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NOVEL THERAPIES IN ACUTE KIDNEY INJURY

A THESIS PRESENTED BY

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For

The degree of Doctor of Philosophy.

DECLARATION:

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. Information derived from the work of others has been acknowledged in the text and a list of references is given.

1st July 2014

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(Signature) **(Date)**

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Abbreviations

AA	Antimycin A
AGE	Advance glycation end product
AIP	Apoptosis inducing factor
ARF	Acute renal failure
AKI	Acute kidney Injury
APAF1	Apoptosis protease activating factor 1
AST	Aspartate aminotransferase
ATN	Acute tubular necrosis
ATP	Adenosine Triphosphate
Bad	Bcl-2 associated death promoter
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-X_L	B-cell lymphoma extra large
Bid	Bcl-2 interacting protein
Bfl-1	B cell lymphoma-2–related gene expressed in fetal liver-1
BMP-7	Bone morphogenic protein-7
CARDs	Caspase activation and recruitment domains
CKD	Chronic kidney disease
Cyt c	Cytochrome c
CXCL-1	Chemokine ligand-1
dATP	deoxyadenosine triphosphate
DEDs	Death effector domains
DISC	Death inducing signaling complex
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
ESRD	End stage renal disease
ERK	Extracellular signal regulated kinases

eNOS	Endothelial nitric oxide synthases
FAD	Flavin adenine dinucleotide
GHRH	Growth hormone releasing hormone
GFR	Glomerular filtration rate
GM-CSF	Granulocyte-macrophage colony stimulating factor
HBSS	Hank's balanced salt solution
HK-2	Human kidney 2
H_2O_2	Hydrogen peroxide
HNE	Hydroxynonenal
IAP	Inhibitor of apoptosis
ICAM-1	Intercellular adhesion molecule 1
IRF-1	Interferon regulatory factor-1
IFN-α	Interferon-α
IRI	Ischaemia reperfusion injury.
IL-1α	Interleukin-1α
IL-1β	Interleukin-1β
IL-2	Interleukin-2
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
IL-18	Interleukin-18
iNOS	Inducible nitric oxide synthase
MIP-2	Macrophage inflammatory protein-2
MCP-1	Monocyte chemotactic protein-1
MPO	Myeloperoxidase
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
$NaNO_2$	Sodium nitrite
Nec-1	Necrostatin-1
NF-κB	Nuclear factor kappa B
NLRs	NOD-like receptors

NO	Nitric oxide
NOS	Nitric oxide synthase
NO_2	Nitrogen dioxide
NO_2^-	Nitrite
NO_3^-	Nitrate
$O_2^{\bullet -}$	Superoxide
$OH^{\bullet}$	Hydroxyl radical
OGDH	2- oxoglutarate dehydrogenase
$ONOO^-$	Peroxynitrite
PAR	poly (ADP-ribose)
PARP-1	poly (ADP-ribose) polymerase-1
PBS	Phosphate buffer saline
PBS-T	Triton X-100 in phosphate buffer saline
PCR	Polymerase chain reaction
PDH	Pyruvate dehydrogenase
PTECs	Proximal tubular epithelial cells
PRRs	Pathogen recognition receptors
rhBMP-7	Recombinant human bone morphogenic protein-7
RHIM	RIP homotypic interaction motif
RIP1	Receptor interacting protein1
RIP3	Receptor interacting protein3
RLRs	RIG-1 like receptors
ROS	Reactive oxygen species
RRT	Renal replacement therapy
Smad	Contraction of sma and mad proteins
Smurf1	Smad ubiquitin regulatory factor1
SS	Serum starvation
TAL	Thick ascending loop of henle
TGF-β	Transforming growth factor-β
TK	Transketolase
TLRs	Toll-like receptors

TNF-α	Tumor necrosis factor-α
TRADD	TNFR-associated death domain
TRAF-2	TNF receptor-associated factor-2
XOR	Xanthine oxidoreductase

