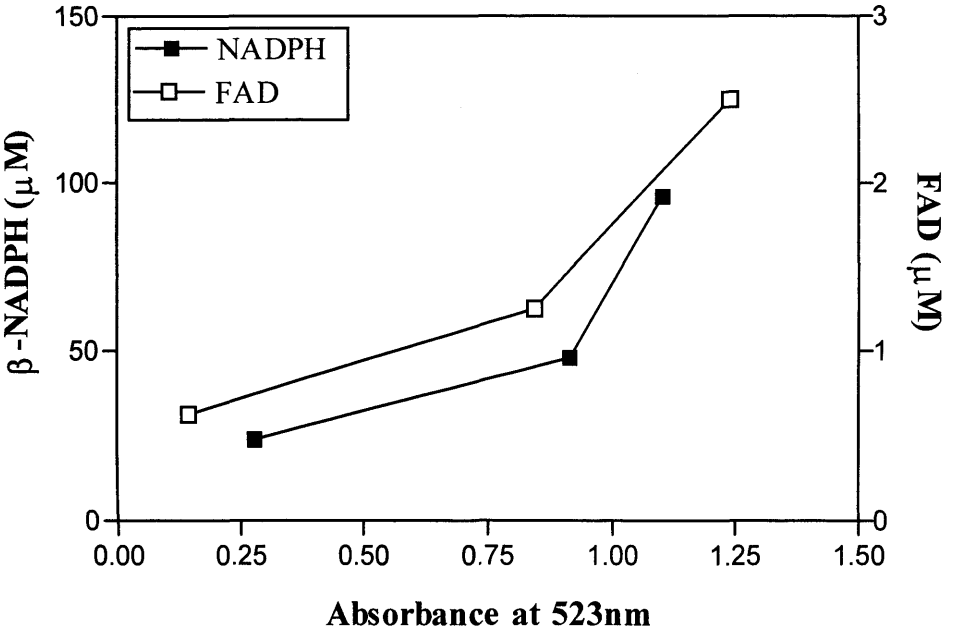


Figure 3.4iv The effect of decreasing co-factor concentration on the efficiency of NRase (0.056U) conversion of nitrate (100μM) to nitrite as measured by sample absorbance at 523nm.

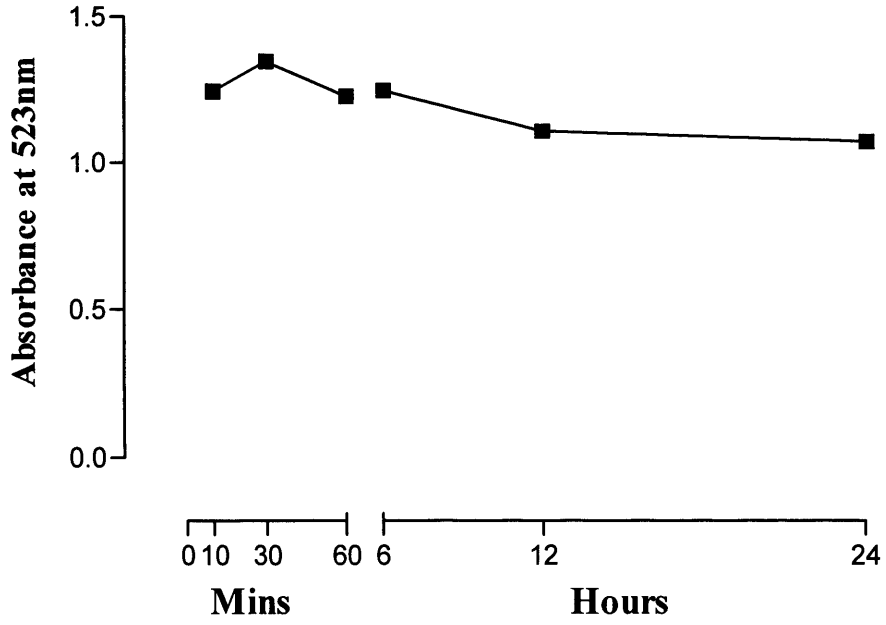


Figure 3.4v Effect of the duration of the nitrate-enzyme system incubation on the subsequent reduction to nitrite. There was no advantage from prolonged incubation beyond 10min.

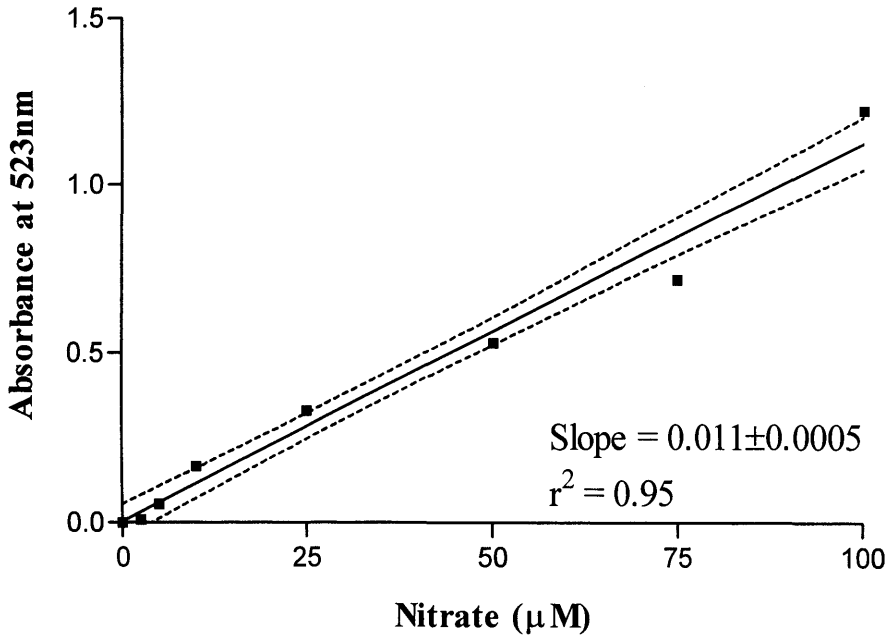


Figure 3.4vi Standard curve for the conversion of nitrate to nitrite using NRase in an end-point assay (mean with 95% CI). Slope is $y = nx \pm n_1$

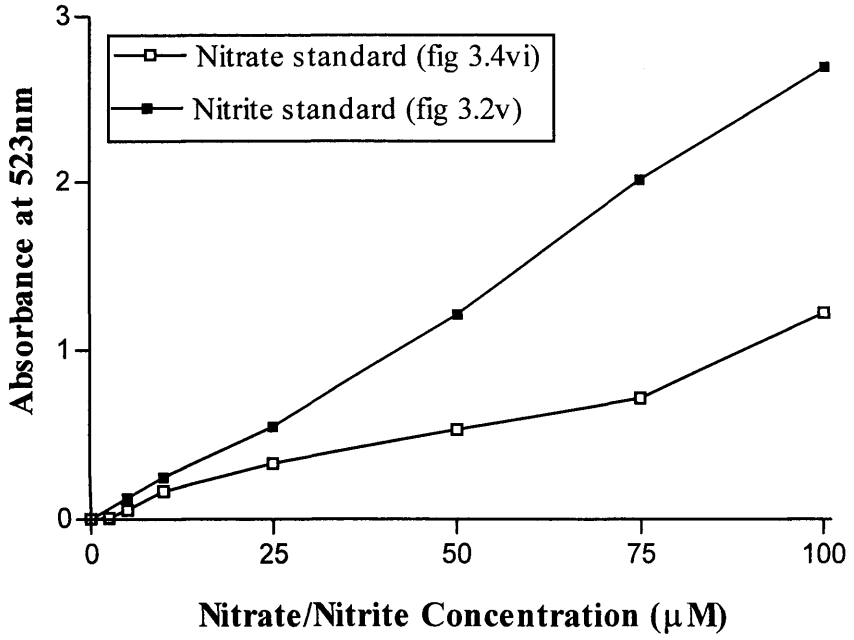


Figure 3.4vii Comparison between the nitrite standard and nitrate (to nitrite via end point assay) standard. The slope ($y = nx$) of the former is 0.027 whilst the latter is 0.011 indicating an efficiency of conversion of nitrate to nitrite of about 40%.

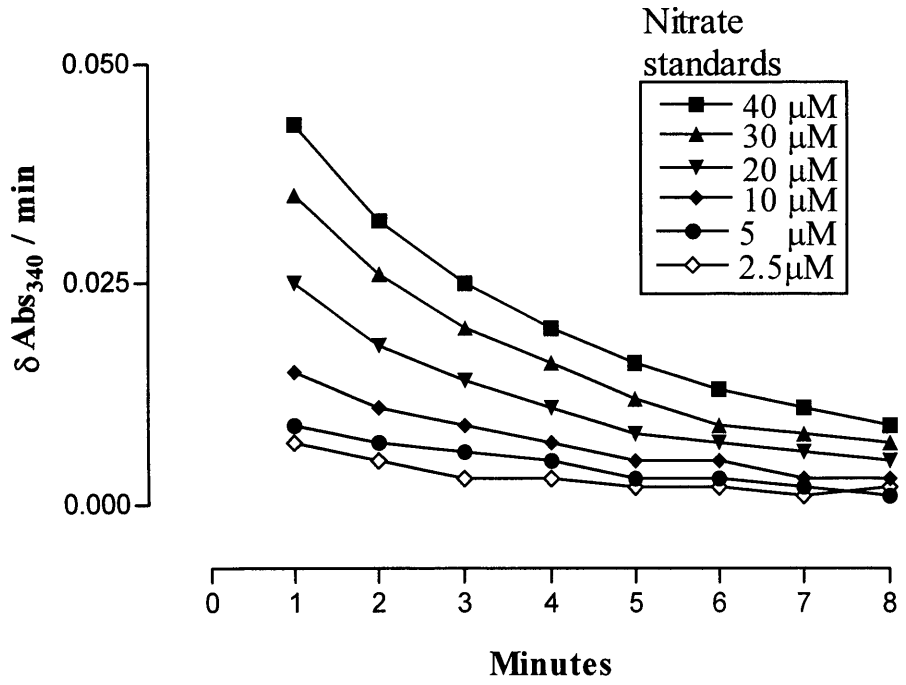


Figure 3.5i Reaction kinetics for the reduction of nitrate by NRase over eight minutes. Each point represents the change in absorbance over the preceding minute, not the absolute value of the absorbance. (in fact the rate of oxidation of β-NADPH is being measured, which is equimolar with the reduction of nitrate).

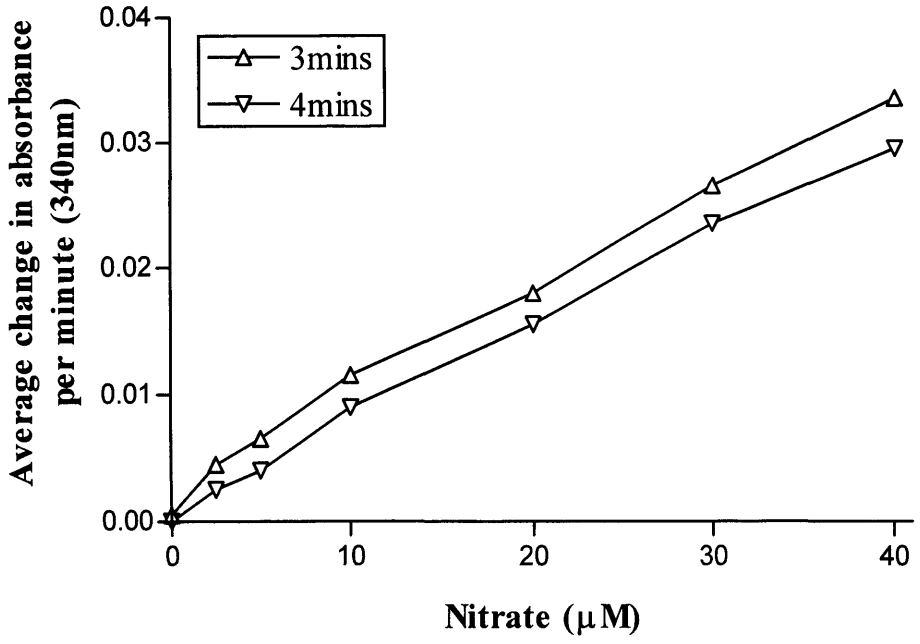


Figure 3.5ii The average rate of change per minute in absorbance as nitrate is reduced by nitrate reductase. This was measured over either three or four minutes in total, with little difference between these two time-points in terms of assay sensitivity.

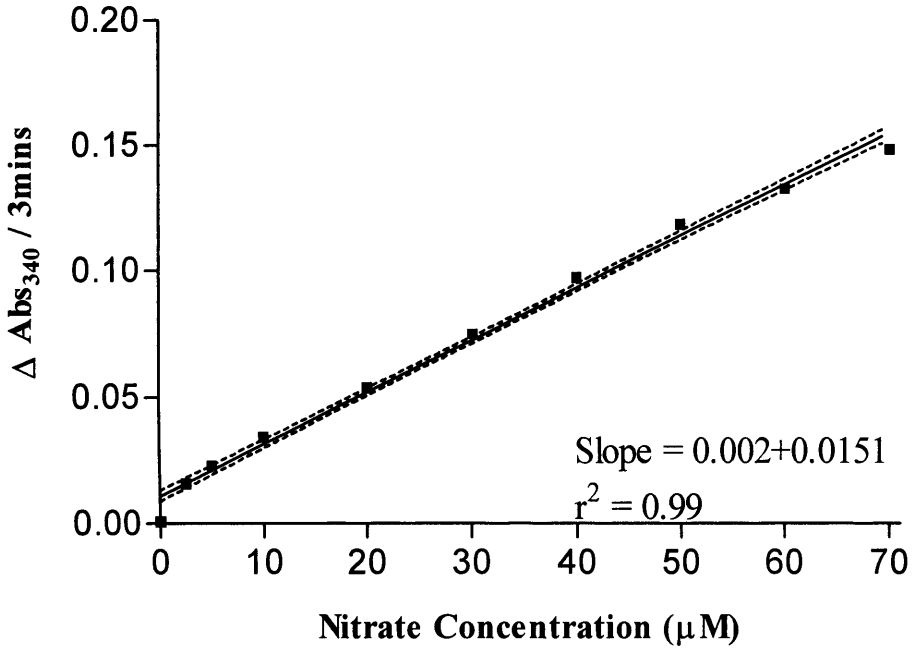


Figure 3.5iii Nitrate standard curve. This is based on the kinetic assay whereby nitrate is reduced by nitrate reductase and this is monitored spectrophotometrically over three minutes. Each point is the mean of five consecutive readings (95% CI shown). Slope is $y = nx \pm n_1$.

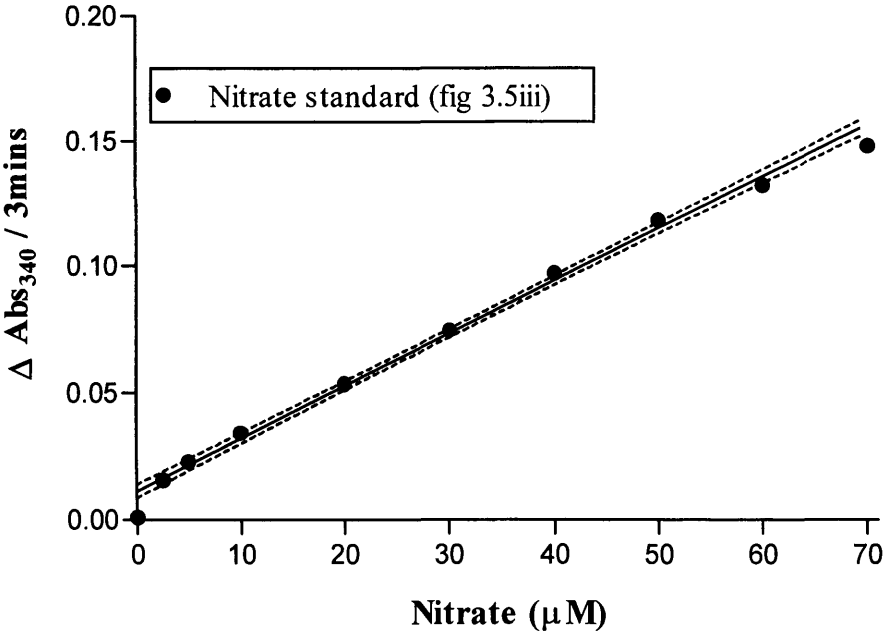


Figure 3.5iv Comparison between the nitrate standard curve (using nitrate in distilled, deionised water, closed circles with no connecting line) and nitrate standard curve using nitrate dissolved in diluted renal homogenate (shown as solid line with 95% CI). This demonstrates there is no endogenous inhibitors of the kinetic assay present within renal homogenate (see text).

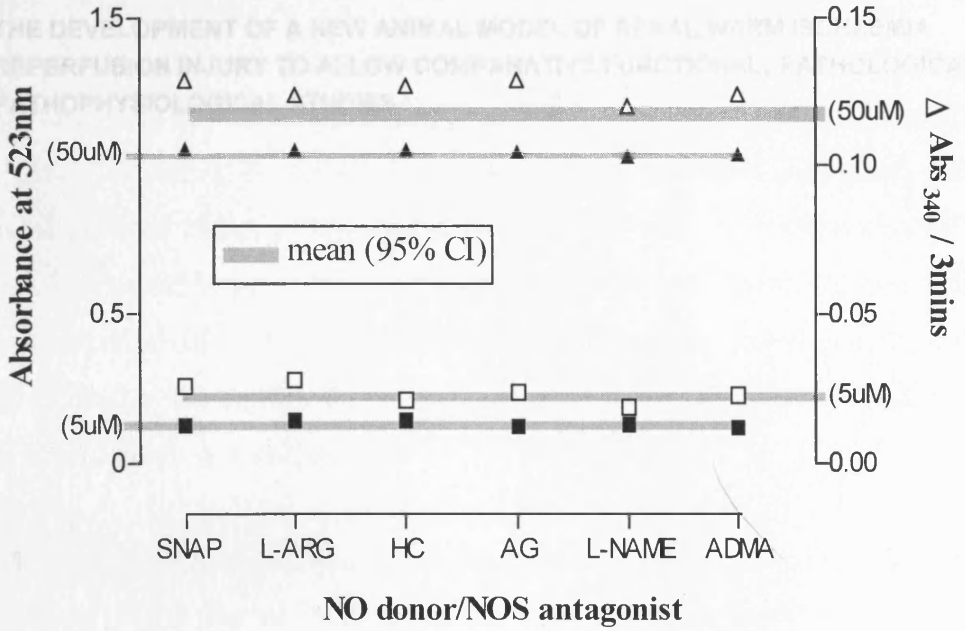


Figure 3.5v Plot demonstrating the effect of NO donors and NOS inhibitors on both the nitrite (left-y axis) and nitrate (right-y axis) assay. Extending from the relevant axis is the range (mean ±95%CI) for both 50μM and 5μM nitrite and nitrate standards. Plotted on this is the test results obtained from investigating the effect of NO donors or NOS inhibitors on the nitrite (closed symbols) and nitrate (open symbols) assays. As can be seen the test values lie close to the standard values.

Chapter 4

THE DEVELOPMENT OF A NEW ANIMAL MODEL OF RENAL WARM ISCHAEMIA REPERFUSION INJURY TO ALLOW COMPARATIVE FUNCTIONAL, PATHOLOGICAL AND PATHOPHYSIOLOGICAL STUDIES

4.1 INTRODUCTION

The standard model for investigating *in vivo* renal damage has involved performing a unilateral nephrectomy, subjecting the remaining kidney to the study insult and then monitoring the renal function of this sole kidney (Jablonski et al, 1983). For the purpose of the proposed study however this model has a major deficiency, namely the unavailability of renal tissue during ischaemia and reperfusion, except by culling the animal. This would entail increasing the number of animals in each group simply in order to obtain sufficient tissue for subsequent analysis, which was undesirable. Whilst it would be possible to biopsy the kidney, not only would this cause problems of haemostasis but more importantly the kidney would be injured in a way that would be difficult to standardise. It was therefore necessary to devise a new model of WIRI to overcome these problems.

The most suitable method for determining renal function also had to be established. Options ranged from measuring serum creatinine and urea, calculating the creatinine clearance or determining the glomerular filtration rate. This latter course was taken, as GFR is the most accurate measure of renal function. The method chosen to measure the GFR was based on that described by Nankivell et al (1992) whereby the renal clearance of a radiolabelled compound, in this case technetium-99m diethylene triamine penta-acetic acid (^{99m}Tc DTPA) is determined. This technique has the advantage of being relatively non-invasive (in comparison to the gold standard inulin clearance estimation of GFR). Furthermore, as this isotope has a half life of only six hours, not only is the isotope easy to dispose of (stored for eight half lives before disposing of as normal waste) but the assay can be repeated after several days without interference from any residual radioactivity.

Any compound used to assess the GFR must possess the following properties:

(a) non-bound to plasma proteins or erythrocytes

(b) physiologically inert

(c) freely filterable at the glomerulus

(d) neither synthesised nor destroyed by the tubules

(e) neither reabsorbed nor excreted by the tubules

Whilst ^{99m}Tc DTPA does in fact undergo some protein binding (and is thus not filtered in the glomerulus) this fraction accounts for only a small amount (<4%) of the total injected activity (Klopper et al, 1971).

In essence, a known activity of ^{99m}Tc DTPA (Amersham International) is injected into the peritoneal cavity of each rat and this is quickly absorbed into the systemic circulation. Then 50µl of plasma is obtained at two accurately noted time periods (approximately 45mins and 90mins after the i.p. injection) in order to measure the radioactivity (counts per minute). Using two plasma samples rather than one avoids the necessity to make assumptions about the volume of distribution and increases the accuracy of the technique (O'Reilly et al, 1979). The GFR was determined using a slope intercept method calculated on a computer program (see 4.2b).

4.2 MATERIALS AND METHODS

4.2a Operative Procedure

Adult male Wistar rats weighing approximately 400-600g were used in all experiments. These were out-bred animals from Charles River stock (Charles River, Margate, UK) that were bred in-house by the Biomedical Services Department (University of Leicester, UK). Following weaning all animals were allowed free access to standard rat chow (UAR, Huntingdon, UK) and tap water *ad libitum*; and housed three to a cage. The animals were cared for in accordance with the Animals (Scientific Procedure) Act 1986 and all procedures carried out in line with the Animal Licence conditions.

Groups of six animals were used for each arm of the experiment. The operative set-up included two anaesthetic gas vaporisers, and using twin face masks per vaporiser it would have been possible to have four animals anaethetised at the same time, although two groups of three was preferred (Figure 4.2i). Each animal underwent induction individually in an anaesthetic box with 5% halothane (Zeneca, Wilmslow, UK) in oxygen (BOC, Manchester, UK) at 2 litres per minute flow. Following this each rat was weighed, tail marked, placed supine on a heated mat to maintain body temperature and connected to the anaesthetic circuit (2% halothane in oxygen) via a face mask with gas scavenger. Three millilitres of sterile normal saline were injected

subcutaneously in the nape of the neck to replace the ensuing surgical losses from evaporation and blood loss and the abdomen clipped, prepared with chlorhexidene in alcohol and covered with a sterile keyhole drape.

A midline laparotomy was performed, the bowel exteriorised and abdominal cavity exposure maintained with a self-retaining retractor. The viscera were examined to exclude any abnormal pathology and then the infra-renal vena cava and kidneys prepared. Using minimal dissection a retroperitoneal window was made both superiorly and inferiorly to the renal vascular pedicle sufficient to allow a Diffenbach vascular clamp (Bolton Instruments, Bolton, UK) to be placed across each of the renal pedicles to occlude it (Figures 4.2ii-iii). The peritoneum was opened over the infra-renal vena cava just proximal to the origin (Figure 4.2iv). Using a 25G needle one millilitre (1ml) of sterile saline was infused over one minute and the needle withdrawn (Figure 4.2v). Saline was infused i.v. to standardise these experiments with the subsequent protocol for infusion of nitric oxide manipulating agents where saline was used as the vehicle. A small patch of Surgicel (J&J, Ascot, UK) was applied to the puncture site with light pressure to achieve haemostasis. Twenty minutes after infusion the clamps were applied to the renal pedicles.

The commencement of renal ischaemia was confirmed visually before the bowel was replaced in the peritoneal cavity and the abdomen closed loosely over a swab moistened in sterile water. At the end of the designated warm ischaemic period (group times = 15, 30, 45 or 60 minutes) the abdominal pack was removed, the clamps released and reperfusion verified visually. During reperfusion (group times = 20 or 80 minutes) the bowel was again covered with a moistened swab and the wound edges loosely apposed. At the end of reperfusion a left (L) nephrectomy was performed by ligating the vascular pedicle with 4/0 vicryl (Ethicon, Edinburgh, UK) and then stripping the renal capsule in-order-to facilitate defatting the organ. When the kidney was free of all fat it was weighed (Maettler AE120), cut into six equal segments containing cortex and medulla and snap frozen in liquid nitrogen.

Four additional groups were also investigated (Table 4.2i). A control group (C) which underwent an identical operative procedure and nephrectomy but without renal ischaemia, a sham group (S) which underwent an identical laparotomy with neither

the renal ischaemia nor nephrectomy and a normal group (N) for baseline values that underwent no operative procedure. One further group was also investigated for comparison with the standard model of renal warm ischaemia (Jablonski et al, 1983). This group (J_{45}) underwent an identical operative procedure except only the right renal pedicle was occluded (45 min) and following reperfusion (20 min) a left nephrectomy was immediately performed.

GROUP (**min** = duration of ischaemia)	***PROCEDURE*** or **REPERFUSION TIME (min)**		
Normal	*Not anaesthetised, no laparotomy. Culled on day 7*		
Sham	*Anaesthetised with laparotomy and renal pedicle manipulation. Culled day 7*		
Control	*Anaesthetised, laparotomy & (L) nephrectomy. Culled day 7*		
J_{45}	*Unilateral (R) renal ischaemia for 45 min then immediate (L) nephrectomy*		
15 min	**20 min**	**80 min**	**Day 7**
30 min	**20 min**	**80 min**	**Day 7**
45 min	**20 min**	**80 min**	**Day 7**
60 min	**20 min**		**Day 7**

Table 4.2i Outline of the experimental groups investigated in developing the new model of renal warm ischaemia reperfusion injury. For full details see preceding text.

Having secured haemostasis the abdomen was closed in layers using mass closure with 3/0 vicryl (Ethicon) and a continuous sub-cuticular skin closure with 3/0 vicryl. Each animal received a 30μg bolus of buprenorphine (R&C, Hull, UK) intramuscularly for postoperative analgesia prior to recovery.

Renal function was assessed on the second and seventh days postoperatively. Following this second GFR the animals were culled by stunning and neck dislocation and the remaining kidney harvested and the capsule stripped. The kidney was weighed, cut into six segments containing cortex and medulla and half of the upper pole saved in 10% formalin for subsequent histological examination before the remaining tissue was snap frozen.

4.2b Measurement Of The Glomerular Filtration Rate

As stated earlier, the GFR was measured on the second and seventh days following renal ischaemia reperfusion injury. In addition to delineating the effects of warm ischaemia on subsequent renal function it was also important to establish a baseline reference with which to compare the effect of subsequent nitric oxide manipulation on renal function.

The ^{99m}Tc DTPA was prepared (from stock solution) on the day by the Department of Radiopharmacy, Leicester Royal Infirmary. Under sterile conditions seven aliquots of approximately 0.2ml were suspended in 0.9% normal saline in 2ml syringes to give a final volume of 0.5ml with an activity of 20-25MBq. The activity was accurately measured in a gas ionisation calibrator (Capintec CRC10B), the time noted (T_0) and these syringes transported to the Biomedical Services Unit (BSU) in a shielded lead container.

An assistant held each rat supine by the scruff of the neck whilst the isotope was injected i.p. to the left of the animal's midline (Figure 4.vi), a stopwatch was started on injecting the first animal with a one minute hiatus between each animal. After all six animals had been injected the final aliquot was used as the standard and injected into one litre (1000ml) of tap water in a volumetric flask. The syringes (with the needles attached) were then immediately transported back to Radiopharmacy and the residual activity measured to allow the injected dose to be calculated after correction for decay. Forty five minutes (T_1) after injection the first rat was removed from its cage, wrapped in a towel and the tail tip amputated with a scalpel. Whilst the rat was restrained, 1ml of mixed blood was collected (Figure 4.2vii) in a 1.5ml Eppendorf which had been pretreated with 20µl of sodium heparin (5000U/ml) as an anticoagulant. The time of each individual blood sample was noted, the Eppendorfs placed in a microcentrifuge (Whatmann Force 16) and spun at 15000g for 6 minutes. Of the resultant plasma, 50µl was pipetted into a capped plastic tube. Meanwhile the volumetric flask containing the standard was inverted several times to ensure adequate mixing of the (heavy) ^{99m}Tc DTPA in the water then a 50µl sample was pipetted into a tube and the time noted. Exactly the same procedure was followed at 90 minutes (T_2), except the tail tip did not need to be reamputated, merely the clot removed with a swab.

These fourteen tubes (two from each rat and two standards) were transferred immediately to the gamma counter (Philips PW4800) which was programmed for ^{99m}Tc DTPA (program 7). This calculated the average counts per minute (cpm) over a total count time of 10 minutes per tube. The GFR could then be calculated from the cpm using a slope intercept method (Nankivell et al, 1992):

(1) $GFR = Ik / C_o$

I= injected activity

k=exponential slope of plasma clearance

C_o=zero time intercept of clearance curve

CR=count rate

(2) $$I = \frac{(\text{net standard CR}) \times (\text{net animal dose}) \times 1000 \text{ (for dilution)}}{(\text{net standard dose})}$$

(3) $$k = \frac{(\ln CR_1) - (\ln CR_2)}{(T_2 - T_1)}$$

(4) $C_o = \exp (\ln CR_2 + (T_2 \times k))$

4.2c Renal Histopathology

The tissue preserved in formalin was batch processed by dehydration and then embedded in paraffin. Sections were cut at 4μm and stained with haematoxylin and eosin (H&E) for histological examination. Examination and scoring of these was performed on a blinded basis by an experienced renal histopathologist. The slides were shuffled to avoid bias from fatigue and graded on two separate occasions with an arbitrary score of 0-4, where 0 was normal (table 4.2ii). At the first assessment the factors scored were cortical and medullary tubular damage, variation in nuclear shape, presence of mitotic figures and glomerular perfusion (i.e. erythrocyte count) (Furness et al, 1993). The second assessment concentrated on cortical tubule damage which was graded separately for tubular dilation, debris in the lumen of the tubules and the presence of apoptotic or pyknotic nuclei. Evidence of interstitial oedema, interstitial lymphocytic infiltration and nucleated cells marginating in the vasa recta was also sought.

	PARAMETER ASSESSED	ABNORMALITY				
		Normal	Mild	Definite	Severe	+necrosis
Cortical tubules	Epithelium	0	1	2	3	4
	Dilation	0	1	2	3	
	Debris	0	1	2	3	
	Nuclei	0	1	2	3	
	Perfusion	0	+1 /-1			
	Mitoses	...	...	count	...	...
	Apoptosis	0	1	2	3	
Medullary tubules	Epithelium	0	1	2	3	

Table 4.2ii The grades and range of parameters used to assess the degree of renal tubular damage at day seven following warm ischaemia.

4.2d Statistical Analysis

Analysis of variance (ANOVA) with Dunnets comparisons was used for all data, except for analysis of the histological data where the Kruskal-Wallis test with Dunns comparisons was used. Significance was taken as $p<0.05$ and both a paired and unpaired t-test was used where appropriate. Results are expressed as mean(sem) except for histological data which is expressed as median (95% CI).

4.3 RESULTS

4.3a Body Weight of Each Group of Experimental Rats

All rats were weaned at two weeks and then kept three to a cage for a further six weeks until their body weight was approximately 500g (Figure 4.3i). The mean weight for the 66 rats used for this part of the experiment was 513(5.7)g.

4.3b Survival Following Increasing Durations of Renal Warm Ischaemia

Day two survival after 15 min warm ischaemia was 92% (11/12, the one death being perioperative), 100% after 30 min (12/12), 100% after 45 min (12/12), and 100% after 60 min (6/6). At day seven the survival was: 92% (11/12) in the 15 min group, 92% (11/12) in the 30 min group, 83% (10/12) in the 45 min group and 33% (2/6) in

the 60 min warm ischaemia group. In view of this poor survival the planned 60 min warm ischaemia and 80 min reperfusion group was not undertaken. The J_{45} group had 100% survival (6/6) to day seven. With the exclusion of the one death perioperatively all other deaths occurred between day three and six (Table 4.3i).

4.3c Change In Renal Weight Following Warm Ischaemic Damage

Section 4.2a described how and when each kidney was removed and weighed. All measurements were wet weights, as any attempt to warm dry the kidney would almost certainly have affected subsequent assay accuracy. The alternative of freeze drying would have been ideal, not only for measuring renal dry weight but also by eliminating several steps in the later processing of the kidney and by preserving the tissue for subsequent analysis, but unfortunately these facilities were not available.

In the normal and sham groups renal weight was measured at day seven as neither of these groups underwent early nephrectomy. The left kidney weight was used for 'early' measurements (to correlate with the left kidney weight measured following nephrectomy after 20 or 80min reperfusion). The right kidney weight (obtained at the same time) was used for day seven comparison. The control renal weights represent left kidney weight peri-operatively and right kidney at day seven (both without ischaemia). The J_{45} values indicate left kidney weight following 45 min right renal warm ischaemia and 20 min reperfusion and the right kidney weight at day seven.

The control renal weight for rats of this size was 1.70(0.09)g. Following WI and twenty minutes reperfusion (Figure 4.3ii) renal weight increased in all groups in line with the duration of ischaemia, significantly in the 60 min group ($p<0.01$). By 80 min reperfusion (Figure 4.3iii) renal weight had returned towards control values. By day seven renal weight was again elevated over control in line with the duration of warm ischaemia (Figures 4.3iv & 4.3v) reaching significance in the J_{45}, 45 & 60 min groups ($p<0.01$).

4.3d The Effect of Renal Warm Ischaemia on Glomerular Filtration Rate

At day two there was progressive impairment of the GFR seen with an increasing duration of warm ischaemic injury (Figure 4.3vi). Compared to the control this was

significant in the 30, J_{45}, 45 and 60 min groups ($p<0.01$). The control (one kidney) GFR was impaired when compared to the normal (two kidney) animals at day two through to day seven ($p<0.01$) and the sham group at day seven ($p<0.05$). By day seven there was an improvement in renal function in all the ischaemic groups although it remained below control values in the 45 & 60 min groups ($p<0.05$ and $p<0.01$ respectively) as well as the J_{45} group ($p<0.05$) (Figure 4.3vii). The recovery in GFR between days two and seven was significant in the 30 min ($p=0.01$),45 min ($p<0.01$) and J_{45} ($p<0.001$) groups, but failed to reach significance in 60 min group due to the excessive mortality by day seven. There was a strong correlation between the GFR at both day two ($r^2 = 0.88$) and day seven ($r^2 = 0.95$) and the duration of warm ischaemia (Figures 4.3viii, 4.3ix).

4.3e Renal Morphology

Mild morphological changes were apparent in those kidneys with 15 or 30 min warm ischaemia. These changes were more pronounced after 45 min (incl. J_{45}) and by 60 min were severe (Figures 4.3x-4.3xiv). Some degree of nuclear variation was seen with increasing warm ischaemia. Overall the cortical tubules demonstrated the most marked changes in all groups with the medullary tubules appearing relatively spared by comparison. Mitotic figures, impaired glomerular perfusion and apoptotic/pyknotic nuclei were only detected in the 60 min and J_{45} groups. The 60 min group alone showed areas of true necrosis with calcified tubular debris. No evidence of interstitial oedema, interstitial lymphocytic infiltration or nucleated cells marginating in the vasa recta was seen in any group. Of all the factors analysed the most sensitive markers of damage were the overall cortical damage and the degree of cortical tubular dilation and debris (Table 4.3ii). In these three categories when compared to the control both 45 and 60 min ischaemia resulted in significant morphological abnormalities ($p<0.01$). Furthermore the 60 min group was damaged in comparison to the 15 min ($p<0.01$) and 30 min ($p<0.05$) groups. A note of caution must be raised with regard to the significance of the histological data. It could be argued that either the Fisher's exact test or Chi-square test would be preferable to Kruskal-Wallis to analyse this data. In practice however the numbers present in each subgroup to perform these tests were too small for any meaningful analysis.

4.4 DISCUSSION

Renal warm ischaemia reperfusion injury in clinical practice is a *sequela* of both systemic hypoperfusion with subsequent circulatory resuscitation and local renal hypoperfusion following either aortic cross clamping or renal transplantation. In abdominal aortic aneurysm (AAA) surgery the proximal extent of the neck of the aneurysm sac in relation to the renal arteries has an important bearing on subsequent morbidity and mortality. Cross clamping the infrarenal or suprarenal aorta is associated with an increased perioperative cardiac and pulmonary demand in addition to an ischaemic insult sustained by the kidneys and possibly the visceral organs and spinal cord. Whilst the majority of AAA repairs allow for infrarenal cross-clamping the consequent altered haemodynamics still results in a significant decrease in renal blood flow (Gamulin et al, 1984). Release of the aortic clamp therefore leads not only to both a systemic reperfusion injury (Khaira et al, 1996), which will effect the kidney as a highly vascularised organ, but also a local renal reperfusion injury.

A lack of suitable organs for transplantation from traditional sources has led clinicians to seek alternatives, in part from non-heart-beating donors (NHBD) (Varty et al, 1994b). The results are encouraging although graft survival is poorer and delayed graft function more prevalent than in kidneys from cadaveric donors. This is a consequence of the prolonged warm ischaemic insult before renal cooling can take place (Castelao et al, 1993; Varty et al, 1994a; Wijnen et al, 1995). Primary non-function is also a significant problem and a reliable preoperative test for tissue viability to prevent transplanting irrevocably damaged organs is urgently needed (Nicholson, 1996).

The experiments presented here were designed to investigate *in vivo* renal warm ischaemia reperfusion injury exclusively. It would be a simple matter, however, to cross-clamp either the supra- or infra-renal aorta instead of the renal pedicles to model the more generalised warm ischaemic damage consequent from aortic aneurysm surgery. This new model offers several advantages over the standard model of unilateral renal ischaemia with immediate contralateral nephrectomy (Jablonski et al, 1983). Firstly, the timeline is more appropriate with bilateral renal ischaemia than predominately unilateral reperfusion. Harvesting one kidney after varying degrees of reperfusion as well as sampling the renal venous blood gives access to tissue for

analysis of the mechanisms of reperfusion injury such as free radicals, nitric oxide and prostanoids (Weight et al, 1996). In addition the cellular markers of injury, e.g. lipid peroxidation, DNA scission and protein nitrosylation can be measured and all this data can then be correlated with the *in situ* kidney which has suffered an identical ischaemic insult. This kidney not only provides tissue to measure the same parameters after seven days (or any other duration) of reperfusion in addition to the morphological damage, but using the simple method described allows the accurate *in vivo* quantification of renal function every second day if required (Nankivell et al, 1992). A further benefit of this model lies in the potential development of a tissue viability test. Those kidneys that turn out to be irrevocably damaged (equivalent to primary non-function) have an identical match in the 'preoperative' state, which can be used to develop a viability assay.

The measurement of GFR as the index of renal function is important as simpler estimations based on the serum creatinine and urea are too insensitive (Jablonski et al, 1983) and are affected by body mass and dietary factors (Tauxe, 1985). The accuracy of the method of GFR determination described is comparable (Peters, 1991) to the gold standard method of inulin clearance (Rehling et al, 1984; Nankivell et al, 1992) but is considerably simpler to perform. In comparison to other radioisotope methods the short half-life (6 hours) of ^{99m}Tc DTPA allows repeated GFR measurements and routine clinical disposal of contaminated waste after storage for eight half-lives (48 hours). Taking two timed samples rather than a single point measure obviates the need to estimate the volume of distribution, a calculation prone to error in renal failure (Hall et al, 1977; O'Reilly et al, 1979).

The initial intention was to score the morphological changes according to an established grading system (Calder et al, 1971) ranging from one (mitoses and necrosis of individual cells) to four (necrosis affecting all three segments of the proximal convoluted tubule). However using this system all the kidneys would have been normal or grade one, even after 60 min warm ischaemia. There was thus a need to develop a more representative histological grading system for this model. The explanation for this discrepancy between the models probably lies in the different duration of renal recovery, seven days in our model and two days for the Jablonski model.

It is likely that the peak at 20min in renal weight represents reactive hyperaemia (Cristol et al, 1993) upon reperfusion, despite the rise in renal vascular resistance and fall in RBF that follows ischaemia (Riley, 1978), rather than oedema as resolution occurs by 80 min. The J_{45} kidneys, although exposed to the injurious systemic effects of reperfusion (primed PMN, cytokines, etc.), had not been primed by an ischaemic insult. It was therefore not surprising that their weights did not differ significantly from control values. At day seven the increase in renal weight must be a consequence of hypertrophy, hyperaemia or oedema or possibly a combination of these factors. Following a unilateral nephrectomy there was little evidence of hypertrophy of the remaining kidney by day seven with control weights increasing from 1.70(0.09)g to 1.75(0.08)g. Of hyperaemia and oedema the latter seems most likely with pro-inflammatory cytokines known to be part of the post-ischaemic picture (Weight et al, 1996). It is interesting to note that, at least as far as renal oedema is concerned, there is a significant difference ($p<0.01$) between this new model and J_{45}.

A second aim of these experiments was to determine the most useful duration of renal warm ischaemia for further studies. In this model there was a good correlation between increasing warm ischaemia and the degree of renal impairment measured both pathophysiologically and morphologically. Altogether, an increase in warm ischaemia led to: (1) An increase in mortality particularly in the 60 min group. (2) An increase in renal weight upon early reperfusion (20 min), presumably as a result of oedema, although this effect had diminished by 80 min. By day seven the weight again reflected the duration of ischaemic damage indicating renal hypertrophy in an attempt to maintain function. (3) An impaired renal function at day two ($r^2 = 0.88$) with partial recovery by day seven and (4) significant morphological changes, especially in the cortical tubules.

Overall, 45 min of ischaemia demonstrated a significant derangement in the measured parameters whilst maintaining an acceptably low mortality rate. A comparison between this and the Jablonski model of 45 min warm ischaemia shows no significant difference between the two groups in any of the measured parameters except renal weight but this new model offers the advantages already outlined. This then would be the ideal group to assess the efficacy of pre- or post-transplantation

regimens including differing preservation media and pharmacological manipulation of ischaemia-reperfusion injury.

In conclusion this new model provides clear advantages in the investigation of renal warm ischaemia reperfusion injury. Comparative functional, morphological and pathophysiological data can be obtained whilst minimising the number of animals required. The 45 min ischaemia group was chosen for further studies addressing potential therapies to ameliorate renal warm ischaemic injury.

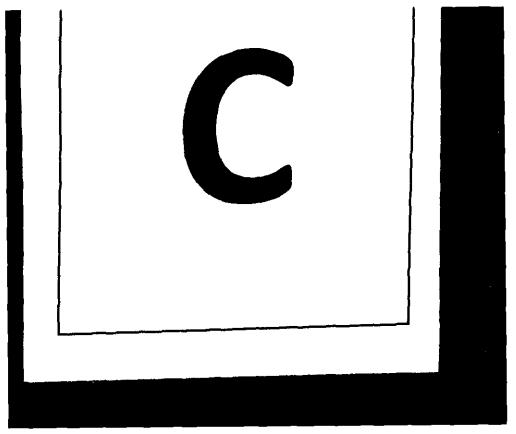

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Figure 4.2i The operative set-up which allowed three animals to be simultaneously anaesthetised under the experimental protocol

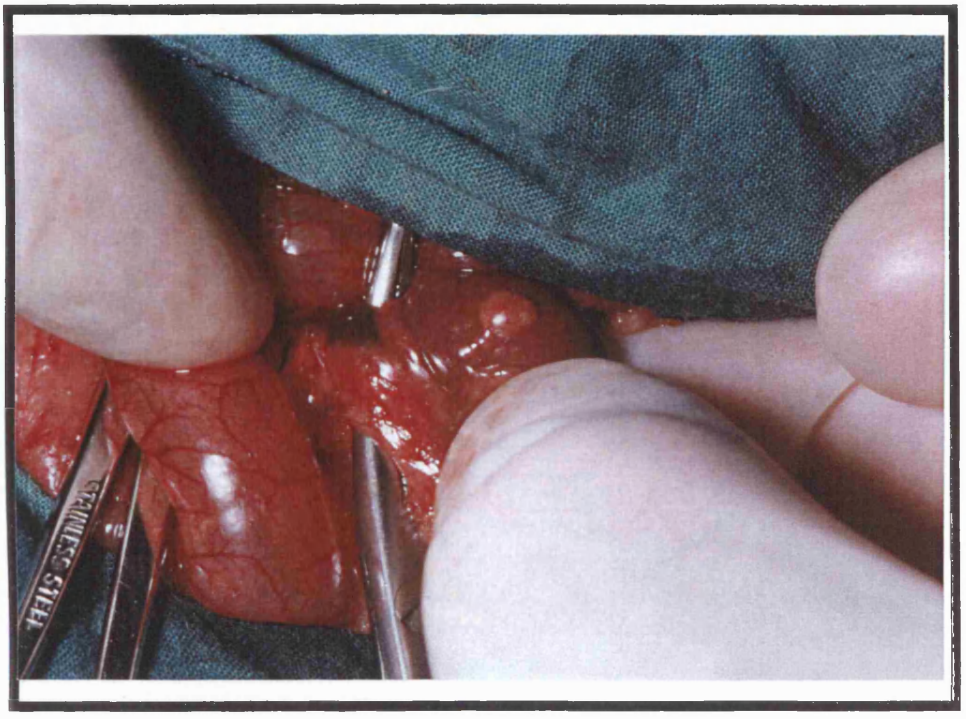

Figure 4.2ii A retroperitoneal window has been dissected behind the left renal pedicle with the kidney retracted laterally

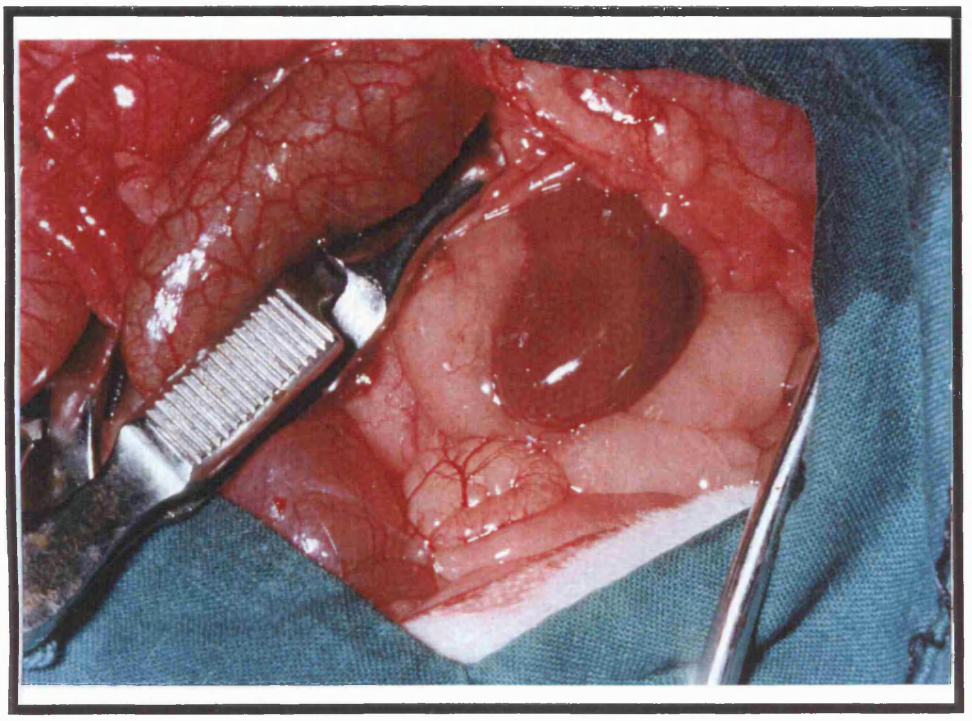

Figure 4.2iii A vascular clamp applied across the left renal pedicle

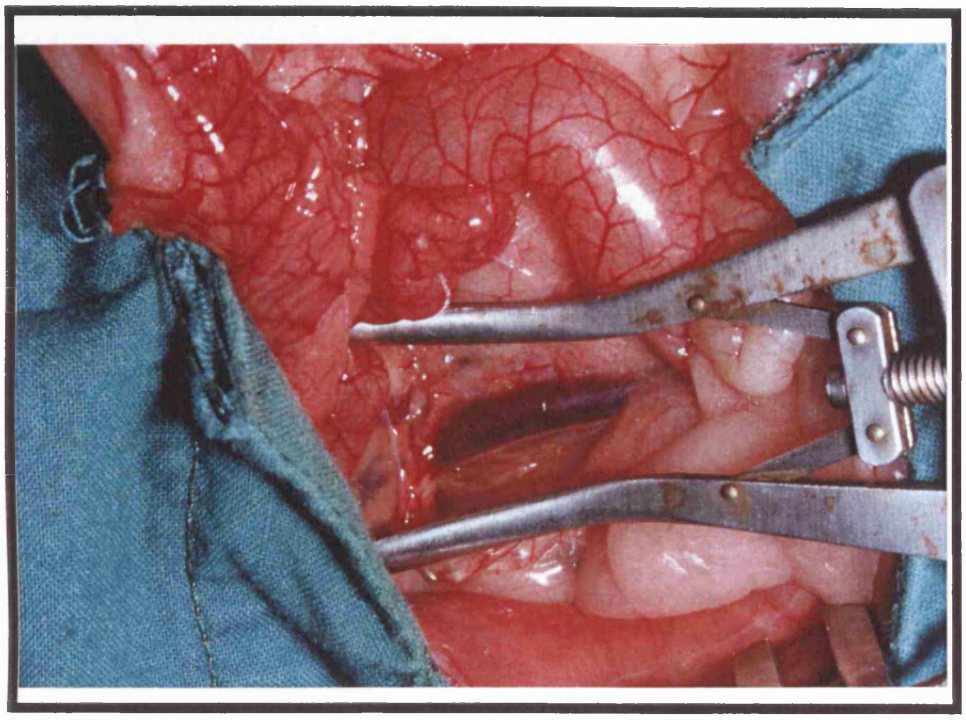

Figure 4.2iv The origin of the vena cava exposed

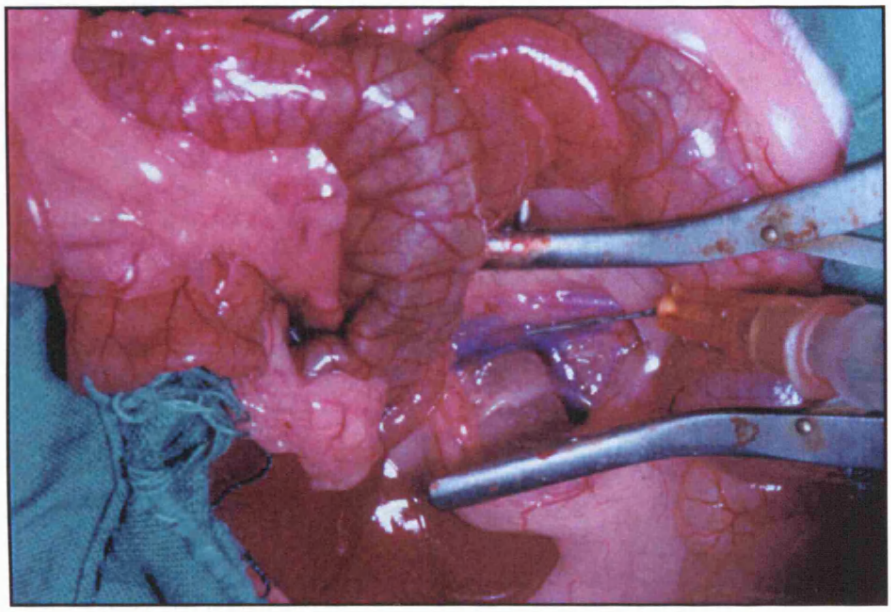

Figure 4.2v Infusion of vehicle (saline) intracavally over one minute using a 25G needle