Units and symbols

g	gram
g	centrifugal force
h	hour
IU	international unit
kg	kilogram
µg	microgram
µm	micrometre
mmol	millimole
µmol	micromole
nmol	nanomole
pmol	picomole
mg	milligram
min	minute
L	litre
mL	millilitre
µL	microlitre
mm	millimetre
mmHg	millimetre of mercury
%	percentage
s	second

Routes of administration and statistical terms.

i.p.	intraperitoneally
s.c.	subcutaneously
i.v.	intravenously
n	number of observations
P	probability, denoting level of significance
ANOVA	analysis of variance

Abstract

Renal ischaemia-reperfusion injury (IRI) is a major cause of acute kidney injury (AKI) which is in turn the leading cause of morbidity and mortality in hospitalized patients. The principle aim of this thesis was to evaluate potential new therapies that might afford protection against IRI in both *in vitro* and *in vivo* settings.

Recent evidence suggests that nitrite (NO_2^-) may play an important role in protecting the myocardium from IRI. Our initial work into the role of NO_2^- in an *in vitro* model of renal IRI in proximal tubular epithelial cells provided evidence that NO_2^- can prevent apoptosis and preserve cell viability. This lead to an *in vivo* study where high NO_2^- concentrations (50 mg/L) were given orally to rats for 7 days prior to inducing renal IRI but no beneficial effects of this treatment were observed.

Another potential treatment identified was thiamine (vitamin B1) and this, like NO_2^- was investigated to see if it had the potential to protect rats from AKI injury. It has been previously recognized that in renal IRI the high energy phosphate ATP is found to be severely depleted whilst is is known that thiamine can play a pivotal role in generating ATP. Furthermore, thiamine has previously been demonstrated to protect against myocardial ischaemic injury and has the ability to reduce myocardial infarct size. *In vitro*, thiamine was found to reduce the degree of apoptosis in cultured HK-2 cells caused by ischaemia whilst *in vivo* it afforded protection against AKI caused by renal IRI by anti-apoptotic, anti-inflammatory and anti-oxidant mechanisms.

Finally, a study into the possible therapeutic role of gene therapy with bone morphogenic protein 7 (BMP-7) in renal IRI was undertaken. Previous work has established that i.v. BMP-7 is able to protect against renal IRI but it has also been associated with ectopic bone formation at the site of injection. Therefore another method to increase circulating BMP-7 was sought. We initially found that BMP-7 gene therapy could attenuate apoptosis and preserves cell viability in an *in vitro* model of renal IRI. However, whilst *in vivo* gene therapy with electroporation of BMP-7 plasmid DNA increased BMP-7 expression in mice serum 2 days post electroporation, it was unable to protect the animals against IRI induced AKI. In rats the direct injection of naked DNA BMP-7 plasmid systematic 2 days prior to renal IRI was able to upregulate BMP-7 expression 4 days later in kidney tissue. Despite this it was unable to afford protection against renal IRI.

Apoptosis and necrosis play a crucial role in the pathogenesis of renal IRI induced AKI. In this thesis we investigated the role of three putative therapeutic agents and their role in apoptosis and necrosis *in vitro* in PTECs and *in vivo* against renal IRI induced AKI. All three therapeutic drugs were able to attenuate apoptosis in PTECs but were unable to protect against necrosis, whilst against renal IRI induced AKI only thiamine was found to be protective. Thiamine appears to hold the most promise and more work needs to be undertaken so that its potential benefit in AKI can be realised.