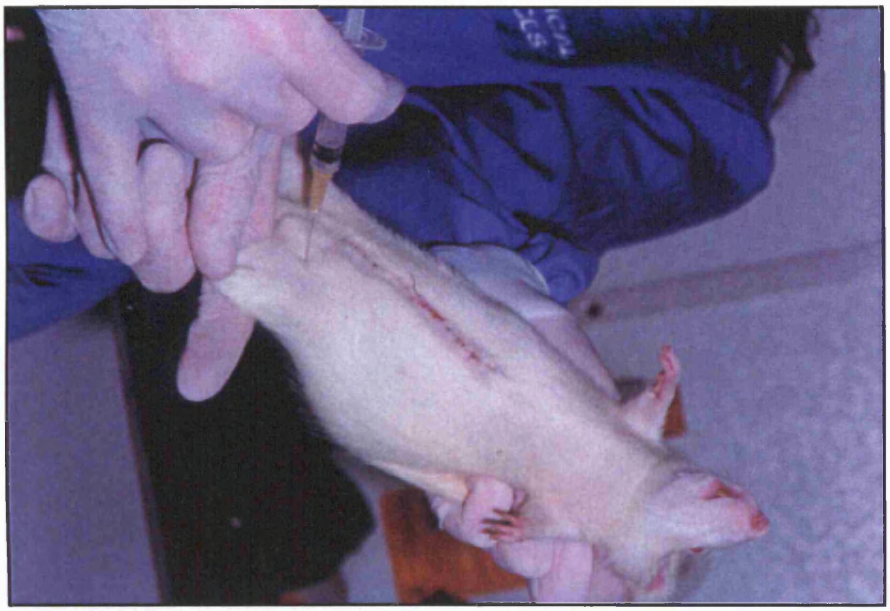

Figure 4.2vi Intraperitoneal injection of the radioisotope to calculate GFR

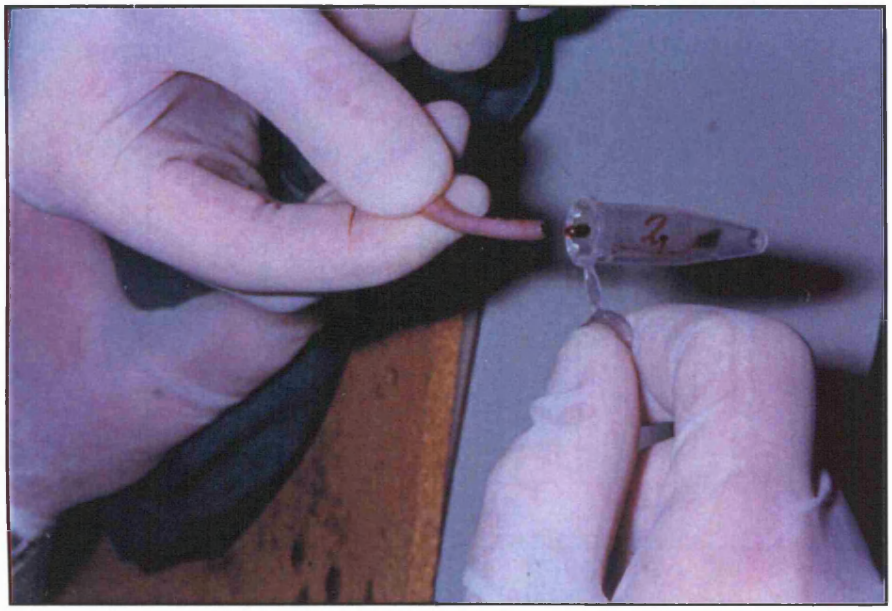

Figure 4.2vii Collection of blood via tail tip amputation for measurement of GFR using the radioisotope clearance technique

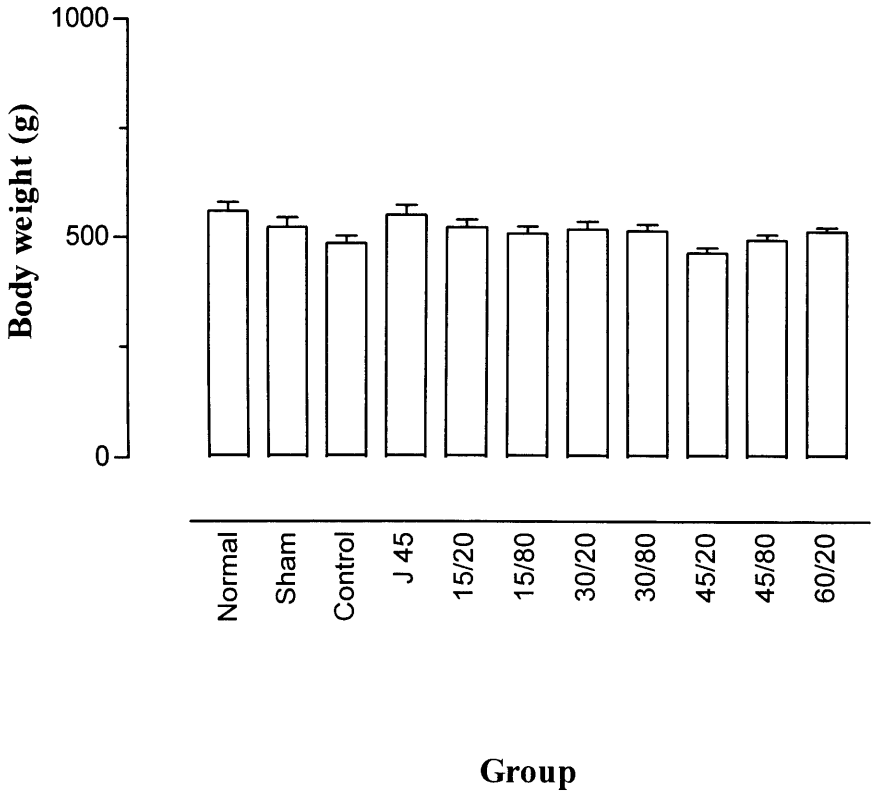

Figure 4.3i Comparison of the body weights of each experimental group in the development of the new model of renal WIRI. Numbered groups depict ischaemic time/reperfusion time. All results are mean(sem).

Group	n	Group Survival						
		Day1	Day 2	Day 3	Day 4	Day 5	Day 6	Day7
Normal	6	6	6	6	6	6	6	6
Sham	6	6	6	6	5	5	5	5
Control	6	6	6	6	6	6	6	6
J_{45}	6	6	6	6	6	6	6	6
15min	12	11	11	11	11	11	11	11
30min	12	12	12	12	12	11	11	11
45min	12	12	12	10	10	10	10	10
60min	6	6	6	5	5	3	2	2

Table 4.3i Survival of animals in each experimental group through to day seven. Groups 15-60 min represent the ischaemic durations and consist of combined data from both 20 & 80 min reperfusion groups (excluding 60min group, see text).

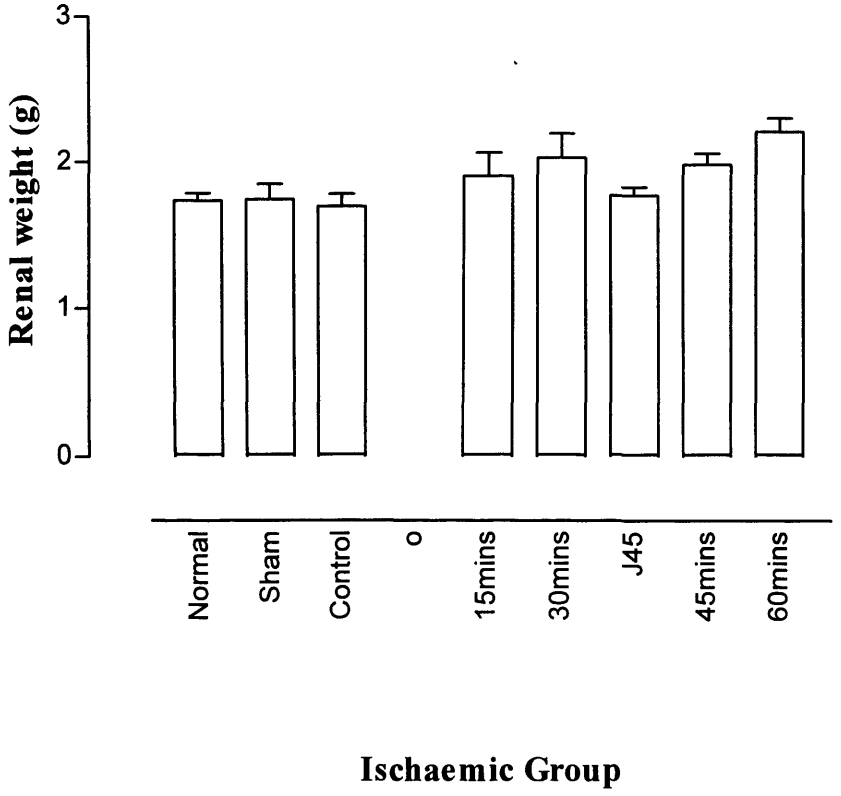

Figure 4.3ii The relationship between the duration of warm ischaemia and renal weight measured after 20 min reperfusion. There is elevation in all groups compared to control, significant in 60 min group ($p<0.01$). All results are mean(sem).

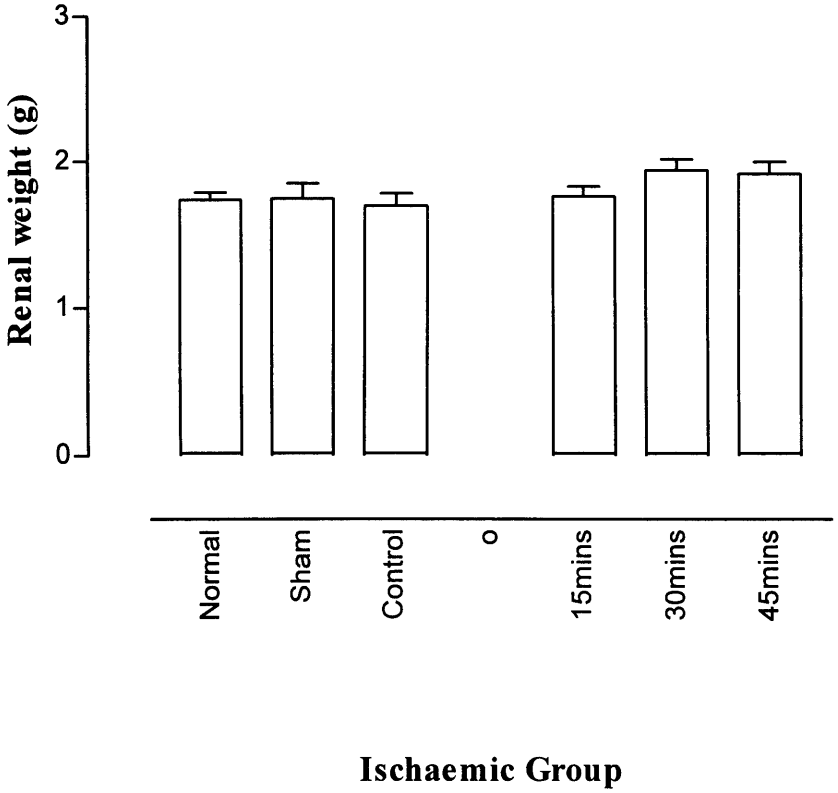

Figure 4.3iii The relationship between the duration of warm ischaemia and renal weight measured after 80 min reperfusion. The increase in weight seen after 20 min has decreased in all groups towards control values. A 60min WI and 80min reperfusion group was not studied in light of the high mortality in the 60/20 group. All results are mean(sem).

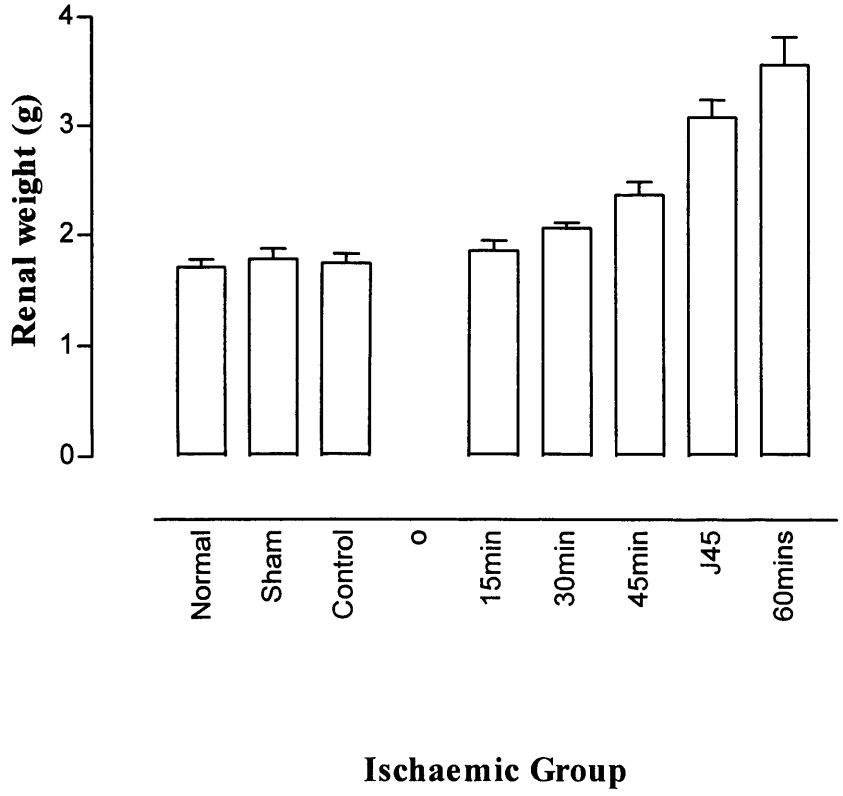

Figure 4.3iv The relationship between the duration of warm ischaemia and renal weight measured at day seven. Renal weight is elevated above control values in all groups, significantly in J_{45}, 45 & 60 min groups ($p<0.01$). There is a significant difference between this new model (45min WI) and the established (J_{45}) model ($p<0.01$). Control values have not changed from day 0 however, suggesting hypertrophy is not the underlying cause for the increase in renal weight. All results are mean(sem).

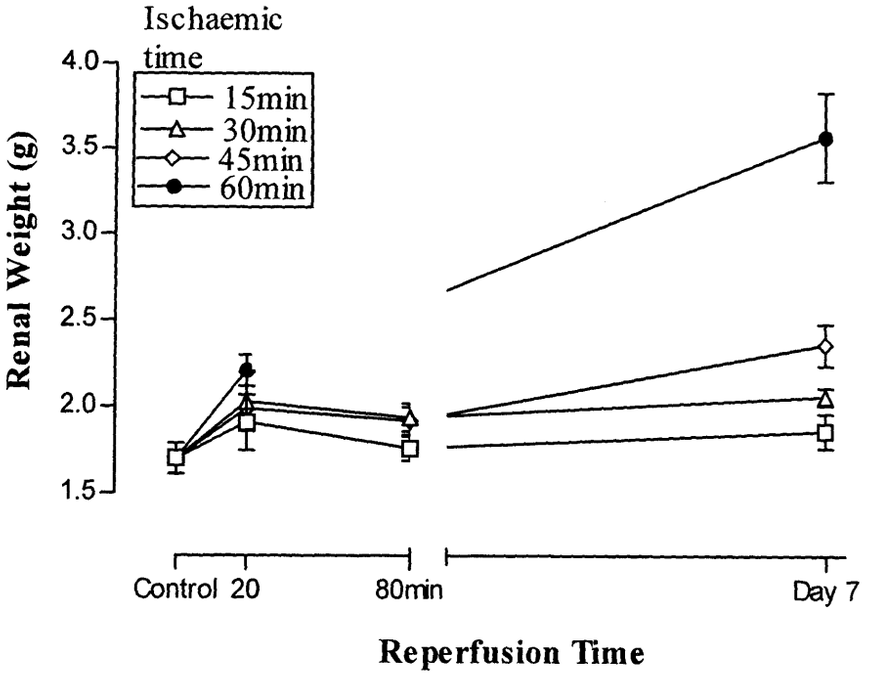

Figure 4.3v Change in renal weight from 20 min reperfusion through to day seven following a warm ischaemic injury. All results are mean(sem).

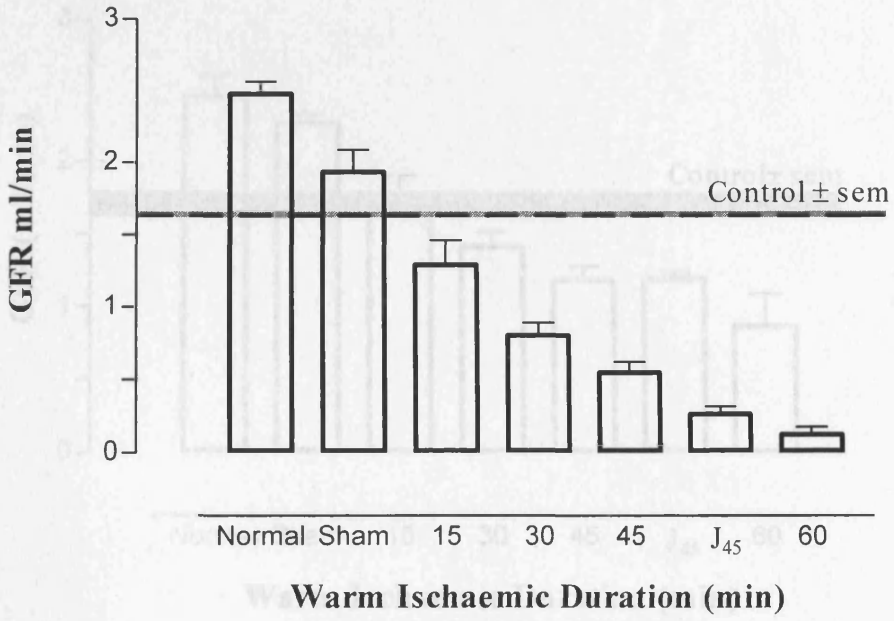

Figure 4.3vi Impairment of renal function at day two following a warm ischaemic insult on day 0. In comparison to the control all ischaemic groups (apart from 15 min) demonstrated impaired function ($p<0.01$) as did the control (one kidney) compared to normal (two kidney) GFR ($p<0.01$). All results mean(sem)

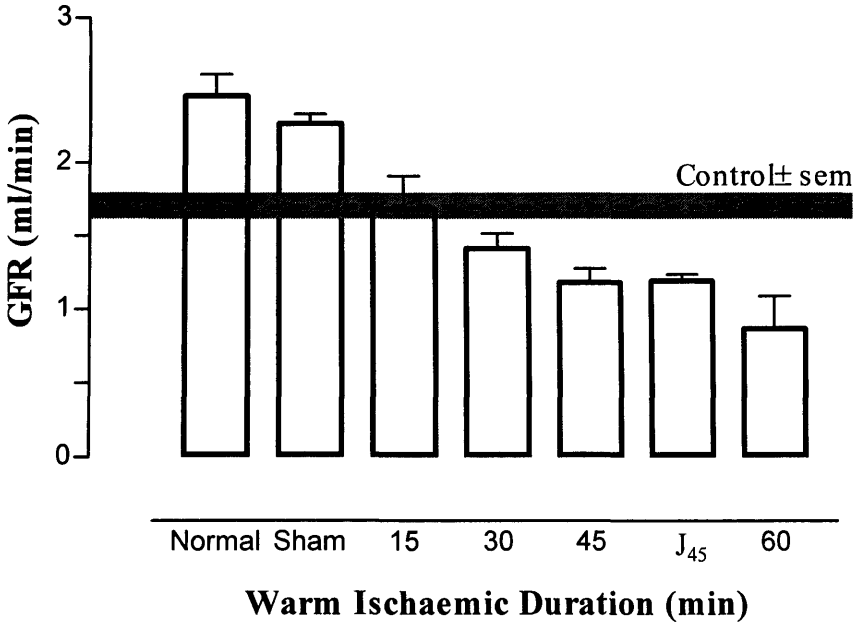

Figure 4.3vii Impairment of renal function at day seven following a warm ischaemic insult on day 7. Renal function remained impaired in animals following 45min ($p<0.05$) and 60min WI ($p<0.01$). Whilst there was no significant difference between normal and sham GFR both were greater than control (one kidney) GFR ($p<0.01$ & $p<0.05$ respectively). All results mean(sem).

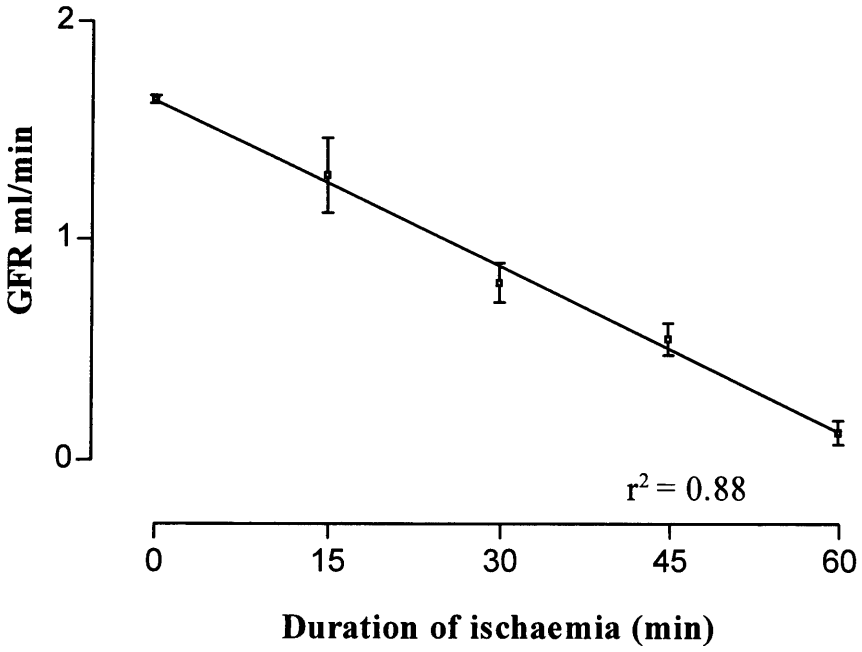

Figure 4.3viii Correlation between the duration of renal warm ischaemia at day 0 and the GFR measured on day two. Group 0 represents the control. All results mean(sem).

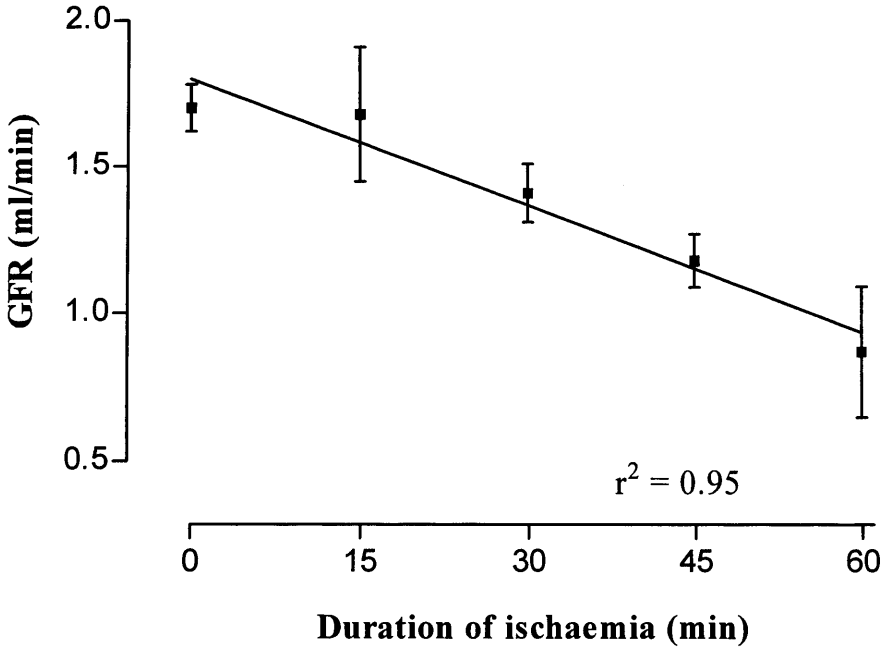

Figure 4.3ix Correlation between the duration of renal warm ischaemia at day 0 and the GFR measured on day seven. Group 0 represents the control. All results mean(sem).

	CORTICAL							MEDULLARY
Ischaemic time (min)	**Epitheliu m**	**Dilation**	**Debris**	**Nuclei**	**Perfusion**	**Mitoses**	**Apoptosis**	**Epithelium**
15	0.58 (0.45-0.73)	0.42 (0.27-0.57)	0.25 (0.12-0.38)	0.08 (0-0.16)	0	0.17 (0.06-0.28)	0	0.50 (0.37-0.67)
30	1.00 (0.87-1.13)	1.00 (0.87-1.13)	0.73 (0.54-0.92)	0.45 (0.29-0.61)	0.09 (0-0.18)	0	0.09 (0-0.18)	0.82 (0.59-1.05)
45	1.60 (1.44-1.76)	1.50 (1.33-1.77)	1.10 (1.00-1.20)	1.10 (0.92-1.28)	0	0	0	1.30 (1.15-1.45)
J_{45}	1.50 (1.28-1.72)	1.33 (1.12-1.54)	1.33 (1.12-1.54)	1.50 (1.28-1.72)	-0.67 -(0.46-0.88)	0.83 (0.52-1.14)	0.50 (0.28-0.72)	1.33 (1.00-1.66)
60	3.50 (3.03-4.10)	3.00	3.00	2.50 (2.01-3.11)	-0.50 -(0.01-1.16)	5.50 (3.05-8.12)	2.50 (2.04-3.23)	2.50 (2.07-3.22)

Table 4.3ii Summary of the group scores for the morphological renal tubular damage seen at day seven following warm ischaemic damage. Results are median (95% CI).

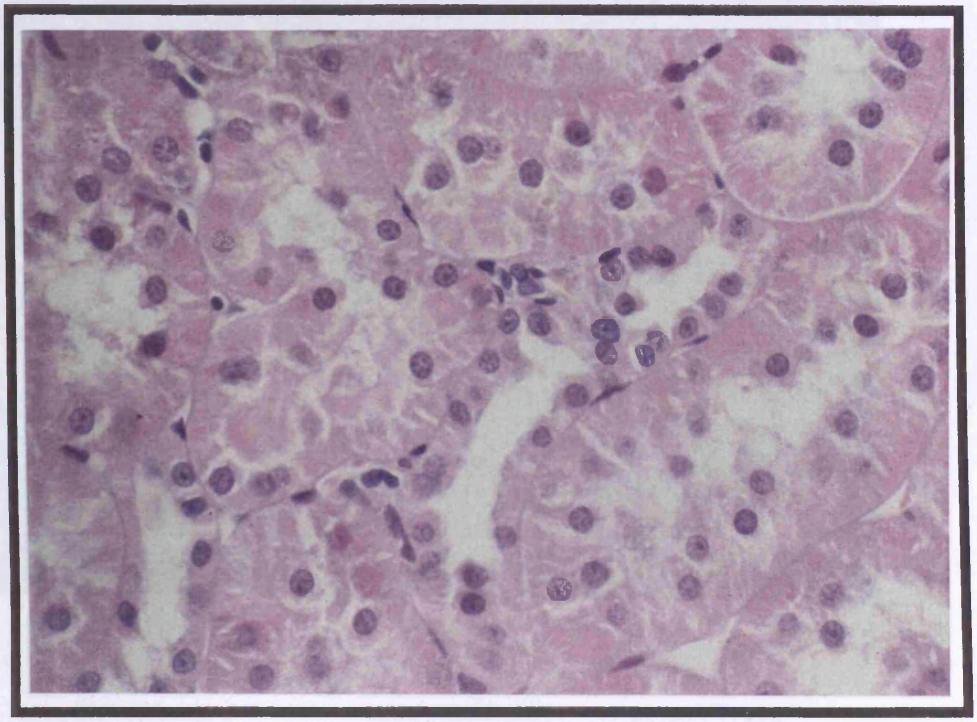

Figure 4.3x Photomicrograph of control renal cortical tubules at day seven (high power field).

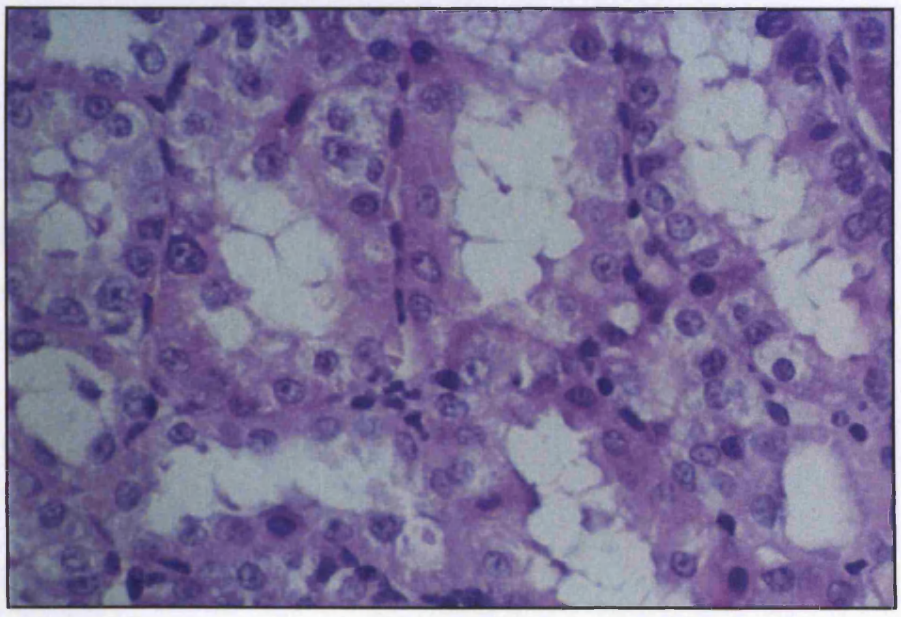

Figure 4.3.xi Photomicrograph of renal cortical tubules at day seven following 15min warm ischaemia (high power field)

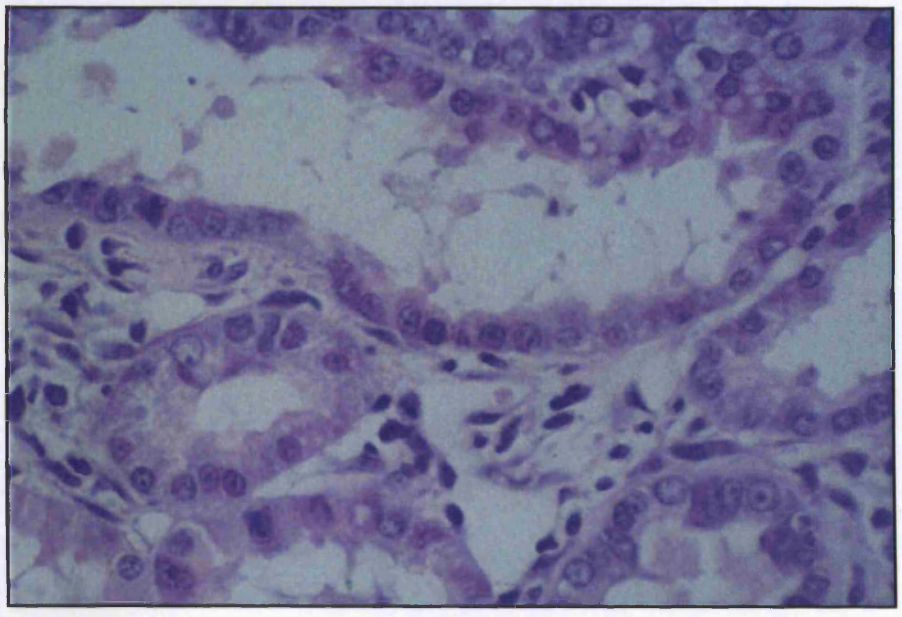

Figure 4.3xii Photomicrograph of renal cortical tubules at day seven following 30min warm ischaemia (high power field)

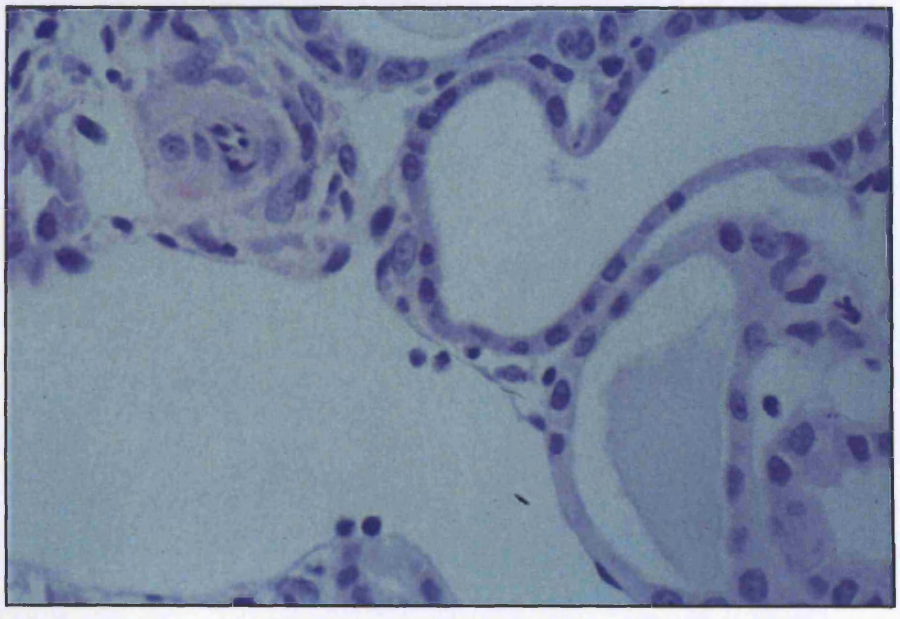

Figure 4.3xiii Photomicrograph of renal cortical tubules at day seven following 45min warm ischaemia (high power field)

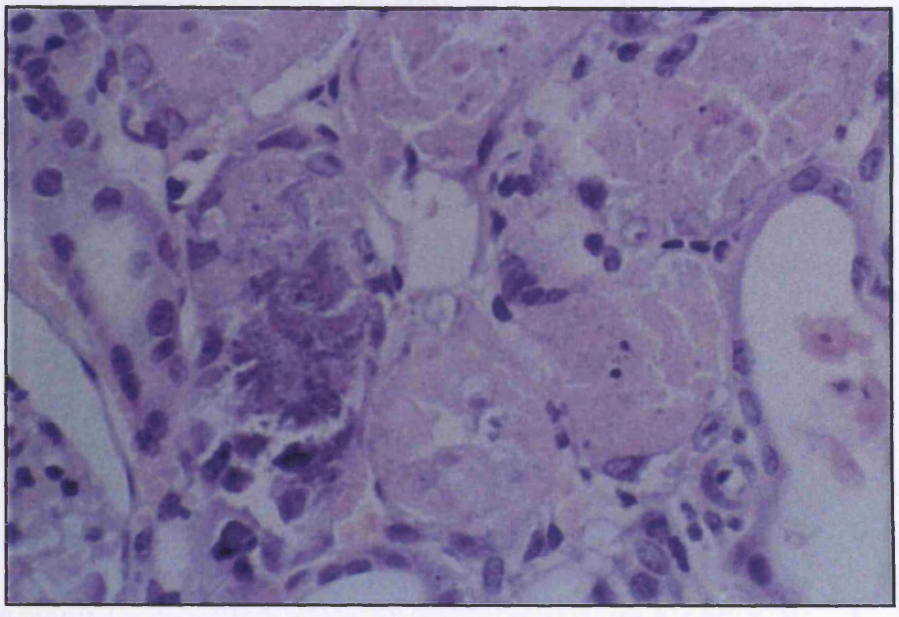

Figure 4.3xiv Photomicrograph of renal cortical tubules at day seven following 60min warm ischaemia (high power field)

Chapter 5

AN INVESTIGATION INTO THE EFFECT OF RENAL WARM ISCHAEMIA AND REPERFUSION ON THE GENERATION OF NITRIC OXIDE AND ROLE OF THIS IN THE EVOLUTION OF THE SUBSEQUENT INJURY

5.1 INTRODUCTION

Research into nitric oxide (NO) has expanded dramatically over the past decade since endothelium-derived relaxing factor (EDRF) (Furchgott and Zawadski, 1980) was first demonstrated to be NO (Palmer et al, 1987). Nitric oxide is a free radical derived from the metabolism of L-arginine (Palmer et al, 1988) under the control of nitric oxide synthase (NOS) which exists in either a constitutive or inducible form. The physiology of endothelial-derived NO in regulating vasodilation through smooth muscle cell phosphorylation (Kiechle and Malinski, 1993) has been clearly elucidated.

A major role for nitric oxide in renal homeostasis has been established with both the constitutive (cNOS) and inducible (iNOS) enzymes expressed. Their distribution within the kidney varies with cNOS predominately localised to the preglomerular vasculature, macula densa (MD) and collecting duct whilst iNOS is more generally expressed with the exception of the vessels and MD (Gabbai et al, 1995). Nitric oxide antagonises the vasoconstrictive effect of angiotensin II on the afferent arteriole (Ito et al, 1991) and helps regulate renal blood flow, glomerular filtration rate (Lahera et al, 1991; Granger et al, 1992) and sodium homeostasis (Javier-Salazar et al, 1995).

The pathophysiology following renal ischaemia reperfusion injury (IRI) is less well established. The complex, interrelated sequence of events that underlies IRI involves priming the endothelium during ischaemia to produce both free radicals and chemoattractants which, upon reperfusion, sequester and activate neutrophils thus amplifying the injury (Weight et al, 1996). There remains continuing uncertainty about the role of NO in renal IRI with theory and experimental evidence offering support for both a toxic and protective role (Yu et al, 1994; Kin et al, 1995; López-Neblina et al, 1995; Conger et al, 1995; Rivas-Cabañero et al, 1995; Yaqoob et al, 1996b). Whilst this partly reflects the different experimental models used, much of the work lacks any quantification of NO. Without this conclusions about the function of NO remain empirical, often based on extrapolating the results of the presumed pharmacological manipulation of NO/NOS. Caution must be exercised however when interpreting the results of experiments when the effect of the pharmacological manipulator is presumed rather than proven, for the structural analogues of

L-arginine can interfere with iron-centred enzymes (Peterson et al, 1992) and even stimulate NO generation in endothelial cells (Archer and Hampl, 1992). Therefore unless NO or NO-metabolites are measured directly the results remain inconclusive.

The following experiments are based on the new model of *in vivo* renal warm ischaemia reperfusion injury described in Chapter 4 and utilises the same normal, sham and control data . This allows the collation of functional (glomerular filtration rate), morphological and cellular (oxidative DNA and protein damage) markers of injury with the simultaneous measurement of renal nitric oxide levels, both early in reperfusion and late in the recovery phase. The changes in renal nitric oxide production following different durations of warm ischaemic damage and reperfusion are presented. The work presented in Chapter 4 demonstrated that 45min of renal warm ischaemia was optimal to maximise the recoverable injury. Using this as the control the effect of manipulating NO/NOS on endogenous NO and subsequent outcome was assessed. The NO/NOS manipulators used were the constitutive and inducible (c+i) NOS inhibitors L-NAME and ADMA, both at 50mg/Kg body weight (Vallance et al, 1992; Bryant et al, 1995; McPherson et al, 1995). Two iNOS inhibitors, the selective AG at 50mg/Kg (López-Belmonte & Whittle, 1995) and the non-selective HC at 20mg/Kg (Radomski et al, 1990; Zhang et al, 1997) and two NO donors: SNAP at 2.5mg/Kg (López-Neblina et al, 1995) and L-ARG at 300mg/Kg (Parrella et al, 1991; Myers et al, 1995).

5.2 MATERIALS AND METHODS

5.2a New Model of Renal Warm Ischaemia Reperfusion Injury

Studies were performed using the novel experimental model of *in vivo* renal warm ischaemia-reperfusion injury described in detail in section 4.2. Briefly, adult male Wistar rats (400-600g) were anaesthetised with halothane and a midline laparotomy performed and the infra-renal vena cava and both renal vascular pedicles were exposed. Twenty minutes prior to placing an occlusive vascular clamp across each pedicle, one millilitre of normal saline (vehicle) was infused into the vena cava over one minute. Following bilateral renal ischaemia and reperfusion a left nephrectomy was performed with the kidney immediately sectioned, snap frozen and then stored at -70°C until analysis. Three other groups were analysed: normal (for baseline values),

sham (laparotomy, vehicle infusion and renal pedicle dissection without ischaemia or nephrectomy) and control (laparotomy, vehicle infusion and left nephrectomy without ischaemia). The GFR was measured on the second and seventh postoperative days using a ^{99m}Tc-DTPA clearance technique (Nankivell et al, 1992). After the day seven GFR the animal was culled, a right nephrectomy immediately performed and part of kidney preserved in formalin with the remainder snap frozen. The formalin-fixed specimens were stained with haematoxylin and eosin (H&E) and the damage graded on an arbitrary scale from 0-4 (where 0 was normal).

Once the effect of warm ischaemia and reperfusion on renal NO generation had been established the role of NO in the genesis of this injury could be investigated. Using the 45 min WI and 20 min reperfusion group as the control (C_{45}), NOS activity was manipulated pharmacologically and NO metabolites as well as the functional, morphological and cellular parameters were measured. Groups of six animals were prepared as previously described. Following anaesthesia and laparotomy both the renal pedicles and vena cava were prepared. Twenty minutes (20mins) prior to renal pedicle occlusion one of the following compounds (Table 5.2) were infused over one minute (1min) per cava in a total volume of one millilitre (1ml) with sterile saline. The protocol outlined earlier in this section was followed with reperfusion for twenty minutes prior to (L) nephrectomy and then recovery, GFR, culling and (R) nephrectomy as previously described.

COMPOUND	ACTION	QUANTITY	MANUFACTURER
Sodium nitroprusside (SNAP)	NO donor	2.5mg/Kg	Sigma, H4001
L-Arginine (L-ARG)	NO precursor	300mg/Kg	Sigma, A5006
N^G-nitro-L-arginine methyl ester (L-NAME)	c+iNOS blocker	50mg/Kg	Sigma, N5751
Dimethyl arginine (ADMA)	c+iNOS blocker	50mg/Kg	Sigma, S0501
Hydrocortisone (HC)	iNOS blocker	20mg/Kg	Sigma, D4268
Aminoguanidine (AG)	iNOS blocker	50mg/Kg	Sigma, A7259

Table 5.2 Manipulators of NO/NOS used to pretreat animals prior to ischaemia

5.2b Measurement of Renal Nitroxides

Kidney sections were thawed on ice, weighed and homogenised with chilled (4°C) distilled, deionised water to achieve a concentration of 20% w/v. The homogenates were then clarified by freeze-thaw cycling (O'Donovan et al, 1995) from -196°C to 4°C then centrifuged at 15000g for 15 min at 4°C and the supernatant decanted.

Renal nitrite was determined using the end-point assay (Green et al, 1982). Equal volumes (500µl) of homogenate and Griess reagent were mixed by vortexing and incubated at 60°C for 15 min. The absorbance of the resultant azo dye was measured spectrophotometrically and the nitrite concentration derived from the calibration curve.

Renal nitrate was calculated utilising the kinetic assay (Gilliam et al, 1993). Renal homogenate supernatant was diluted 1:7 with 0.14M potassium phosphate buffer. The protein content was calculated using the bicinchoninic acid reaction (see Appendix B). A mixture of 400µl of buffer, 300µl of distilled, deionised water and 250µl of sample was incubated at 25°C for 2min before adding 25µl FAD (2.5µM final concentration) and 8µl of β-NADPH (96µM final concentration). Then 17µl of nitrate reductase was added, mixed thoroughly, and the rate of change in absorbance over three minutes (δA_{3min}) at 25°C measured spectrophotometrically (344nm). The δA_{3min} was then used to calculate the renal nitrate concentration from the calibration curve. The protein content was calculated using the bicinchoninic acid test (see Appendix B). Two separate sections of each kidney were analysed and the mean of these readings, expressed as nanomoles nitrate/ mg protein, used in subsequent analysis.

5.2c Quantification of Oxidative DNA Damage by HPLC-ECD (see Appendix A)

The DNA was first isolated and extracted (Fraga et al, 1990. Herbert et al, 1996). Briefly, renal samples were thawed, homogenised in extraction buffer (0.1M Trisma base, 0.1M NaCl, 20nm EDTA) and centrifuged at 2500rpm for 15min at 10°C and the supernatant discarded. The pellet was resuspended (20mM Trisma base, 20mM EDTA, 1.5% w/v sarkosyl and then 10mM Tris-HCl, 1mM EDTA) and 250µg of Ribonuclease A and 2mg of Pronase E added with incubation overnight at 37°C.

Following the addition of 10mM Tris-HCl, 1mM EDTA and 7.5M sodium acetate the DNA was precipitated with ice-cold 100% ethanol, spooled and rinsed in ethanol for 10min. The concentration of DNA was calculated spectrophotometrically (260nm) and known (60μg) aliquots of DNA freeze-dried.

These aliquots were later reconstituted in water, heated to 100°C and rapidly cooled prior to digestion with nuclease P_1 and alkaline phosphatase at 37°C. Three nucleotide standards were prepared: 2'-deoxyadenosine (dA, range: 0-15μM), 2'-deoxyguanosine (dG, range: 0-50μM) and 8-oxo-2'-deoxyguanosine (8-oxodG, range: 0-30nM). Samples and standards were analysed on a 150mm × 4.6mm Hypersil ODS (C_{18}) column with a 3μm particle size. The injection loop was 100μl with a column flow rate of 1ml/min at ambient temperature. The dA and dG peaks were detected in UV range (254nm) and 8-oxodG peak electrochemically at 800mV (2nA sensitivity) with the system software calculating the peak areas for dA, dG and 8-oxodG in the base standards and dG and 8-oxodG in the samples. A calibration curve for each base standard was constructed allowing the sample base concentration to be calculated (and expressed as 8-oxodG/10^5 dG).