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CHAPTER 1

INTRODUCTION

1.1: Acute kidney injury (AKI)

Acute Kidney Injury (AKI) was previously known as Acute Renal Failure (ARF) (Webb and Dobb, 2007) and is characterized by a rapid reduction in kidney function resulting in a failure to maintain fluid, electrolyte and acid-base homoeostasis and is typically diagnosed by an accumulation of end products of nitrogen metabolism (urea and creatinine) or decreased urine output, or both. This complex condition can be due to (i) pre-renal AKI (in around 55% of cases), involving diseases that can cause renal hypoperfusion leading to renal dysfunction without renal parenchymal damage (ii) intrinsic AKI (~40%), including disease that directly insults the renal parenchyma; and (iii) post-renal AKI (~5%), resulting from diseases associated with urinary tract obstruction (Brady, 1998).The major cause of intrinsic AKI is acute tubular necrosis (ATN) which is caused by ischaemic injury or a toxic insult to the kidney caused by varied distinct pathomechanisms (Webb and Dobb, 2007).It results in severe and persistent hypoperfusion of the kidneys (as seen in pre-renal AKI).

AKI is a common, harmful, and life threatening condition which may be treatable if detected early. A minor reduction in the kidney function has a detrimental effect on the prognosis of AKI (Chertow *et al*., 1998). It is clinically important to detect and treat AKI as this may improve outcomes. Recently two similar definitions of AKI based on serum creatinine and urine output (RIFLE and AKIN) have been proposed and validated. Firstly, the Acute Dialysis Quality Initiative Group (Bellomo *et al*., 2004) has classified AKI according to the severity and duration of degree of acute renal dysfunction. This classification of AKI is known as RIFLE criteria (Table 1).

Table 1: AKI classification according to RIFLE category (Bellomo *et al.*, 2004)

RIFLE Category	Serum creatinine or GFR criteria	Urine output (UO) criteria
Risk (R)	Increased creatinine x 1.5 or GFR decrease > 25%	UO < 0.5 mL/kg/h ≥ 6 h
Injury (I)	Increased creatinine x 2 or GFR decrease > 50%	UO < 0.5 mL/kg/h ≥ 12 h
Failure (F)	Increased creatinine x 3 or GFR decrease > 75% or an absolute serum creatinine ≥ 354 μmol/L with an acute rise of at least 44 μmol/L	UO < 0.3 mL/kg/h ≥ 24 h or anuria ≥ 12 h
Loss (L)	Persistent ARF = complete loss of renal function > 4 weeks	
ESRD (E)	Complete loss of renal function > 3 months	

Secondly, the Acute Kidney Injury Network (AKIN) group (Mehta *et al.*, 2007) has proposed rectifications to the RIFLE criteria. They are as follows in Table 2:

Table 2: AKIN criteria (Mehta *et al.*, 2007).

Stage 1	Increase in serum creatinine ≥ 26.2 μmol/L or increase to ≥ 150 – 199% (1.5 – 1.9 fold) from baseline	UO<0.5 mL/kg/h ≥ 6 h
Stage 2	Increase in serum creatinine to 200 – 299% (>2 – 2.9 fold) from baseline	UO<0.5 mL/kg/h ≥12 h
Stage 3	Increase in serum creatinine to ≥ 300% (≥ 3-fold) from baseline or serum creatinine ≥ 354 μmol/L with an acute rise of at least 44 μmol/L or initiation of RRT	UO<0.3 mL/kg/h ≥24 h or anuria ≥ 12 h

Multiple studies have shown the validity of both RIFLE and AKIN criteria to identify the groups of hospitalised patients that have an increased risk of death and/or need for renal replacement therapy (RRT) (Hoste *et al*., 2006, Uchino *et al*., 2006, Ostermann and Chang, 2007, Bagshaw *et al*., 2008, Joannidis *et al*., 2009, Thakar *et al*., 2009). One study has directly compared RIFLE criteria with and without the AKIN modification (Joannidis *et al.*, 2009) and showed that AKI classified by either criterion was associated with similarly increased hospital mortality rates. Surprisingly, the two sets of

AKI criteria detected diverse patients. The RIFLE criteria did not detect 9% of patients that were detected by AKIN criteria. Likewise, the AKIN criteria missed 26.9% of the patients detected by RIFLE criteria. Most of the cases found by RIFLE criteria and missed by AKIN included 30% RIFLE-Injury(R-I) and 18% RIFLE-Failure (R-F). While the patients detected by AKIN and missed by RIFLE were 90% in stage 1. Mortality rate of patient detected by RIFLE and missed by AKIN was R-I group 37% and R-F group 41%, but mortality rate of patients detected by AKIN stage 1 and missed by RIFLE was twice high when compared with patients without AKI. This analysis therefore identified the importance of using both sets of criteria for the diagnosis and staging of AKI (Joannidis *et al.*, 2009, John, 2012, Citerio *et al.*, 2013).

The loss of tubular epithelial cells, a dilated and flattened epithelium, and the presence of Tamm-Horsfall protein-rich casts are the main histological findings in ischaemic AKI. This may be caused by IRI of the kidney due to kidney transplantation (Aydin *et al*., 2007), sepsis (Ahlstrom *et al*., 2006, Ronco *et al*., 2008), cardiopulmonary (Mangano *et al*., 1998) and aortic bypass surgery (Kazmers *et al*., 1997). AKI is very common in critically ill patients and all other hospital based patients and is often secondary to extra renal events and is the leading cause of morbidity and mortality in all hospitalised patients (Bonventre, 1993). Furthermore, research indicates that hospitalised patients who develop AKI have a poor prognosis with mortality ranging from 10% to 80% (Hou *et al*., 1983, Shusterman *et al*., 1987). It has now been recognised that even a very small rise in serum creatinine in a variety of clinical settings is associated with a likely outcome of AKI (Praught and Shlipak, 2005). Multiple studies show that the population incidence of AKI in the UK ranges from 172 per million population (pmp) per year from

early data up to 486-630 pmp/year identified more recently (Feest *et al*., 1993, Stevens *et al*., 2001, Hegarty *et al*., 2005). Furthermore, the mortality rate is around 50% in patients with AKI and multiple organ failure and if RRT is required the mortality reaches around 80% (Liano *et al*., 1998). The mortality rate is further enhanced by severe complications of AKI such as sepsis, bleeding and respiratory failure (Levy *et al.*, 1996). A Veterans Affairs/National Institutes of Health Acute Renal Failure Trial Network (ATN) study involving patients with multi-organ dysfunction or failure indicates that the all-cause mortality by day 60 after admission was 51% - 54% (Palevsky *et al.*, 2008). A recent study involving 2,901 patients from 17 Finnish Intensive Care Units (ICU) showed an AKI incidence rate of nearly 40%. Hypovolemia, diuretics, artificial colloids and CKD were identified as major independent risk facts for developing AKI in ICU. This study further showed that the reversibility of AKI is highly dependent on its stage at presentation. There is 30% resolution on the following day for AKI stage 1 and 87% AKI persistence in patients with stage 3 (Nisula *et al*., 2013, Citerio *et al*., 2013). Despite recent advances in our understanding of AKI and its pathogenesis the therapeutic options for such patients are very limited.

Research has been conducted in order to identify novel early biomarkers for AKI such as serum neutrophil gelatinase associated lipocalin (NGAL), IL-18, cystatin-c and KIM-1. NGAL is an iron-transporting protein (Bao *et al*., 2010) that is produced in the kidney distal nephron and many other tissues and organs and is normally filtered and reabsorbed by the proximal tubule. Iron has been proposed to play an important role in protecting the proximal tubule from injury. Studies suggest that intravenous (i.v) administration of purified recombinant NGAL results in uptake by proximal tubular cells

where it enhances proliferation, attenuates apoptosis and provides significant functional and pathological protection in murine models of renal IRI (Mishra *et al*., 2004, Mori *et al*., 2005, Bonventre and Yang, 2011, Citerio *et al*., 2013). NGAL as a maker has been shown to be able to help in detecting the occurrence of AKI after 48 h, before any significant increase of serum creatinine was seen and has also indicated the requirement of RRT in critically ill patients (Cruz *et al*., 2010). Its main drawback as a marker is the influence of systemic inflammation and the resulting increase in serum NGAL concentrations even in the absence of AKI (Martensson *et al*., 2010). Further research shows that NGAL does not seem to be effective as an early marker in critically ill patients suffering from contrast-induced AKI (Valette *et al*., 2013).