5.2d Quantification of the Carbonyl Content (see Appendix B)

The carbonyl content was measured as an index of oxidative protein damage according to the method of Levine *et al* (1990). Kidney samples were homogenised in EDTA and centrifuged at 2500rpm. The supernatant was decanted, mixed with 10mM 2,4-dinitrophenylhydralazine and the protein precipitated by the addition of 20%w/v trichloroacetic acid. This was centrifuged at 13000g and the pellet washed and resuspended in 6M Guanidine. After a further centrifugation at 13000g to remove insolubles the absorbance at 360nm was determined spectrophotometrically. The carbonyl content could be calculated using the known molar absorption coefficient (MAC = 22000$M^{-1}cm^{-1}$) and expressed per milligram of protein following protein concentration determination by the bicinchoninic acid reaction.

5.2e Statistical Analysis

Analysis of variance (ANOVA) with Dunnets comparisons was used for all data except histological where the Kruskal-Wallis test with Dunns comparisons were used

to establish any significant difference (taken as $p< 0.05$) between the study groups. Both a paired and unpaired two-tailed t-test used where appropriate. Results are expressed as mean(sem).

5.3 RESULTS:

AN INVESTIGATION INTO THE EFFECT OF DIFFERING DURATIONS OF RENAL WIRI ON FUNCTIONAL, MORPHOLOGICAL & PATHOPHYSIOLOGICAL INDICES

5.3a Survival & Renal Weight

This data can be found in section 4.3b & 4.3c.

5.3b Renal Nitroxides

There was no difference between the basal level of renal nitrate in the normal, sham and control groups. After 20 min reperfusion nitrate had increased in all groups: significantly in the 30 min ($p<0.05$) and 45 min groups ($p<0.01$) when compared to the control (Figure 5.3i). By 80 min reperfusion the elevation in NO was significant in all groups ($p<0.05$) (Figure 5.3ii). There was a strong positive correlation ($r^2 = 0.97$) between the duration of WI (15-45min) and renal NO at 20 min reperfusion (Figure 5.3iii).

At day seven (Figure 5.3iv) nitrate remained elevated above control values ($p< 0.01$) in all groups except 60 min WI. The day seven values were also significantly higher than those of early (20 min) reperfusion in the 15min and 30min ($p<0.05$) groups (Figure 5.3v). Only two animals survived to day seven in the 60 min group and those that died had no elevation in NO_3 levels after 20min reperfusion.

In none of the samples analysed were there any detectable levels of nitrite.

5.3c Renal Function

There was progressive impairment in the GFR at day two, significant in the 30, 45 and 60 min ischaemic groups ($p<0.01$). By day seven renal function had improved although it remained impaired in the 45 and 60 min groups ($p<0.05$ and $p<0.01$ respectively). The recovery in GFR between days 2 & 7 was significant in the 30 ($p=0.01$) and 45 min ($p<0.01$) groups. There was a strong correlation between the

GFR at both day two ($r^2 = 0.88$) and day 7 ($r^2 = 0.95$) and the duration of warm ischaemia (see section 4.3d).

5.3d Renal 8-oxodeoxyguanosine Levels

The DNA damage increased with the duration of warm ischaemic damage during the early phase of reperfusion (20 min). This was significant in the 15 ($p< 0.05$), 45 ($p< 0.001$) and 60 min ($p< 0.05$) groups. By 80 min reperfusion the 8-oxodG levels had returned towards control values and this persisted to day seven (Figure 5.3vi).

5.3e Renal Carbonyl Content

The renal carbonyl content (CC) did not significantly alter from control levels at any stage of reperfusion except in the 15 min WI group after 80 min reperfusion ($p<0.01$) (Figure 5.3vii). The CC increased from 20-to-80 min reperfusion, significantly in the 15 ($p< 0.01$) and 30 min ($p= 0.01$) groups.

5.3f Histopathology

Morphological damage ranged from mild (15 & 30 min warm ischaemia) to severe (60 min) with cortical rather than medullary tubules demonstrating the most marked changes (see section 4.3e). The 60 min group alone showed areas of true necrosis with calcified tubular debris as well as mitotic figures, impaired glomerular perfusion and apoptotic/pyknotic nuclei. The most sensitive indices were the overall cortical damage and the degree of cortical tubular dilation and debris. Damage after 45 and 60 min ischaemia was significant ($p<0.01$).

5.4 RESULTS

THE EFFECT OF PHARMACOLOGICALLY MANIPULATING NO/NOS PRIOR TO WARM ISCHAEMIA ON SUBSEQUENT RENAL FUNCTIONAL, MORPHOLOGICAL & PATHOPHYSIOLOGICAL INDICES

5.4a Mortality

There were no perioperative deaths in any of the groups. By day two there had been one death (1/6) in the L-ARG, L-NAME and AG groups. By day seven there had been three additional deaths in the L-NAME group (total 4/6) and single fatalities in the SNAP and ADMA groups.

5.4b Body Weight of Each Experimental Group

Body weight was measured as described in section 4.3a. The groups were well matched (Figure 5.4i) with an average weight of 423(7.4)g. The comparative control for this part of the experiment, C_{45} (45min WI and 20min reperfusion), had a group weight of 464(11.5)g.

5.4c Renal Weight

After 20 min reperfusion (Figure 5.4ii) renal weight had increased above baseline in all groups. Although none of the kidneys in the pretreated group were as heavy as the C_{45}, this was not significant. By day seven (Figure 5.4iii) only in the AG group was the renal weight significantly altered (lower) in comparison to C_{45} ($p<0.05$).

5.4d Renal Nitroxides

Renal homogenate levels of nitrate rose significantly from baseline values of 95(5.9) nmol/mg protein to 208(17.3) nmol/mg protein after 45min ischaemia and 20min reperfusion (C_{45} $p<0.01$). This rise was abolished by pretreatment with both the c+iNOS inhibitors ($p<0.05$) whilst a comparable rise was seen in the other groups (Figure 5.4iv). By day seven (Figure 5.4v) nitrate levels remained elevated in C_{45} at 241(12.5) nmol/mg protein. At this stage nitrate levels were comparable in the iNOS groups but were significantly lower in the NO donor ($p<0.01$ in SNAP and $p<0.05$ in L-ARG) and c+iNOS groups ($p<0.01$ in L-NAME and $p<0.05$ in ADMA).

Nitrite remained below recordable limits in all groups.

5.4e Renal Function

Following 45min warm ischaemia the renal function of the solitary kidney at day two, 0.55(0.07) ml/min, was significantly impaired in comparison to baseline, 1.64(0.05) ml/min. Pretreatment with both NO donors and iNOS inhibitors improved the GFR (Figure 5.4vi), significantly ($p<0.01$) in all but the L-ARG group, whilst L-NAME further impaired the GFR ($p< 0.05$). By day seven (Figure 5.4vii) functional damage was ameliorated only by pretreatment with L-ARG ($p<0.05$) and worsened ($p<0.01$) by c+iNOS blockade.

5.4f Renal 8-oxodeoxyguanosine Levels

After twenty minutes reperfusion renal levels of 8-oxodG were significantly elevated in control at 14.6(0.9) 8-oxodG/10^5dG ($p<0.01$) compared to the baseline level of 2.0(0.7) 8-oxodG/10^5dG. Levels of DNA damage were not significantly raised over baseline however in any of the pretreated groups (Figure 5.4viii). By day seven (Figure 5.4ix) control levels had returned towards baseline but 8-oxodG was elevated in both the c+iNOS and iNOS inhibitor groups (all $p<0.01$).

5.4g Renal Carbonyl Content

Levels of renal homogenate oxidative protein damage in the C_{45} group, 41.4(1.9) nmol/mg protein, after 20 min reperfusion were unchanged from baseline values. Levels were elevated above C_{45} in the SNAP ($p<0.01$) and L-ARG groups (Figure 5.4x) but were significantly reduced in all NOS inhibitor groups ($p<0.01$). By day seven (Figure 5.4xi) levels of carbonyl were below control in the L-ARG and SNAP groups, significantly in the latter ($p<0.01$), but showed increased damage in all NOS inhibitor groups ($p<0.01$, except for AG).

5.4h Renal Histopathology

The morphological indices of renal damage were significant in the control group (C_{45}) in comparison to baseline ($p<0.01$) at day seven. Of the parameters assessed, both tubular dilation/debris and overall cortical damage were the best markers of damage. Whilst damage was lessened in the NO donor groups and increased in the c+iNOS groups this failed to reach significance (Figure 5.4xii). Representative photomicrographs of each group are presented in Figures 5.4xiii-xviii.

5.5 DISCUSSION

In the clinical setting renal warm ischaemia reperfusion injury is a consequence of both systemic hypoperfusion with subsequent circulatory resuscitation and local renal WIRI. Whilst the role of NO in normal renal homeostasis has been well established the role in ischaemia reperfusion injury remains contentious. This is a result not only of genuine variance between different organs but also factors related to the model used (e.g. *in vivo* vs. *in vitro*, the temporal effects of the duration of ischaemia and reperfusion and species-specific variance) and a lack of quantification of the post-ischaemic NO level.

The classical model of ischaemia reperfusion injury whereby ischaemia 'primes' the tissue for OFR-mediated damage during reperfusion is clearly not the whole story. *In vitro* studies have demonstrated a rise in NOS activity early in ischaemia (Yacoob et al, 1996a) and any resultant disequilibrium between NO and OFRs (Yacoob et al, 1996b) may be important in determining the degree of post-ischaemic damage. Nitric oxide is a free radical which can injure the actin cytoskeleton (Salzman, 1995) via ATP depletion and both directly damage DNA (Nguyen et al, 1992) and inhibit the subsequent repair (Laval and Wink, 1994). Renal epithelial cell adhesion may also be impaired through actin degradation which affects integrin-ligand binding (Kellerman and Bogusky, 1992; Kroshian et al, 1994). During reperfusion NO and OFRs are able to interact with probably the most significant reaction between NO and superoxide (O_2-) (Gryglewski et al, 1986) with the resultant elimination of both radicals. Such quenching will be cytoprotective (Cooke and Tsao, 1993) only if the product, peroxynitrite (ONOO), is less toxic than the reactants. In fact ONOO is rapidly protonated and then decays ($t_{1/2}$ <2s) generating the highly toxic hydroxyl radical Beckman et al, 1990; Beckman et al, 1993).

It was important to quantify the renal nitric oxide level and compare this with cellular markers of injury at the same timepoint as well as the functional and morphological data. Direct measurement of NO is possible (Ignarro et al, 1987. Chung and Fung, 1990. Gustaffson et al, 1991) although difficult in the *in vivo* environment. Several NO probes have been described (Malinski and Taha, 1992; Tsukahara et al, 1994) although their use has tended to be restricted to *in vitro*/*ex vivo* studies. The indirect

measurement of NO via the metabolites NO_2/NO_3 is an established technique (Green et al, 1982; Gilliam et al, 1993) for *in vivo* studies (Mueller et al, 1994).

The experiments presented clearly demonstrate that following WIRI *in vivo* renal nitric oxide production is elevated. The lack of measurable nitrite is unsurprising as in biological solution it is rapidly oxidised (Zeballos et al, 1995) to NO_3. After 20 min of reperfusion following 15 to 45 min warm ischaemia NO increased by between 24-119% respectively with a further increase at 80 min reperfusion. There was a continued upward trend in nitric oxide levels towards the seventh day, being around 125% greater than baseline values. This is broadly in line with the results of a model of norepinephrine-induced renal failure (Conger et al, 1995) which found NOS/NO activity maximal at one week. Other *in vitro* renal studies have suggested elevated NO either during ischaemia (and falling on reperfusion) or after one hour reoxygenation (Yacoob et al, 1996b; Rivas-Cabañero et al, 1995). The rapid alteration in NO levels also reflects work on post-ischaemic coronary arteries although in this bioassay NO was found to diminish with reperfusion (Ma et al, 1993).

Interestingly the generation of NO following 60 min ischaemia did not follow this general pattern. Although levels were elevated over baseline they were always lower than those in the other groups, significantly so at day seven. Whether this represents a genuine decrease in NOS activity, possibly secondary to impairment of enzyme function from the prolonged hypoxia, or more rapid quenching of NO with the other free radicals generated upon reperfusion is not clear. The former would appear more likely as the effect continued to day seven although *in vitro* studies have not demonstrated this effect (Yu et al, 1994). One important conclusion is that models which extrapolate the results of the pharmacological manipulation of NOS to conclusions about NO generation (without quantifying NO/NOS activity) may be misleading. Furthermore whilst neither urine nor serum measurements of nitrate can be used as an index of NO production in renal failure (Conger et al, 1995) this clearly is not the case for renal homogenate as the group with the most impaired renal function (60min WI) was associated with the lowest levels of NO_3.

It is of note that the carbonyl content was not elevated above control values although this has been reported previously in cerebral homogenate (Krause et al, 1992). It is possible that the duration of ischaemia and/or reperfusion was not sufficient to demonstrate oxidative protein damage in this model (Liu et al, 1993; Folbergrova et al, 1993), and certainly the CC increased between 20 and 80min reperfusion in each group. Furthermore, the overall levels of carbonyl detected are considerably higher than seen either in plasma (Quinlan et al, 1994; Odetti et al, 1996) or cerebral homogenate (Forster et al, 1996) and it is possible that other forms of oxidative damage are being detected (Levine et al, 1990).

In this model there was a strong correlation between the duration of warm ischaemia and the functional, cellular and histological markers of renal injury, i.e. (i) the GFR at both day 2 ($r^2 = 0.88$) and day 7 ($r^2 = 0.95$), (ii) the 8-oxodeoxyguanosine levels at 20 minutes reperfusion ($r^2 = 0.88$) and (iii) the morphology at day seven ($r^2 = 0.89$). These are therefore excellent markers of the degree of damage sustained, although it would not be justified to extrapolate these results alone to reach conclusions about a causal role for nitric oxide. Once the baseline change in renal NO during WIRI had been established however the results from the quantified NO/NOS manipulation provided strong evidence.

The early rise in NO upon reperfusion was increased by the provision of exogenous NO, although this failed to reach significance. Similarly attempts to augment this with L-ARG (at least in the dose given) were unsuccessful. This data also supports the previously reported study suggesting that NOS activity was maximal (Conger et al, 1995) soon after reperfusion. The absence of an elevated nitrate level in these groups is presumably a function of the timing. Sampling at twenty minutes of reperfusion reflects an 85min delay from infusion meaning a single dose of SNAP would have been metabolised (Vesey et al, 1990) and the nitroxides redistributed and excreted. Furthermore the reaction kinetics of L-ARG suggest that substrate deficiency should not, at least initially, affect NOS activity (Forstermann et al, 1994) in the kidney (Walder et al, 1992). Thus pre-treatment may not increase NO levels at this time point. Therefore although the provision of exogenous NO (SNAP) prior to ischaemia did not significantly elevate renal NO levels at 20min reperfusion the degree of oxidative protein damage was increased at this time suggesting that NO

may be injurious earlier in reperfusion. Levels of DNA damage however were improved in all groups. One reason for this may be that the alteration in NO:O_2- balance is relatively sparing of the intracellular environment. The increased oxidative stress from NO donors may occur in the first few minutes, when O_2- production is also maximal (Paller & Neuman, 1991). At this stage the cell is relatively protected from the effects of peroxynitrite because of the low intracellular pH (Pryor & Squdrito, 1995). Membrane proteins however would not be spared on this basis accounting for the elevated CC seen. Oxidative DNA damage (8-oxodG) was lessened both early and late, whilst protein damage (CC) was raised early in reperfusion levels but fell below control by day seven. This early rise in oxidative damage has previously been shown for lipids (Cristol et al, 1996; López-Neblina et al, 1996) but not in protein.

Pretreatment with c+iNOS but not iNOS inhibitors prevented the early rise in NO suggesting a predominant role for cNOS-derived NO. Certainly the early lack of effect from iNOS blockade on NO levels is in line with the reported induction time of around two hours (Radomski et al, 1990). This however is at odds with the significant decrease in early oxidative damage seen. Although this may be as a result of poor selectivity of the iNOS blockers (Griffiths et al, 1993; Knowles & Moncada, 1994; Marletta, 1994) causing low-level cNOS blockade (Lopez-Belmonte & Whittle, 1995) it is more plausibly due to a non-NOS effect of the blockers used (Ohrui et al, 1992; Eaton et al, 1993). Pre-treatment with the c+iNOS inhibitors significantly reduced NO not only early in reperfusion but through to day seven, i.e. long after the metabolism of these structural analogues of L-arginine. Previous *in vivo* work has shown that cNOS activity is important in maintaining post-ischaemic renal blood flow (Cristol et al, 1993) with Shoskes et al (1997) demonstrating increased cNOS protein expression (although decreased enzyme activity) following IRI through to day 14.

The intermediate phase of reperfusion (day two) was monitored by renal function alone. That NO donors improved and c+iNOS inhibitors impaired function was therefore perhaps unsurprising. The results seen in the iNOS groups were however unexpected. Pretreatment with both the specific and non-specific iNOS inhibitors significantly improved renal function at day two. It can be inferred that NO from the

induction of NOS is injurious whilst the upregulation of cNOS (prevented by pretreatment with L-NAME) is associated with partial amelioration of the intermediate GFR. Whilst the role of endothelial NOS (cNOS) in maintaining RBF has already been alluded to other factors must account for the iNOS picture. The upregulation of iNOS has been related to endothelial dysfunction (Orucevic et al, 1997) and increased microvascular permeability; this would have a particularly deleterious effect on renal function if mirrored in the tubules where iNOS is prevalent. Furthermore iNOS is the leucocyte isoform and inhibition of this might therefore be expected to abrogate neutrophil-mediated secondary damage (Cattell et al, 1994).

By day seven the picture had changed. Nitric oxide levels remained constant and possibly maximal in the control (C_{45}) group through to day seven and were matched by the iNOS inhibitor group. However in both the NO donor and c+iNOS inhibitor groups levels were significantly reduced towards baseline. Reference to the indices of injury at day seven however suggests that this may be for opposing reasons. The NO donor groups demonstrated improved renal function and morphology with less oxidative damage whilst the opposite was true for the c+iNOS groups. It would appear that early antagonism of NOS prevented elevation of NO throughout reperfusion with an associated impairment of functional, morphological and pathophysiological parameters. This would appear analogous to the 60 min WI group where greater injury was associated with reduced NO. The iNOS inhibition data, which is comparable to C_{45} values at day seven, suggest that it was the cNOS inhibition that was crucial. Kidneys pretreated with NO donors were less injured at day seven and consequently had lower (i.e. closer to baseline) NO levels. The manner in which early NO ameliorated secondary injury was not investigated in this study but it is likely to reflect the interaction of NO with both the vascular endothelium and neutrophils. A lack of NO has been shown to contribute to post-ischaemic vasospasm (Wang et al, 1997) and endogenous NO is known to be an important factor in both the maintenance of renal blood flow after ischaemia-reperfusion and the attenuation of microvascular dysfunction (Kukrose et al, 1994) with a concomitant reduction in endothelial-neutrophil interaction. This may be fundamental to the prevention of secondary injury as neutrophils are known to exacerbate post-ischaemic renal failure

(Linas et al, 1992; Linas et al, 1988) and NO has been demonstrated to inhibit neutrophil chemotaxis, O_2- production and enzyme release (Geffner et al, 1995).

Two conclusions therefore seem to stem from these findings. Firstly the early burst of NO during reperfusion was cytotoxic. Secondly, however, inhibiting this rise led to more severe late damage suggesting the prolonged elevation of NO is cytoprotective and in this regard cNOS rather than iNOS appears pivotal. Inducible NOS played a significant role at the intermediate stage of reperfusion where it appeared to impair renal function. The basis for the duality of action demonstrated by NO may be hypothesised. Early in reperfusion NO is one of several free radicals generated which can interact. Probably the most important of these is the rapid reaction (Blough and Zafirov, 1985) between NO and superoxide (O_2-) which will be cytoprotective (Cooke and Tsao, 1993) only if the product is less toxic than the constituents. The product is in fact peroxynitrite ($ONOO^-$), a short-lived oxidant species that is rapidly protonated and then decays generating the highly toxic hydroxyl radical (Beckman et al, 1990; Beckman and Crow, 1993) thus accounting for the early cytotoxicity associated with the elevated NO. The late cytoprotection may stem from the interaction of NO with leucocytes. The sequestration of activated neutrophils is thought to cause secondary damage (Linas et al, 1996) although it has been suggested that this is less important in the kidney than in some other organs (Simpson et al, 1988; Thornton et al, 1989; Carden et al, 1990a). Following chemotaxis activated neutrophils adhere to the endothelium and cause injury through the release of free radicals and enzymes but all these processes are in fact inhibited by NO (De Caterina et al, 1995; Geffner et al, 1995). It may be therefore that the high levels of NO demonstrated in the post-ischaemic kidney attenuate secondary neutrophil-mediated injury (Egdell et al, 1994; Kukrose et al, 1994; Bouchier-Hayes et al, 1997). This contrasts with the situation seen elsewhere, e.g. heart (Ma et al, 1993) and skeletal muscle (Wang et al, 1997), where there is a reduction in post-ischaemic NO. Thus in both modulating the effects of neutrophils and maintaining renal blood flow (Cristol et al, 1993) NO may be cytoprotective in the longer-term.

In summary, the increase in nitric oxide seen during reperfusion following renal warm ischaemia would appear to play a biphasic role in the subsequent injury. What have previously been seen as divergent results may simply be the result of different

time frames. Nitric oxide would appear to be cytotoxic early in reperfusion, presumably due to a combination of *de novo* toxicity and peroxynitrite formation, and yet this NO burst is essential to ameliorate the secondary injury consequent at least in part from vasospasm, microvascular dysfunction and neutrophil-mediated damage. Furthermore these results suggest that the different isoforms of nitric oxide synthase have opposing functions in the post-ischaemic kidney with cNOS cytoprotective and iNOS injurious.

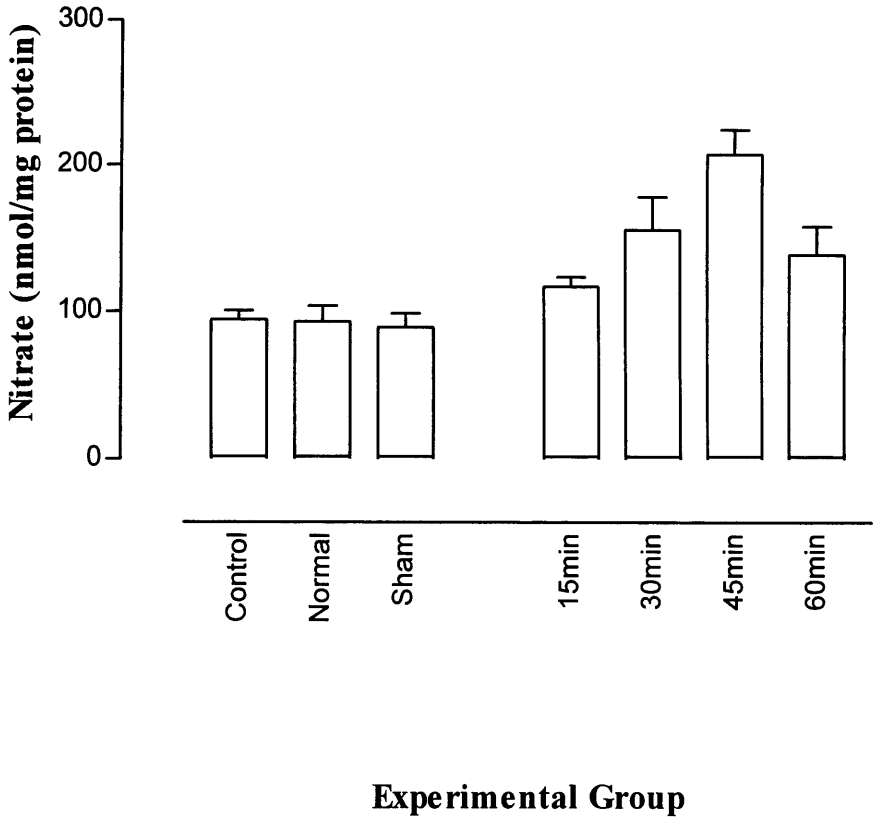

Figure 5.3i Relationship between an increasing duration of renal warm ischaemia and nitrate at 20min reperfusion. Compared to the control nitrate was significantly elevated in the 30min ($p<0.05$) and 45min ($p<0.01$) groups. Results are mean(sem).