Urinary NGAL was once considered to be a reliable parameter for detecting AKI with sepsis (Bagshaw *et al*., 2010) but research conducted on 100 patients with systemic inflammation and the lowest stage of AKI found a lack of specificity for AKI prediction and a very weak link with RRT requirement (Glassford *et al*., 2013, Citerio *et al*., 2013). Urinary NGAL monomeric form is derived from neutrophils and renal tubular cells whilst NGAL homodimer is generated only by neutrophils whilst NGAL heterodimer is produced in the renal tubular cells only. Unfortunately there is currently no test available to evaluate heterodimer NGAL thereby limiting its diagnostic potential (Glassford *et al*., 2013, Citerio *et al*., 2013).

KIM-1 is a phosphatidylserine receptor in the proximal tubule that recognizes and directs apoptotic cells to lysosomes in proximal tubular cells. Furthermore, in the proximal tubular cells KIM-1 mediates the phagocytosis of necrotic cells and oxidized

lipoproteins (Bonventre and Yang, 2011). Recent studies demonstrate that KIM-1 facilitates the clearance of apoptotic debris from the tubular lumen and may play an important role in limiting the immune reaction by phagocytosis of apoptotic bodies. This might be a mechanism for limiting the pro-inflammatory response (Ichimura *et al*., 2008, Citerio *et al*., 2013, Bonventre and Yang, 2011). Research suggest that the ectodomain of KIM-1 can shed into the urine of human and rodent kidneys with renal injury and can serves as a biomarker for the early diagnosis of AKI in humans and rodents (Bonventre and Yang, 2002, Bonventre and Yang, 2011). However, despite all these new biomarkers currently under study for the early detection of AKI, serum creatinine is still considered clinically as a biomarker for AKI diagnosis (Praught and Shlipak, 2005, Khwaja, 2012).

The management of AKI is dependent on the identification and treatment of the underlying cause. This routinely includes the avoidance of substances that are toxic to the kidneys known as nephrotoxins. These include non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen, iodinated contrast media and antibiotics such as gentamicin. Although there is a significant amount of experimental research carried out on AKI with drugs like dexamethasone (Kumar *et al*., 2009), furosemide (Cantarovich *et al*., 2004), low dose dopamine (Bellomo *et al*., 2000), theophylline (Ix *et al*., 2004), erythropoietin (Patel *et al.*, 2004), haemoxygenase inducer (Takahashi *et al.*, 2004, Sikorski *et al*., 2004), hepatocyte growth factor (Nagano *et al.*, 2004) and pyrrolidine dithiocarbamate (Chatterjee *et al.*, 2003a) but none is documented as being an effective treatment for AKI. Stem cell therapy is a novel approach and research indicates that bone marrow stem cells have the ability to repair ischaemic renal tubular injury but the

clinical implementation of this procedure will take time (Kale *et al.*, 2003).

Briefly, there is an urgent need for novel specific strategies with respect to prevention, early identification and treatment of established AKI in order to significantly improve the dismal outcome of this clinical syndrome.

1.2: Renal ischaemia reperfusion injury:

1.2.1 Pathophysiology and basic mechanism

One of the leading causes of AKI is renal ischaemia reperfusion injury (IRI). Renal IRI is caused by any impairment in the delivery of oxygen and nutrient to the kidney and any impairment in the removal of waste product from the kidney. Evidence suggests that in renal IRI there is a severe reduction in oxygen supply to tissue compared to its demand and accumulation of waste product of metabolism. Due to this imbalance the tubular epithelial cells progress to apoptosis and necrosis with organ functioning impairment of water and electrolytes balance and reduced excretion of waste products of metabolism. There are many known pathophysiological mechanisms that contribute to renal IRI (Bonventre and Yang, 2011).

Normally, when there is a fall in renal perfusion pressure the kidneys maintain a relatively constant normal blood flow and GFR, even when the mean arterial pressure is as low as 80 mmHg. This property of the kidneys is known as autoregulation. This is achieved by the production of prostaglandins which cause a decrease in afferent arteriolar resistance and angiotensin II leading to an increase in efferent glomerular

arteriolar resistance (Abuelo, 2007). However, when renal perfusion pressure is very low, autoregulation is lost and afferent arteriolar constricts due to vasoconstrictors which leads to a significant decrease in renal blood flow and GFR, causing pre-renal azotaemia. This results in a decrease in blood flow and perfusion pressure in the post-glomerular capillary bed which perfuses the renal tubules. With increasing severity and duration ischaemia causes structural and functional tubular injury and alteration in the microcirculation leading to the clinical syndrome of AKI. Co-morbid conditions such as hypertension, diabetes mellitus, CKD, atherosclerosis and NSAIDS cause an impaired afferent arteriolar vasodilatory response. Sepsis, hypercalcaemia, radiocontrast agent, calcineurin inhibitors can cause afferent arteriolar vasoconstriction as a primary response and angiotensin converting enzyme or angiotensin receptor blockers are associated with inability to increase efferent arteriolar resistance. All of the above mentioned conditions are associated with increased risk of AKI, even with moderate hypoperfusion (Abuelo, 2007).

During renal ischaemia there is tubular dysfunction and impaired salt reabsorption (Kwon *et al*., 1999, Abuelo, 2007) that leads to an increase in sodium delivery which enhances glomerular vasoconstriction and decreases glomerular filtration rate (GFR) through tubuloglomerular feedback (TGF) (Brezis and Rosen, 1995). When there is increase of distal delivery of solutes to the macula densa it results in feedback signals to the glomerulus to decrease GFR by afferent arteriole constriction. After renal ischaemic injury, tubular epithelial cell reabsorption of sodium is impaired, which results in increased distal delivery of NaCl and subsequent activation of TGF. This decreased renal blood flow and GFR result in oliguria and, in severe cases, anuria (Srichai *et al.*,

2008).

The kidneys receive 25% of the cardiac output, the outer medullary region of the kidney is very vulnerable because it receives <10% of the blood delivered to the kidney via the vasa recta; the majority of that flow being directed to the renal cortex. The partial pressure of oxygen in the renal cortex is about 50 mmHg whilst in contrast the outer medulla has a partial pressure of oxygen in the 10 to 20 mmHg range (Brezis and Rosen, 1995). Recent evidence suggests that the enhanced vasoconstriction that occurs with small vessel occlusion is due to endothelial-leukocyte interactions caused by enhanced expression of ICAM-1 on damaged endothelial cells and their counter receptors on leucocytes and the activation of the coagulation system. This leads to a local compromise of the microcirculation and regional ischaemia, especially in the outer medulla. Furthermore, arteriolar vasoconstriction reduces the tubular blood flow to the outer medulla and is reduced further by local oedema (Bonventre and Yang, 2011). During ischaemic AKI, there is tubular necrosis in the outer stripe of the outer medulla (the straight portion of proximal tubules) and marked congestion in the inner stripe of the outer medulla (Bonventre, 1993). Furthermore the outer medullary region contains the S3 segment of proximal tubules and the medullary thick ascending limb of the loop of Henlé (TAL) and both of these are sensitive to hypoxia, but their role in renal IRI induced renal is unclear.