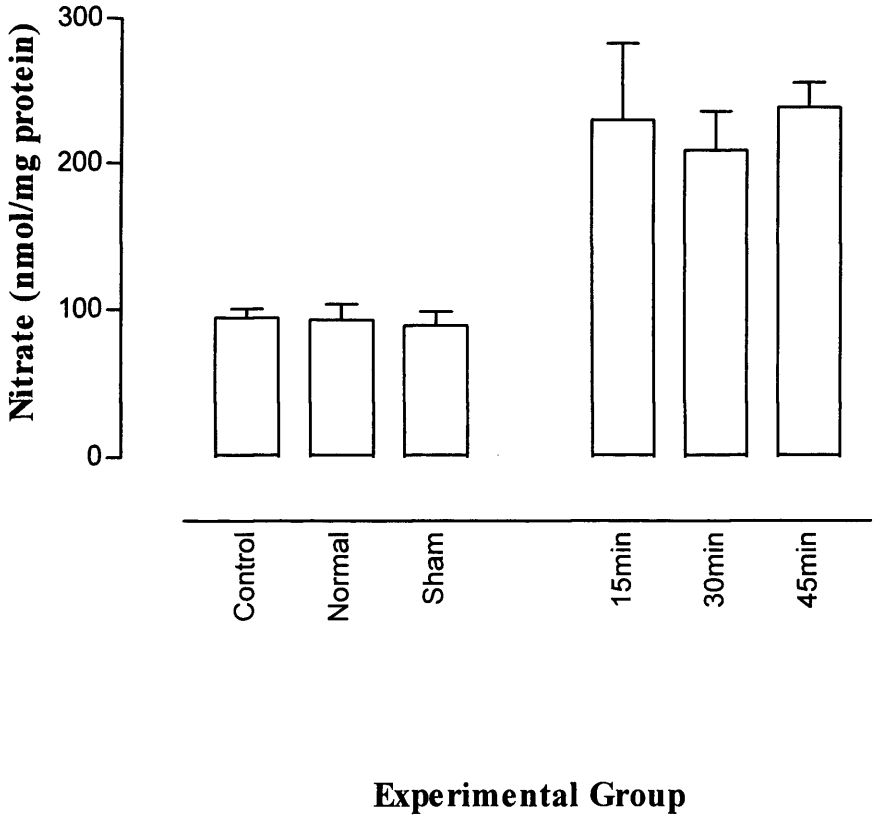

Figure 5.3ii Relationship between an increasing duration of renal warm ischaemia and nitrate at 80min reperfusion. Compared to the control nitrate was significantly elevated in all WI groups ($p<0.05$). Results are mean(sem).

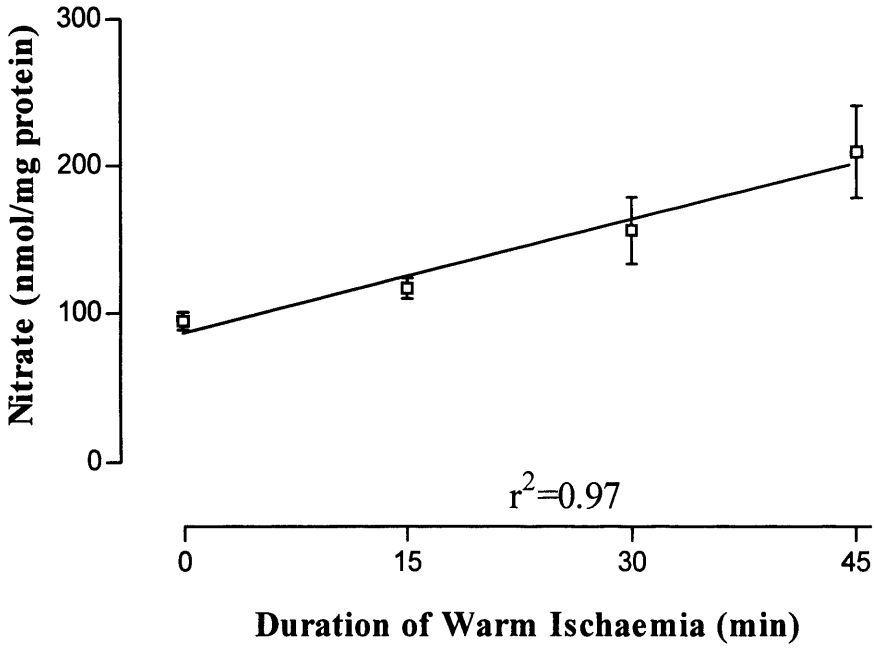

Figure 5.3iii Positive correlation between the duration of WI from 15-45 min and renal nitrate at 20 min reperfusion.

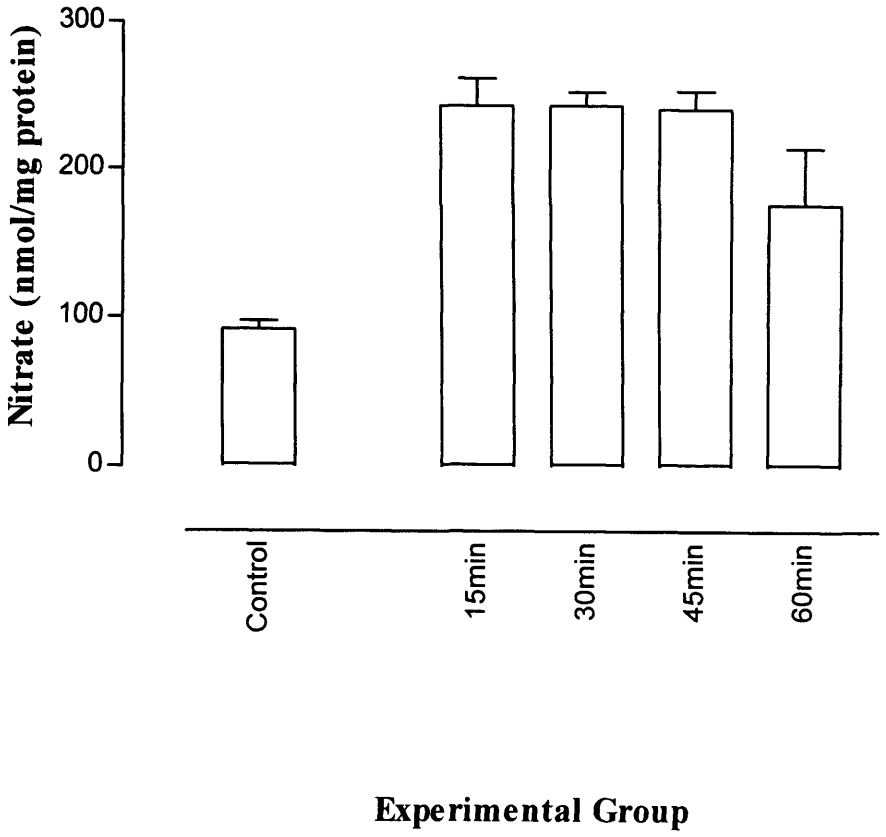

Figure 5.3iv Relationship between increasing duration of renal warm ischaemia and nitrate at the seventh day post ischaemia. The elevation in nitrate was significant ($p<0.01$) in all groups except 60 min WI. Results are mean(sem).

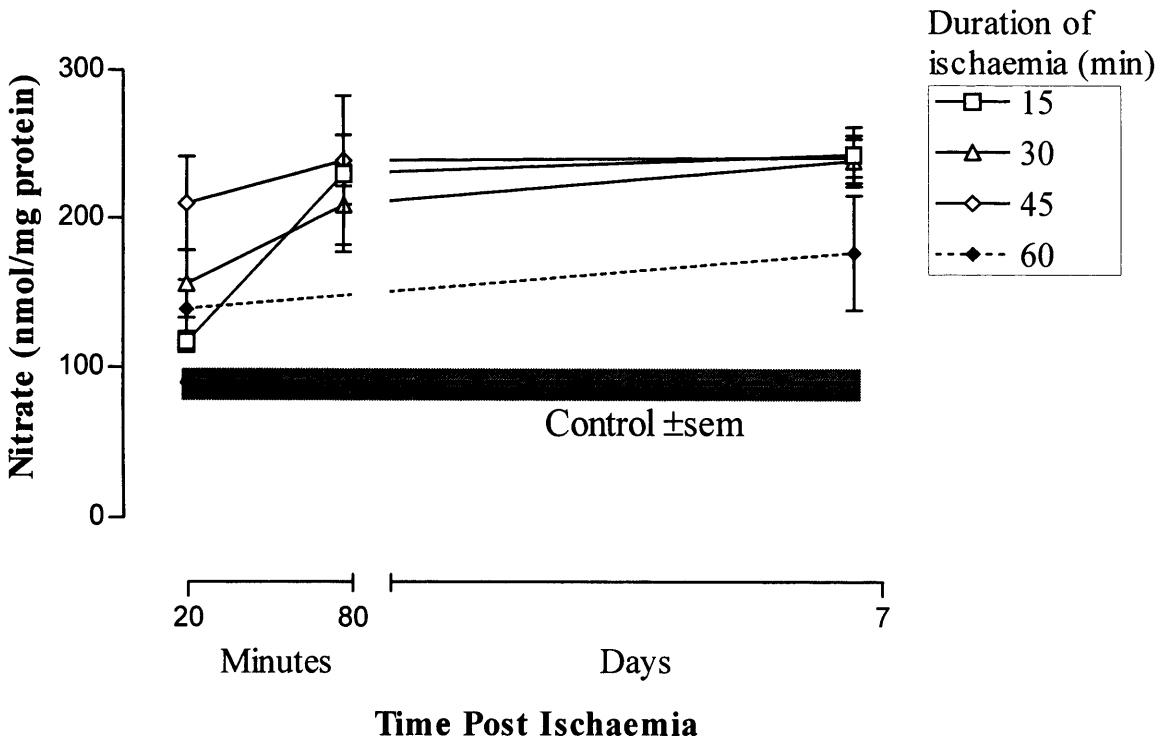

Figure 5.3v Change over time in renal NO following WI injury. Results are mean(sem).

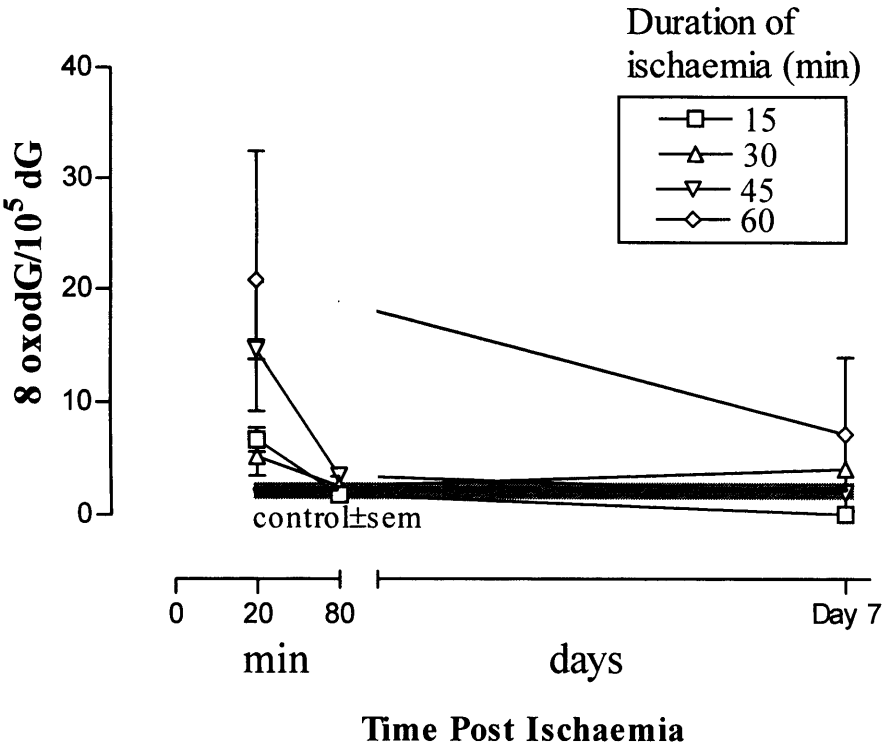

Figure 5.3vi Change over time in levels of oxidative DNA damage following WI injury. The elevation at 20 min was significant in the 15 ($p<0.05$), 45 ($p<0.01$) and 60 min ($p<0.05$) groups. Results are mean(sem).

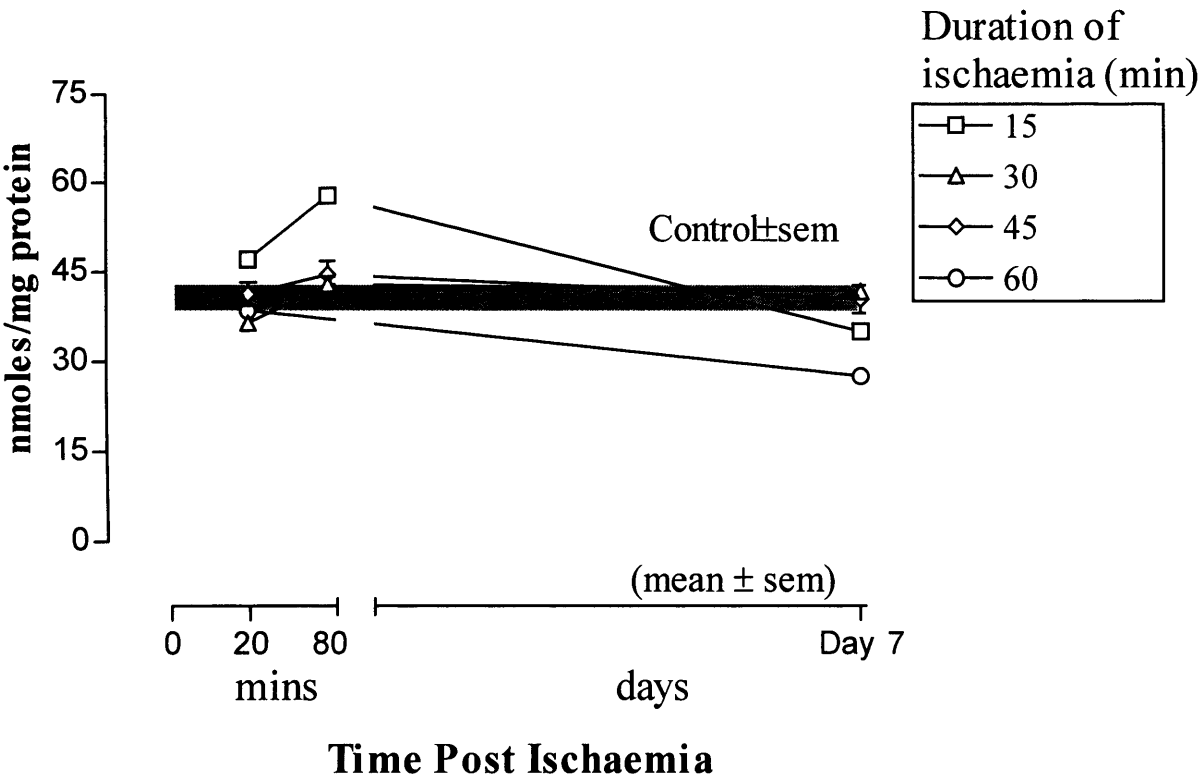

Figure 5.3vii Change over time in levels of oxidative protein damage (carbonyl content) following WI. Results are mean(sem).

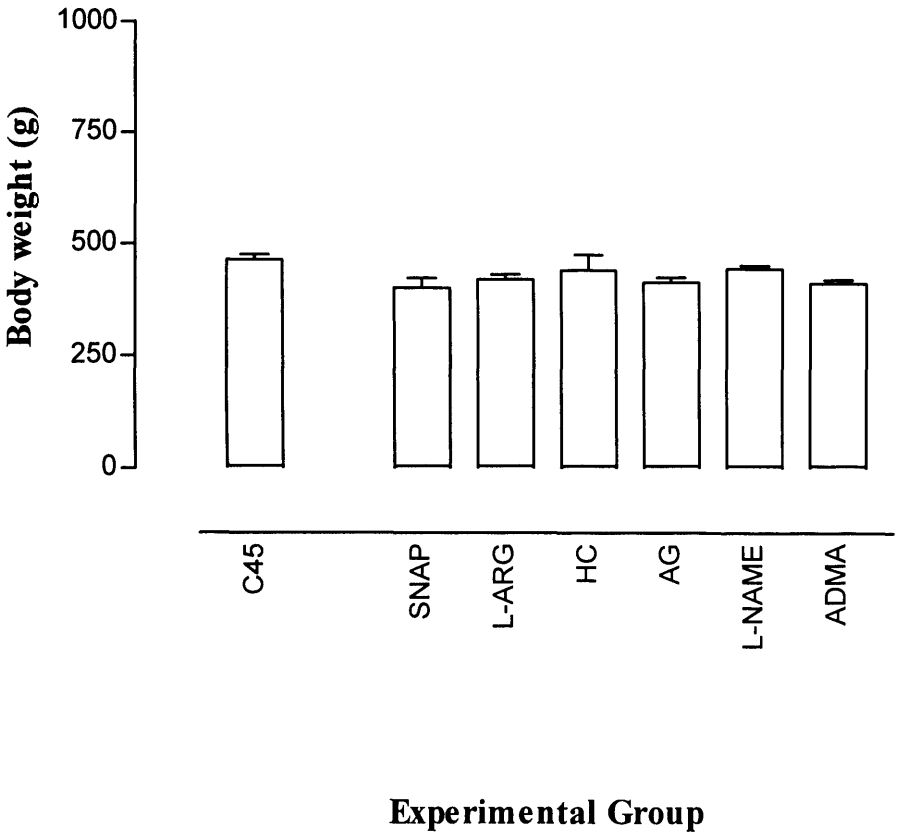

Figure 5.4i Comparison of the body weights of each experimental group used in the pharmacological manipulation of NOS. Also depicted is the group weight for the control group C_{45}. All results are mean(sem).

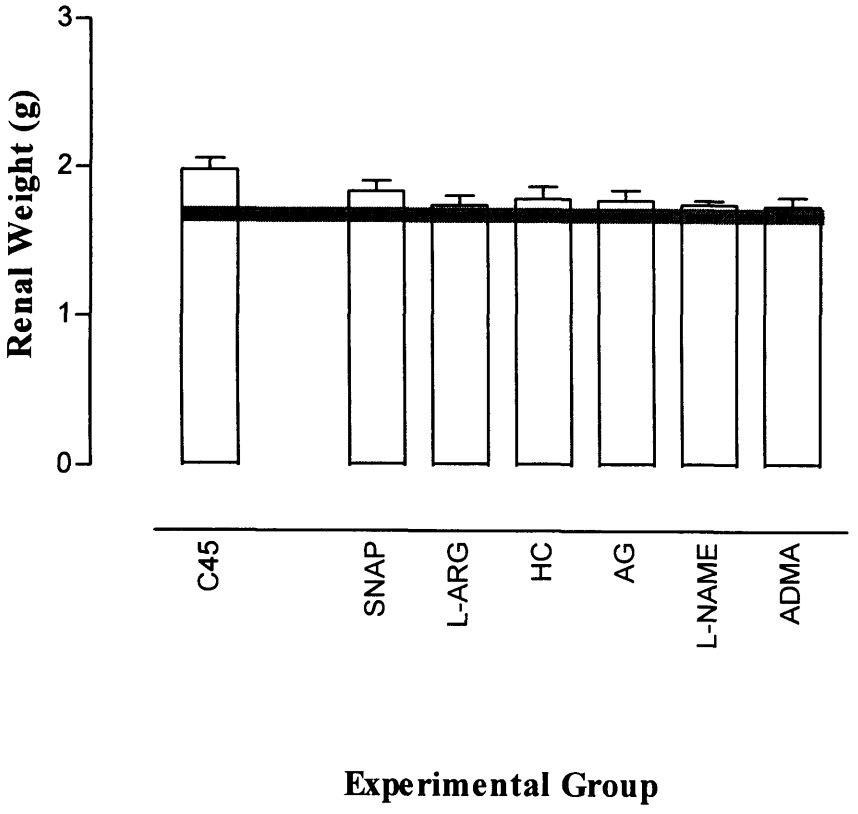

Figure 5.4ii Change in renal weight at 20 min reperfusion in the experimental groups. The dashed line represents baseline values (from the control group). All results are mean(sem).

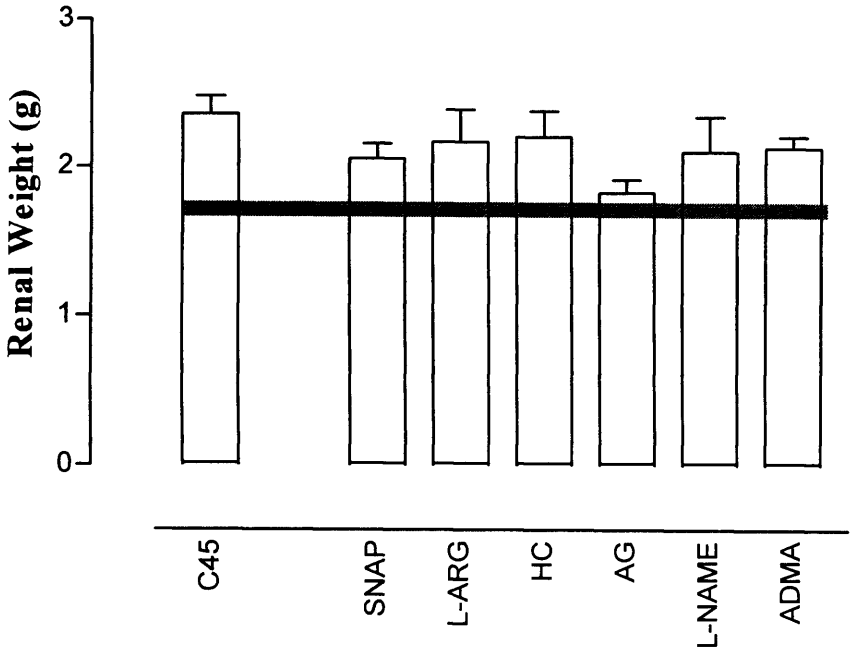

Figure 5.4iii Change in renal weight at day seven in the experimental groups in comparison to C_{45} and baseline values (dashed line). The increase in weight seen following WIRI was abolished by pretreatment with AG ($p<0.05$). All results are mean(sem).

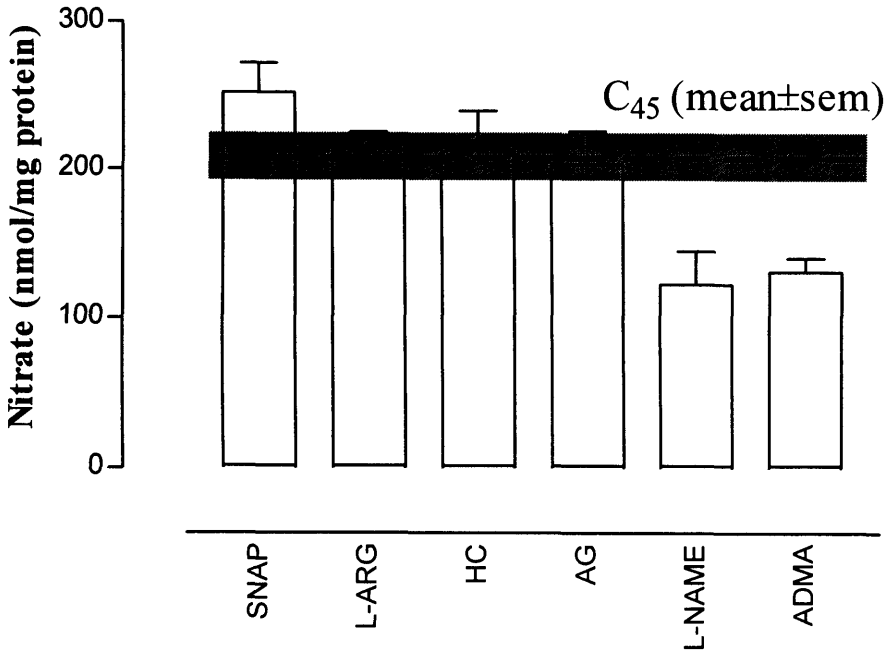

Figure 5.4iv The effect of pretreatment with pharmacological manipulators of NO/NOS on renal homogenate nitrate at 20 min reperfusion. Both L-NAME and ADMA significantly reduce endogenous NO production ($p<0.01$). All results are mean(sem).

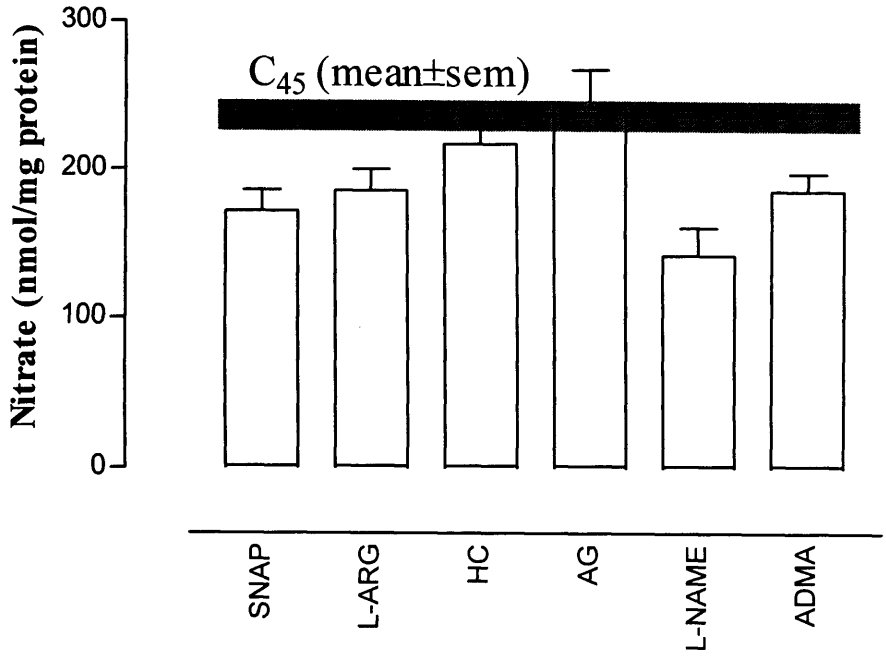

Figure 5.4v The effect of pharmacological manipulation of NO/NOS on renal homogenate nitrate at day 7. Pretreatment with the NO donors SNAP ($p<0.01$) & L-ARG ($p<0.05$) and the c+iNOS inhibitors L-NAME ($p<0.01$) and ADMA ($p<0.05$) significantly reduce endogenous NO production. All results are mean(sem).

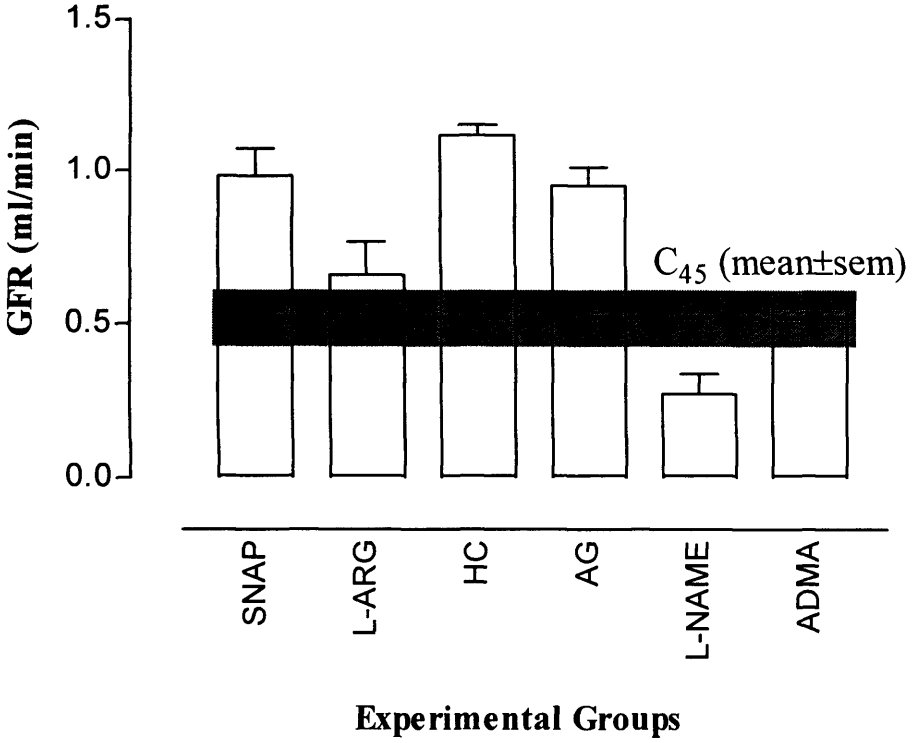

Figure 5.4vi The effect of pretreatment with pharmacological manipulators of NO/NOS on renal function at day two. The improvement was significant ($p<0.01$) in the SNAP, HC and AG groups whilst GFR was impaired ($p<0.05$) in the L-NAME group. All results are mean(sem).

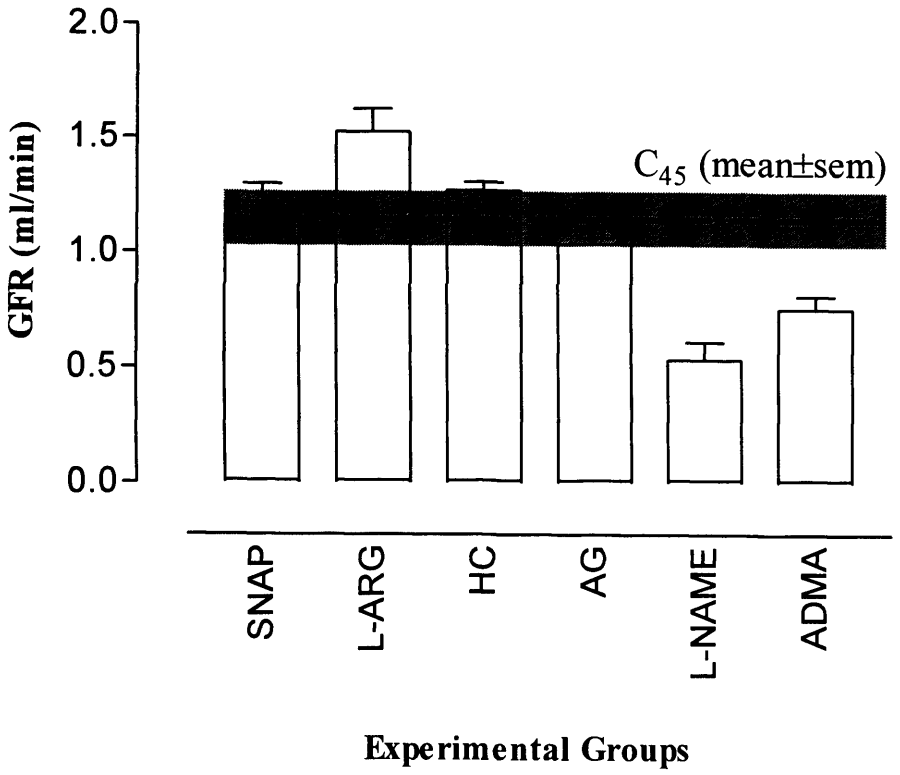

Figure 5.4vii The effect of pharmacological manipulation of NO/NOS on renal function at day seven. Only pretreatment with L-ARG led to a sustained improvement in GFR ($p<0.05$) whilst function was impaired ($p<0.01$) in both c+iNOS blocker groups. All results are mean(sem).

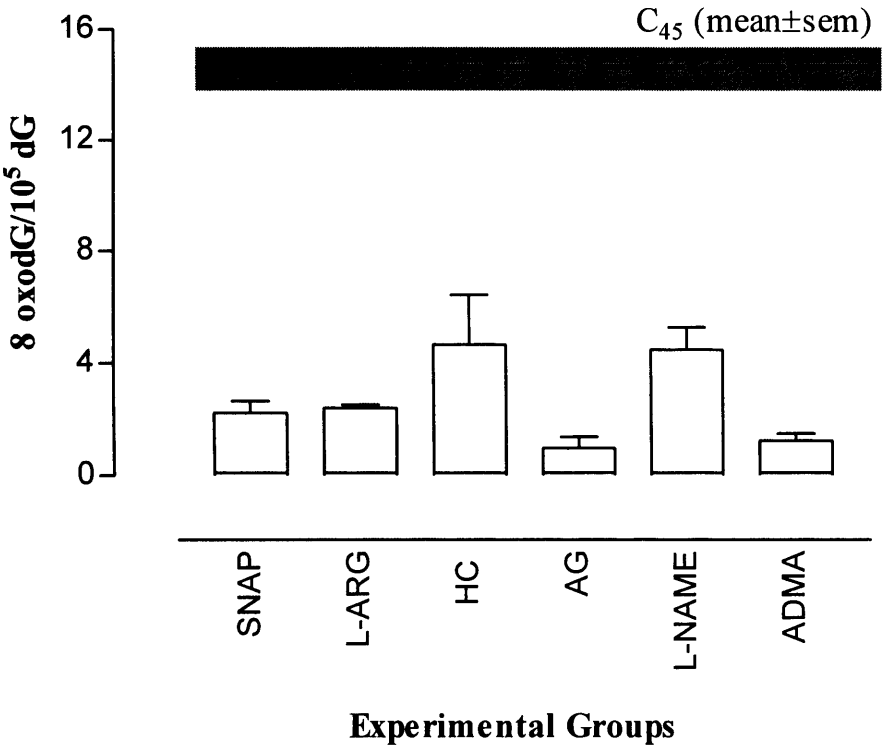

Figure 5.4viii The effect of pretreatment with pharmacological manipulators of NO/NOS on oxidative DNA damage at 20 min reperfusion. Damage was significantly reduced in all groups ($p<0.01$). All results are mean(sem).

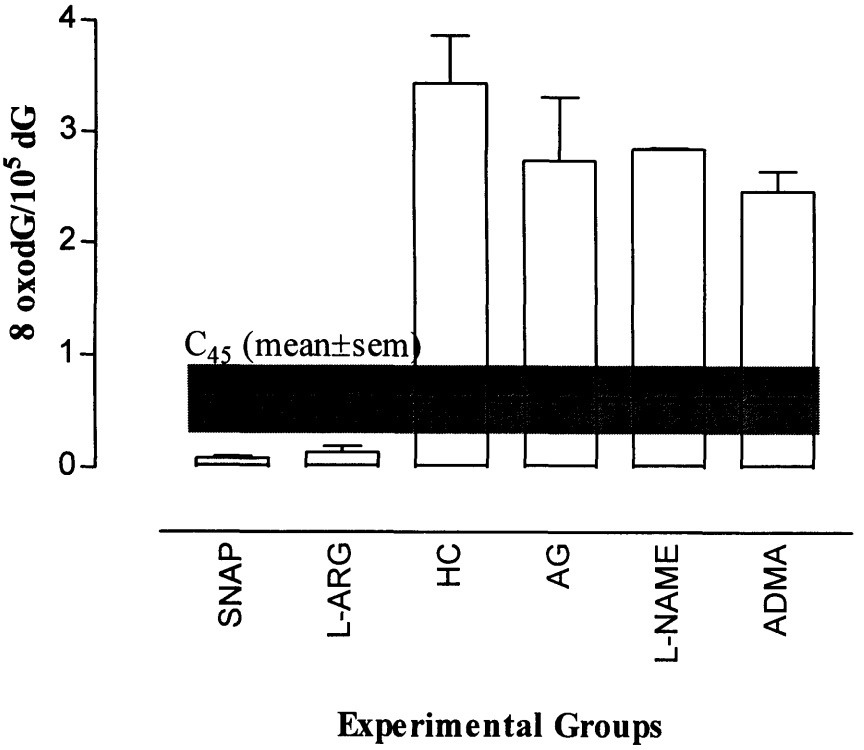

Figure 5.4ix The effect of pharmacological manipulation of NO/NOS on oxidative DNA damage at day seven. Only pretreatment with L-ARG and SNAP prevented a significant elevation ($p<0.01$) in damage. All results are mean(sem).

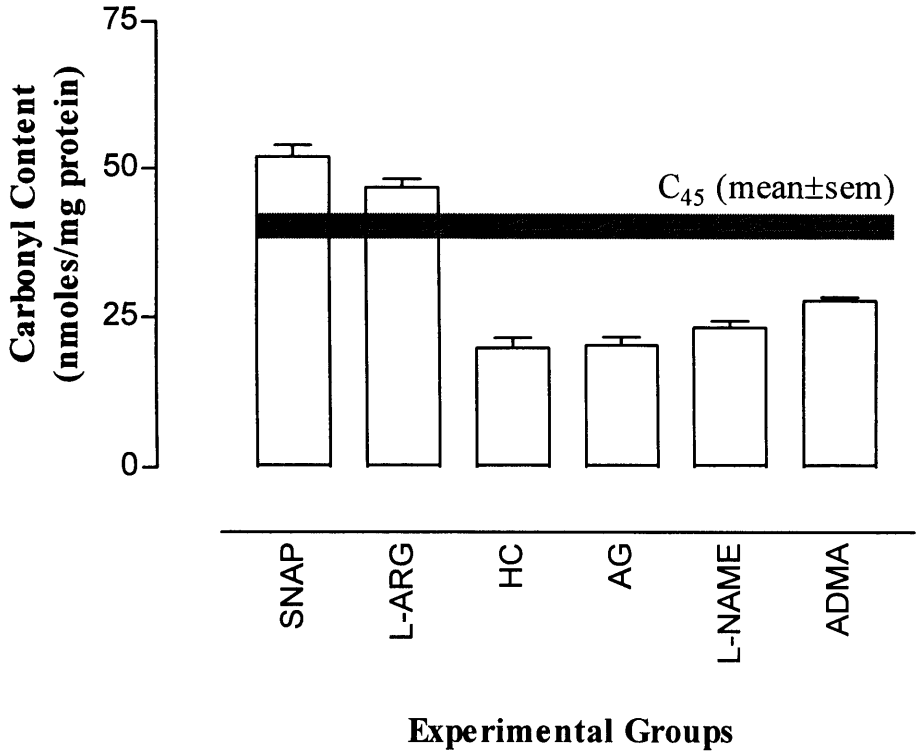

Figure 5.4x The effect of pretreatment with pharmacological manipulators of NO/NOS on oxidative protein damage at 20 min reperfusion. Damage was significantly reduced in all NOS inhibitor groups ($p<0.01$) but increased in the NO donor groups, significantly in the SNAP group ($p<0.01$). All results are mean(sem).

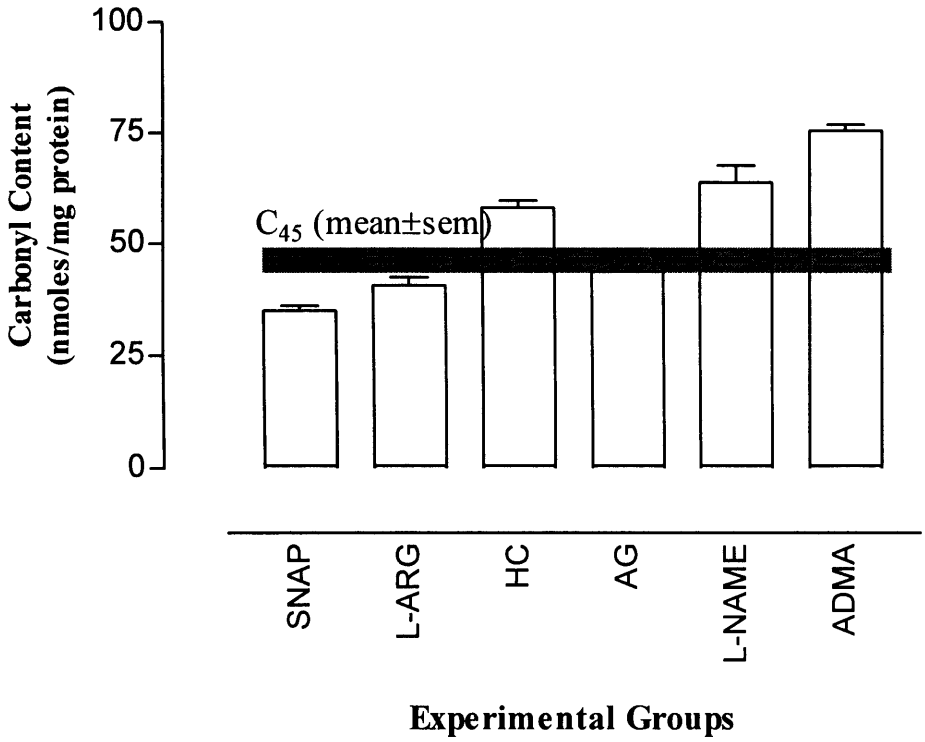

Figure 5.4xi The effect of pharmacological manipulation of NO/NOS on oxidative protein damage at day seven. Only pretreatment with L-ARG and SNAP ($p<0.01$) lowered the CC. It was raised in the NOS inhibitor groups, significantly ($p<0.01$) in all except the AG group. All results are mean(sem).

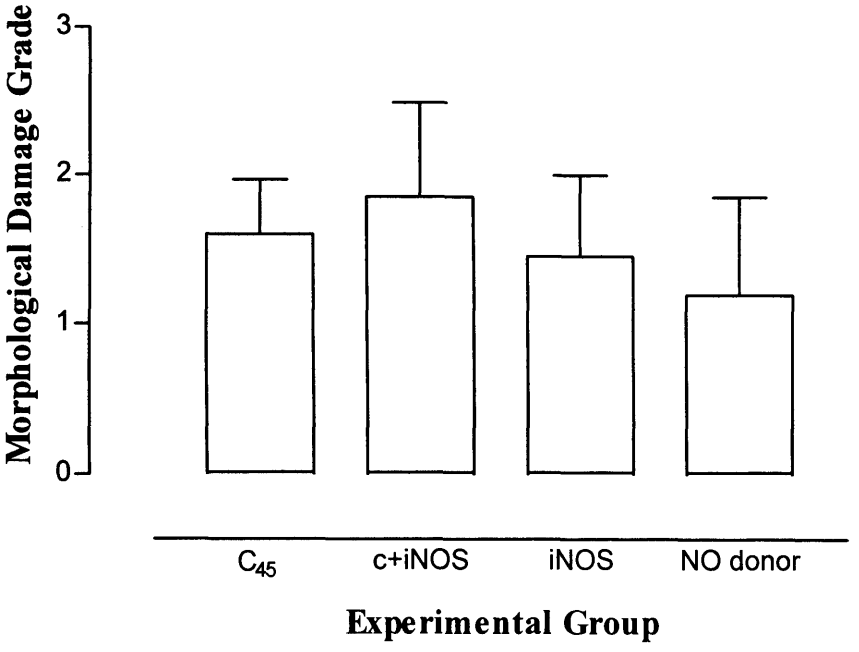

Figure 5.4xii Comparison between pretreatment groups and morphology at day seven. The NO donor (SNAP & L-ARG), iNOS (HC & AG) and c+iNOS (L-NAME & ADMA) groups do not differ significantly from control values. All results are median(95% CI).

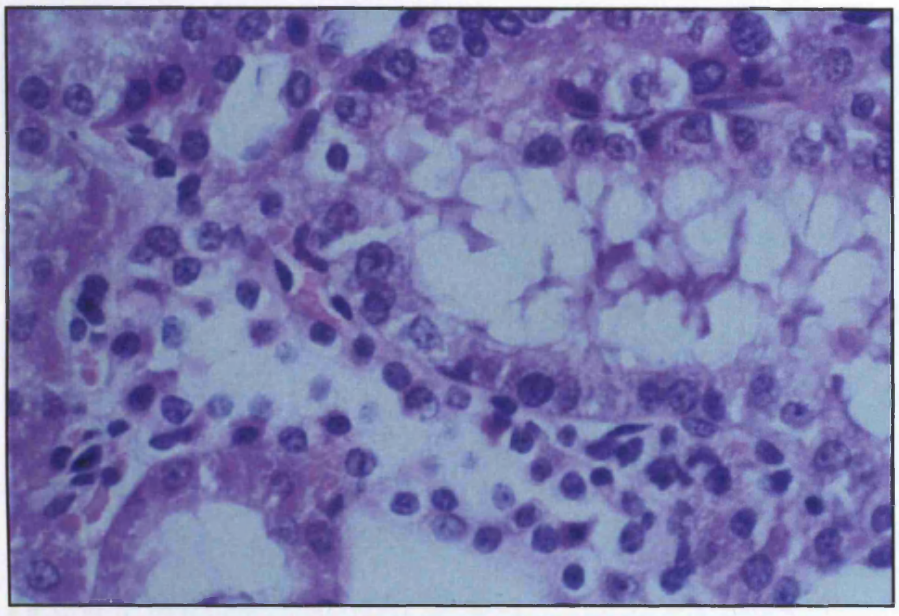

Figure 5.4xiii Photomicrograph of renal cortical tubules at day seven following pre-treatment with sodium nitroprusside (high power field)

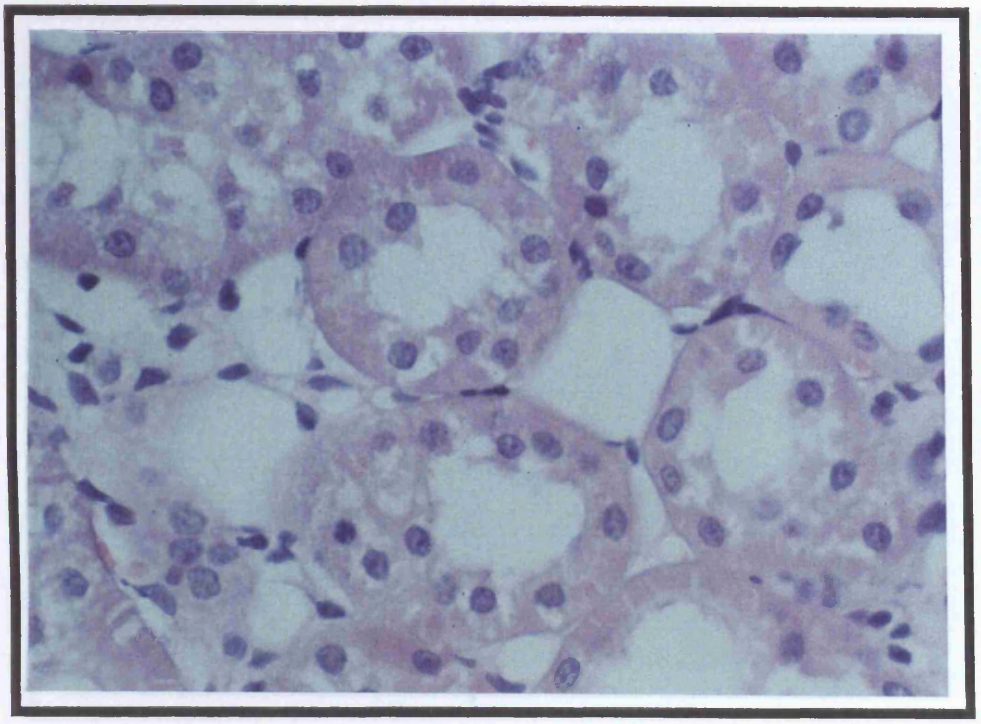

Figure 5.4xiv Photomicrograph of renal cortical tubules at day seven following pretreatment with L-arginine (high power field).

Figure 5.4xv Photomicrograph of renal cortical tubules at day seven following pretreatment with hydrocortisone (high power field).

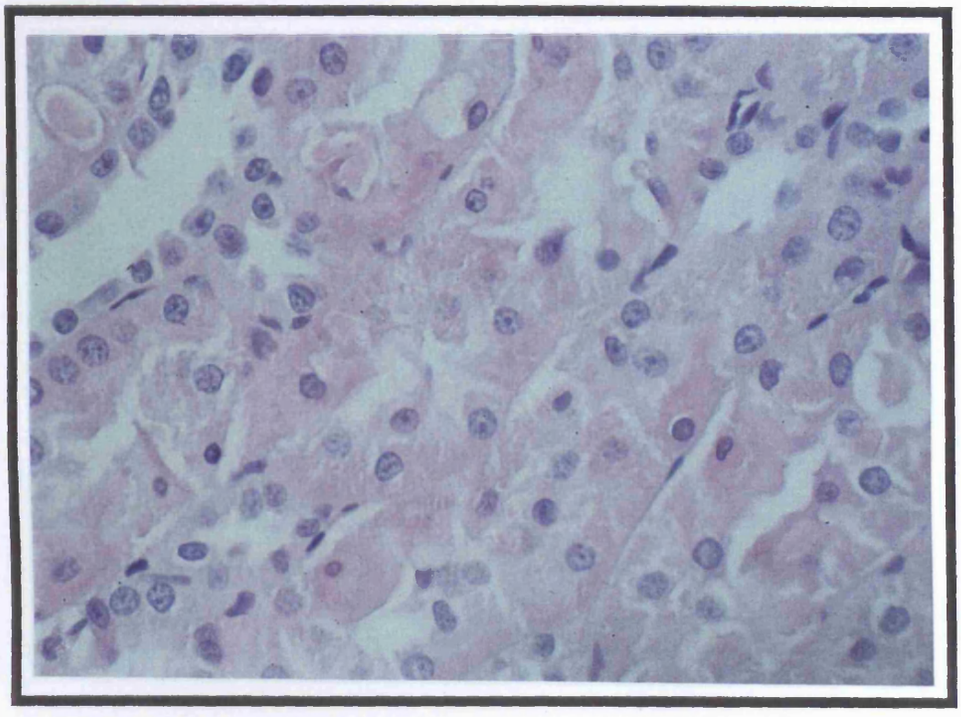

Figure 5.4xvi Photomicrograph of renal cortical tubules at day seven following pretreatment with aminoguanidine (high power field).

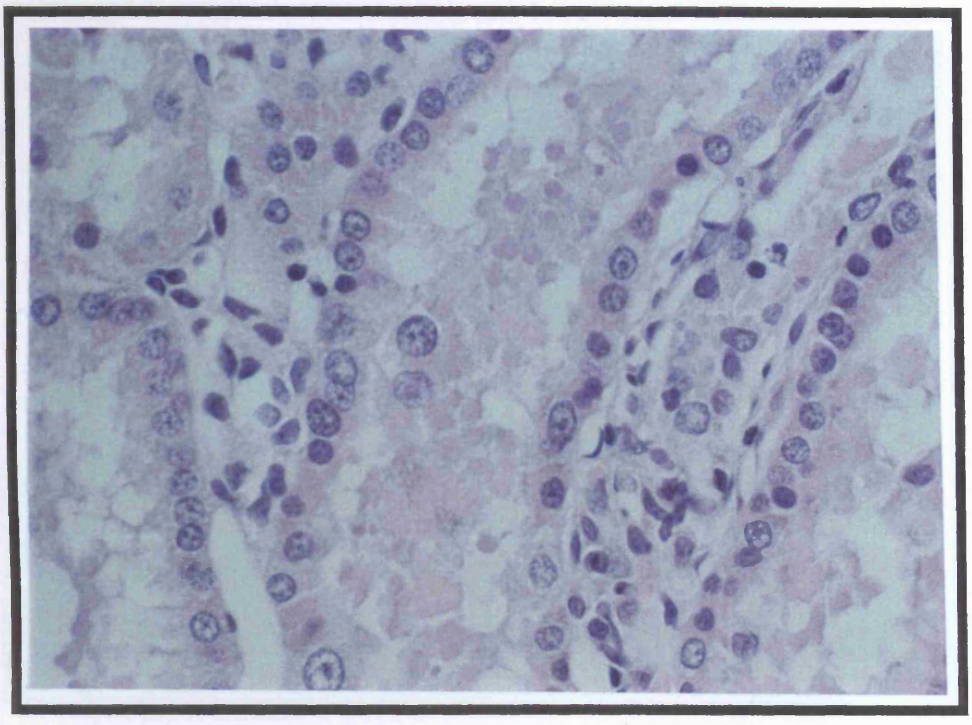

Figure 5.4xvii Photomicrograph of renal cortical tubules at day seven following pretreatment with dimethylarginine (high power field).

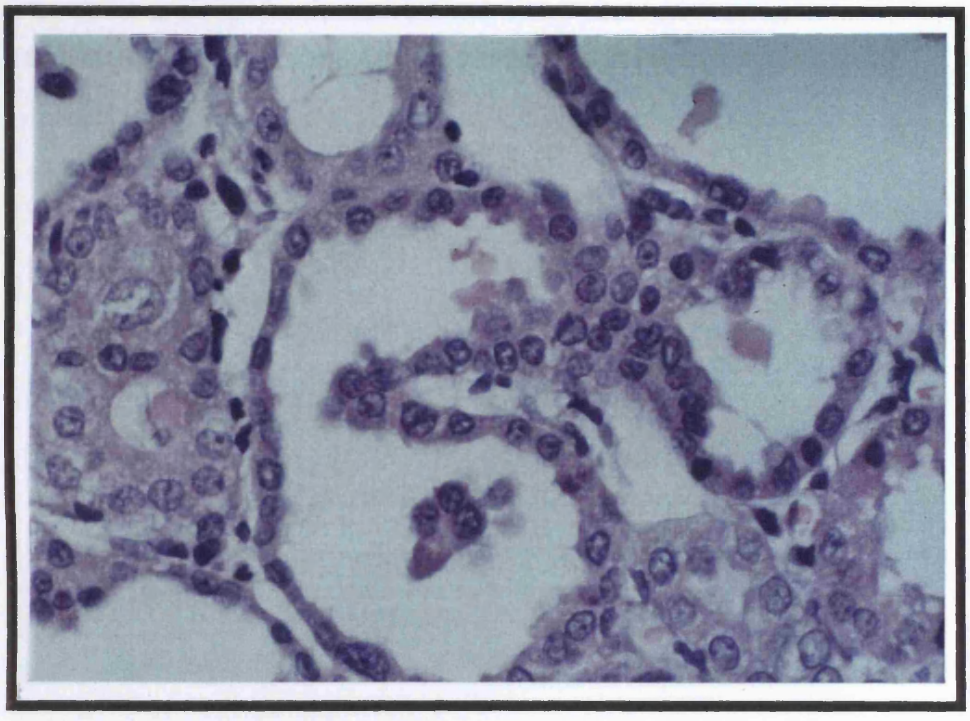

Figure 5.4xviii Photomicrograph of renal cortical tubules at day seven following pretreatment with N^{G}-nitro-L-arginine methyl ester (high power field).

Chapter 6

SUMMARY, CONCLUSIONS AND PROSPECTS FOR FURTHER RESEARCH

6.1 SUMMARY AND CONCLUSIONS

The underlying theme to this work was to investigate the role of nitric oxide in renal reperfusion injury. In order to achieve this it was necessary to quantify both NO and the degree of injury but the literature was notable for the absence of any cogent model to base this study upon. It was therefore necessary to develop and validate the required techniques in a new model before commencing the experimental work 'proper'.

The experimental Chapters of this thesis therefore began with the development of an assay to quantify NO in renal homogenate. The indirect route of measuring nitrite and nitrate was chosen for reasons of both simplicity and general availability and this appears to have been justified by the subsequent marketing of a commercial assay (Cayman Chemicals) using similar techniques. Whilst the nitrite assay was relatively straightforward to develop the nitrate assay proved more difficult and the original end-point assay was abandoned in favour of a more robust kinetic assay. Both methods were extensively validated prior to use in an experimental setting. This prolonged testing led to great familiarity with the techniques and an overall coefficient of variation of less than five percent was achieved. With these methods verified the best mode of preparing the kidney for analysis needed to be determined. Samples were to be snap frozen when harvested and thus the processing of freeze-dried samples would have been ideal but this facility was not available. Therefore the most germane means of homogenising and clarifying renal homogenate was assessed. The standard method of clarification by acidification and centrifugation proved untenable because of the pH-dependency of the nitrate assay and an alternative means through freeze-thaw cycling was developed.

Once the laboratory techniques had been established the animal model was designed and this forms the basis for Chapter 4. A standard *in vivo* model for investigating renal warm ischaemia reperfusion injury was detailed by Jablonski in 1983. This model however was unsuitable for the current experimental design, mainly because of the unavailability of tissue for analysis. A new model was therefore developed to circumvent this. By introducing bilateral renal warm ischaemia and then bilateral reperfusion before unilateral nephrectomy a kidney could be harvested at any desired

combination of WI/R. The remaining kidney, which had suffered an identical injury, could then be used for functional analysis as well as providing further tissue. Renal function was measured as the GFR using a relatively non-invasive radioisotope clearance technique that permitted repeated measures over several days. The validation of this technique was in three phases, firstly in three different groups of controls, secondly using the new model with increasing durations of warm ischaemia and finally by direct comparison with the Jablonski model. There was a negative correlation between the duration of warm ischaemia and the GFR at both day two ($r = 0.88$) and seven ($r = 0.95$). The grading system used for morphological damage also had to be developed (by Dr Furness) as the changes at day seven were far less severe than that seen with earlier damage as described by Calder et al (1971). Again there was good correlation between the duration of WI and the morphological damage ($r^2 = 0.89$). The other important aspect of the work detailed in this Chapter was the determination of the optimum duration of WI to use in the subsequent experimental work. In terms of group survival, significant but recoverable impairment in GFR and demonstrable morphological damage the 45min WI group was clearly the best. This was therefore chosen as the control (C_{45}) for the NO donor/NOS inhibitor experiments.

The final experimental Chapter is in two parts and utilises those techniques detailed above to investigate both the effect of renal WI on NO production and the effect of pharmacologically manipulating NO on the subsequent injury sustained. The initial section details the effect of the duration of WI on renal NO during early (20 & 80 min) and late (day 7) reperfusion. The levels of oxidative damage (DNA and protein) were also measured at these time points. Renal NO increased from control values in all groups showing strong positive correlation with the duration of WI (15-45 min) at 20 min reperfusion ($r = 0.97$). These elevated levels persisted through to day 7 at about 125% above control values. The exception was the 60 min WI group where NO was only slightly elevated, possibly as a result of the ischaemic injury to NOS. Whatever the reason, however, this emphasises the superiority of quantified NO over empirically reached conclusions. Oxidative DNA damage at 20 min also correlated well ($r = 0.88$) with the duration of WI although measurement of the carbonyl content proved less useful. When combined with the data in Chapter 4 this section gives a picture of the functional, pathophysiological and morphological injury in this model

with the comparative NO level. With these baseline indices determined, the effect of manipulating NO/NOS is described in the second section to Chapter 5. The 45 min WI group was used as the control and the effect of pretreatment (prior to WI) with NO donors, c+iNOS and iNOS inhibitors on the indices of injury was assessed. Early in reperfusion (20 min) c+iNOS, but not iNOS, inhibition decreased NO generation suggesting a predominant role for cNOS. Administration of exogenous NO did not lead to a measurable increase in nitrate although this may be a function of the timing of sampling. Oxidative DNA damage was decreased in all groups whilst protein damage was increased in the NO donor groups and decreased in the NOS inhibitor groups. The intermediate phase of reperfusion (day 2) was assessed by renal function with an improvement seen in the donor groups. The GFR was also improved by iNOS inhibition but impaired by c+iNOS blockade suggesting that cNOS has a protective role in sustaining renal function, possibly through the maintenance of renal perfusion. Quite why iNOS should be injurious is not clear but several possibilities, including increased microvascular permeability and neutrophil-mediated secondary damage, may be pertinent. Late in reperfusion (day 7) NO is reduced in both the NO donor and c+iNOS inhibitor groups although the suggestion is that this may be consequent upon opposing pathophysiology. Certainly at this time point the markers of damage are reduced in the NO donor groups but elevated in the c+iNOS groups. Whilst the iNOS groups shows elevated oxidative protein damage in all other regards they are comparable to the control (C_{45}).

The following conclusions can be drawn from this work. Nitric oxide is elevated in renal WIRI and whilst this is injurious early in reperfusion, inhibition of this rise leads to more profound late damage. In addition the evidence suggests that cNOS upregulation has a protective role whist the corollary is shown by iNOS induction.

6.2 FURTHER RESEARCH

In general terms this work set out to develop existing assays for measuring nitroxides for use in tissue homogenate and as such these methods should be applicable in any model of organ or limb ischaemia reperfusion. The model detailed should also lend itself to investigation of other aspects of the underlying pathophysiology of renal

WIRI such as complement or eicosanoids. One specific use of the model might be in developing a tissue viability assay for use in renal transplantation.

On the more specific aspect of where this work itself could be developed I think four main areas stand out. Firstly, it is now comparatively easy to measure both NOS activation and peroxynitrite. Applying these techniques should confirm the findings described and might elucidate whether synthase function is impaired after 60 min WI or whether free radical generation quenches NO. Secondly, more specific NOS inhibitors, including antisense DNA, are now available and these could be incorporated into this model. Thirdly, the question of NOS activation during the ischaemic phase could be easily investigated *in vivo.* Finally, it was hoped that the use of NO manipulation would ameliorate post-ischaemic renal damage. Although this proved the case to a limited degree it would be sensible to assess if altering the timing and/or duration of the pharmacological manipulation can enhance this effect.

Appendices

Appendix A

QUANTIFICATION OF 8-OXODEOXYGUANOSINE

Using HPLC-ECD Following the Enzymatic Digestion of DNA

Oxygen-derived free radicals can damage DNA in a variety of ways as discussed previously. One of the major products of such damage is 8-oxodeoxyguanosine (8-oxodG) which is the purine base guanidine with the hydrogen atom at position 8 replaced by an oxygen. Two methods have been described to measure this (and other base) species: via high performance liquid chromatography (HPLC) or gas chromatography-mass spectrometry (GC-MS). Both methods are of comparable sensitivity (when electrochemical detection-ECD is combined with HPLC) and it was decided to use HPLC-ECD in the light of concerns about the creation of artefact 8-oxodG during the derivatisation phase necessary for GC-MS (Halliwell & Dizdaroglu, 1992).

The HPLC-ECD technique involves the primary isolation, extraction and quantification of DNA followed by the detection of 8-oxodG within these DNA samples.

Isolation and Extraction of DNA

The following were all prepared fresh on the day of extraction.

Buffers:

1) 5mM trisodium citrate (S4641) = 0.738g
   520mM sodium chloride (S7653) = 0.584g
   (pH 6)

2) 20mM Trisma base (S6791) = 1.214g
   20mM EDTA (E1644) = 3.72g
   1.5% w/v sarkosyl (L5777) = 7.5g
   (pH 8.5)

3) 10mM Tris-HCl (T6666) = 0.395g
   1mM EDTA = 0.186g
   (pH 7.5)

4) 0.1M Trisma base = 0.606g
0.1M sodium chloride = 0.292g
20mM EDTA = 0.373g
(pH 8.0)

5) RNAse'A' 50mM Tris-HCl = 0.394g
10mM EDTA = 0.186g
10mM sodium chloride = 0.0292g
(pH 6.0)

Buffers 1-3 were all made up to 500ml using distilled, deionised water whilst buffer 4 (extraction buffer) and RNAse'A' were made up to 50ml. Buffers 1,2,4 and RNAse'A' were stored at 4°C whilst buffer 3 was kept at room temperature.

Enzymes:

Ribonuclease A. Ribonuclease A was dissolved in RNAse'A' buffer at 1mg/ml and heated in a water bath at boiling point for 15min before cooling to room temperature. The quantity of enzyme required for each day was calculated as follows: (No. samples + 1) ×0.25 = total weight of enzyme (mg).

Pronase E. Pronase E was dissolved in buffer 1 at 4°C to give a 4mg/ml solution. The quantity of enzyme required for each day was calculated as follows: (# samples + 1) ×2 = total weight of enzyme (mg).

The kidney sample was thawed in a small volume of extraction buffer at 4°C before dabbing off the excess buffer and homogenising at 3000rpm for 30sec in 1.5ml of extraction buffer. The homogenate was then centrifuged at 2500rpm for 15 min at 10°C before discarding the supernatant and adding 10ml of PBS, agitating, and then centrifuging as before and discarding the supernatant. The pellet was re-suspended in 1.75ml of ice-cold buffer 1, mixed by inversion and then 2ml of buffer 2 was added with vigorous mixing. A 250µl aliquot of Ribonuclease A in RNAse'A' was added, vortexed and incubated at 37°C for 1 hr. Then 0.5ml of Pronase E in buffer 1 was added and this was incubated overnight at 37°C.

After this incubation 2ml of buffer 3 and 0.5ml of 7.5M sodium acetate were added and mixed by inversion and then the DNA was precipitated by the addition of 18ml

of ice-cold ethanol (E) with the solution inverted until the precipitate appeared. The DNA was spooled onto a pipette hook and immersed in 10ml of 100% ethanol for 30 min and then a further 10 min after rinsing in 10ml of 100% ethanol for 10 min. All ethanol was removed from the DNA by blowing a stream of nitrogen over the sample and the DNA was then placed in 2ml of ultra-pure water and subjected to rotation-inversion overnight.

The concentration of DNA was then calculated spectrophotometrically. Into an Eppendorf was pipetted 680µl of ultra-pure water and 120µl of vortexed DNA. The sample was then thoroughly mixed by vortexing and transferred to a quartz cuvette with 800µl of ultra-pure water used as the control. Five consecutive readings at 260nm were taken for each sample and the average used to calculate the DNA concentration according to the equation {1}. From this the volume of sample containing 60µg DNA can be calculated {2} to allow such aliquots to pipetted into screw top Eppendorfs and freeze-dried until further analysis.

{1} $A_{260} \times 50 \times (20/3) \times 2 = [DNA]$ µg/ml

{2} [DNA] × 60/1000 = volume (µl) of sample containing 60µg DNA

Quantification of 8-oxodeoxyguanosine by HPLC-ECD

Reagents

a) 0.5M sodium acetate (S9513) = 1.5g in 25ml final volume (adjust to pH 4.8 with HCl). Pass through 0.45µm filter.

b) 20mM sodium acetate = 1 in 25 dilution in water of 0.5M stock solution.

c) 1.5M Tris-HCl (T6666) = 5.91g in 25ml final volume (adjust to pH 7.2 with NaOH). Pass through 0.45µm filter.100mM Tris-HCl = 1 in 15 dilution in water of 1.5M stock solution.

d) 1100U/ml Nuclease P_1 = 1mg in 0.5ml 20mM sodium acetate (pH 4.8)

e) Alkaline phosphatase (P4377) = dilute in 100mM Tris-HCl. Prepared fresh on the day of analysis.

The enzyme solutions in (d) and (e) are designed for a 60µg DNA sample. The amount of DNA chosen for analysis must be sufficient to detect baseline levels of 8-oxodG and this can only be determined by experimentation. If a different quantity of DNA was used then it would be necessary to tailor the concentration of these

enzymes accordingly. The basic principles are that there must be 0.22U nuclease P_1/μg DNA and 0.008U alkaline phosphatase/μg DNA and that it is important to keep the volume of each enzyme low (<20μl) to avoid diluting the analytes.

HPLC standard stock solutions and mobile phase

1mM 2'-deoxyadenosine (D7400) = 2.5mg in 10ml water

1mM 2'-deoxyguanosine (D7145) = 2.7mg in 10ml water

0.1mM 8-oxo-2'-deoxyguanosine (Wako Bioproducts, Richmond, USA) =0.6g in 20ml water

These standards were prepared by repeated vortexing until all visual evidence of particulates had disappeared and then filtered through a 0.45μm membrane. The concentration of each standard as stated is only a good approximation and therefore the exact concentration was calculated using the known extinction coefficients (ε) of each base in the ultraviolet spectrum:

8-oxodG (ε_{245}) = 12.3 $mM^{-1}.cm^{-1}$

dA (ε_{260}) = 15.2 $mM^{-1}.cm^{-1}$

dG (ε_{253}) = 13.0 $mM^{-1}.cm^{-1}$

Following this 300μl aliquots were pipetted into Eppendorf tubes and freeze-dried. These samples were then stored at -20°C and reconstituted as necessary to form the HPLC standards in subsequent analyses. The HPLC standards (five per base) were prepared fresh on the day of analysis by diluting the stock solutions to achieve the following range of concentrations in a total volume of less than 200μl: 8-oxodG (0-30nM), dA (0-15μM) and dG (0-50μM), where the zero concentration standard consisted of water only.

The HPLC mobile phase was prepared fresh on the day of analysis as follows:

a) 12.5mM potassium phosphate (P5504) = 5.71g

b) 1mM EDTA = 0.19g

c) Methanol (27,047-4)= 50ml

Reagents (a+b) were dissolved in 450ml of ultra-pure water and the pH adjusted to 5.1 with concentrated hydrochloric acid (H7020). Then 50ml of methanol was added and titrated with further HCl as necessary to maintain pH 5.1. The mobile phase was then passed through a 0.45μm nitro-cellulose filter membrane and de-gassed by sonication prior to use.

Method

The initial phase consisted of DNA digestion. The freeze-dried (60μg) DNA samples were reconstituted in 200μl of water and vortexed briefly before heating to 95-100°C in a water bath and then rapidly cooled by plunging into ice. To each sample was added 9μl of 0.5M sodium acetate and 11.5μl of nuclease P_1 (Calbiochem, Nottingham, UK). This was vortexed and then incubated at 37°C for 60 mins. Then 20μl of 1.5M Tris-HCl and 9.5μl of alkaline phosphatase were added, and the sample vortexed and incubated at 37°C for 120 min.

The samples were analysed on a Hypersil ODS (C_{18}) column with a 3μm silica particle size and of dimension 150mm × 4.6mm, with a guard column (both Phenomenex, Macclesfield, UK) of 30mm × 4.6mm. The injection loop was 100μl with a column flow rate of 1ml/min at ambient temperature. A 60μl aliquot of standard or sample was injected into the column and the dG and dA peaks were detected in the ultraviolet range (254nm) whilst 8-oxodG was detected electrochemically at 800mV (2nA sensitivity).

The in-built system software was used to calculate the peak areas for dA, dG and 8-oxodG in the base standards, dA and 8-oxodG in the oligonucleotide standard and dG and 8-oxodG in the samples. Using the data from the standards a best fit calibration curve (peak area v standard concentration) could be constructed for each base by linear regression and then the concentrations of each sample base could be calculated from the respective peak areas.

Appendix B

QUANTIFICATION OF THE CARBONYL CONTENT

In the preceding Chapters the mechanism through which free radicals are believed to damage proteins was outlined. Briefly the hydroxyl radical is thought to induce the formation of α-carbon radicals which are then oxidised with the generation of a peroxyl radical. This then forms peroxide which subsequently decomposes allowing the chain scission of proteins into carbonyls and amides (Davies & Delsignore, 1986).

In 1990 Levine described a simple method for the measurement of the carbonyl content of plasma. Subsequently the carbonyl content has been used as a marker of plasma protein oxidative damage in a variety of models such as adult respiratory distress syndrome (Quinlan et al, 1994), the effects of cigarette smoke (Reznick et al, 1992; Eiserich et al, 1995) and haemodialysis and renal transplantation (Odetti et al, 1996). More recently several investigators have focussed on organ specific protein oxidation using homogenates of brain (Forster et al, 1996; Dubey et al, 1996) and liver (Rouach et al, 1997) and have verified, in so doing, that the technique for quantifying plasma carbonyl content is suitable for homogenates.

Measurement of Renal Carbonyl Content

Reagents:

10mM 2,4-dinitrophenylhydrazine (D2630) = 1.98mg in 10ml 2M HCl.

20% (w/v) trichloroacetic acid (T6399) in distilled water.

Ethanol : ethyl acetate (E7770) 1:1 (v/v)

6M Guanidine solution (G4505) = 12g in 20ml 20mM potassium phosphate. Adjust to pH 2.3 with trifloroacetic acid.

20mM potassium phosphate (P8709) = dilute 1M stock solution 1:50 with distilled water.

Trifluroacetic acid (T6508).

EDTA =1mg/ml in 0.9% saline

Each kidney sample was analysed in duplicate. A 100mg sample was homogenised in the EDTA and centrifuged at 2500rpm for 5 min and the supernatant decanted. To

0.5ml of supernatant was added 0.5ml of dinitrophenylhydralazine and this was vortexed every 15 min whilst standing for one hour at room temperature. A 0.5ml aliquot of trichloroacetic acid was then added, vortexed and centrifuged at 13000g for 3 min before discarding the supernatant. The pellet was then washed three times to remove any free agent by resuspending in ethanol:ethyl acetate, left to stand for 5 min then centrifuged at 13000g for 3 min. The precipitated protein was then resuspended in 1ml of Guanidine solution and incubated at 37°C for 30 min before centrifuging at 13000g for 1 min to remove any insoluble material. The absorbance of the supernatant was then be determined spectrophotometrically at 360nm using distilled water as zero. As the molar absorption coefficient (MAC) of carbonyl is known ($22000M^{-1}\ cm^{-1}$) the carbonyl content of the sample can be calculated:

a) Carbonyl content (CC) $\mu M \mu l^{-1} = \dfrac{\text{supernatant absorption}}{\text{MAC (i.e. 22000)}}$

b) CC × 500 (i.e. the original vol. of homogenate) = CC μM

c) Carbonyl content / mg protein = $\dfrac{\text{CC } \mu M}{\text{protein content}}$

Protein Determination Assay

Several methods exist to determine protein concentration. This particular test uses the property of protein to reduce alkaline copper (II) to copper (I) in a concentration-dependent manner. The bicinchoninic acid is a chromogenic reagent that reacts with the copper (I) forming a specific purple complex with maximal absorbance at 562nm.

Reagents:

4% copper sulphate pentahydrate}
Bicinchoninic acid solution } Sigma kit BCA-1
Bovine serum albumin 0.1mg/ml }
Distilled water

The copper sulphate and bicinchoninic acid solution were mixed 1:50 to generate the protein determination reagent. A series of protein standards were produced from the albumin ranging from 0-0.02mg protein, with the 0mg protein standard being the

blank for the spectrophotometer. To 100µl of protein standard was added 2ml of protein determination reagent and the tube vortexed and incubated at 37°C for 30 min. The tubes were cooled to room temperature and the absorbance at 562nm measured, using water to zero the spectrophotometer. The absorbance of the blank tube was subtracted from the others to give the net absorbance from protein and a calibration curve then constructed. It was then a simple matter to calculate the protein concentration of each sample using a 100µl aliquot.

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