Some data (Srichai *et al.*, 2008) suggest that TAL epithelial cells had a five-fold higher blood urea nitrogen and creatinine levels upon induction of apoptosis in genetically engineered mice that received targeted injury. This study identifies that there is loss of

urine concentrating ability in these animals that resemble clinical AKI. Furthermore, sodium is reabsorbed in the TAL, which plays an important role in the kidneys capacity to concentrate urine and is necessary for maintaining the counter-current exchange mechanism by generating a hypertonic medullary interstitial. This medullary concentration gradient is lost with the loss of Na-K-2Cl co-transport in the TAL and ultimately leads to an inability to concentrate urine even in the presence of vasopressin (Srichai *et al*., 2008). Renal IRI can be seen to mimic clinical AKI with effects on renal tubular cells, endothelial cells and haematological cells (Friedewald and Rabb, 2004).

During ischaemia, there is reduction or cessation of the blood supply to the kidney that causes hypoxia or anoxia. This insult leads to a massive depletion of adenosine triphosphate (ATP) and causes the release of catecholamines such as adrenaline, noradrenaline and dopamine which enhance contractility to thereby increase the blood flow to the tissue at risk. This leads to intracellular accumulation of ions such as Ca^{2+}, Na^{+} and Cl^{-} with an influx of water that causes the swelling of cells, particularly in the endothelial cells. Simultaneously, there is loss of fluid from the intravascular space resulting in increased blood viscosity which is further enhanced by the leukocytes and all these factors may interfere with the restoration of the microcirculation during reperfusion leading to capillary obstruction (no-reflow phenomenon) (Ames *et al*., 1968, Hakan Parlakpinar, 2013). ATP depletion during ischaemia stimulates many critical alterations in structure, metabolism and tubular dynamics which can cause apoptotic or necrotic cell death (Wiegele *et al*., 1998). These include depletion of cellular energy stores, disruption of the actin cytoskeleton, accumulation of intracellular calcium, accumulation of hypoxanthine, and generation of reactive oxygen species (ROS).

Ischaemic AKI can be sub-lethal resulting in tubular dysfunction, or lethal, resulting in cell death (Edelstein *et al*., 1997). Furthermore, like tubular cells, endothelial cells may also experience similar consequences leading to cellular malformations and endothelial-erythrocyte interactions which enhance erythrocyte sludging in the ascending vasa recta (Friedewald and Rabb, 2004). All of this results in a massive reduction in substrate and oxygen delivery (Friedewald and Rabb, 2004, Mason *et al*., 1989). In addition, there is endothelial dysfunction and recruitment of inflammatory cells (see section 1.3) during ischaemia induced microcirculatory alterations and there is upregulation of intracellular adhesion molecules-1 (ICAM-1) during reperfusion. Reperfusion is essential for the survival of ischaemic tissue as it removes toxic metabolites and restores energy. However, in the last few decades studies have shown that tissue damage is further enhanced by reperfusion (Lieberthal and Levine, 1996, Thadhani *et al*., 1996, Weight *et al.*, 1996) and this phenomenon is known as reperfusion injury or the reflow paradox. This is associated with release of pro-inflammatory cytokine (TNF-α, IL-1β and generation of ROS), the upregulation of adhesion molecules (selectins, ICAM-1, β-integrins) and adhesion of leucocytes to endothelial lining of microvasculature (Menger *et al*., 1999, Hakan Parlakpinar, 2013).

1.2.2: Inflammation during renal IRI

Inflammation plays a major role in the pathophysiology of AKI (Bonventre and Zuk, 2004, Friedewald and Rabb, 2004). During experimental renal ischaemia, ischaemic injury results in functional and structural changes to the vascular endothelium and tubular epithelium. This leads to the infiltration of leukocytes, neutrophil, macrophages,

natural killer cell and lymphocytes into the damaged kidney. The injury forces tubular and endothelial cells to release inflammatory cytokines and chemokines which may enhance inflammation or decrease it. Adherent and infiltrating leucocytes are responsible for the tubular and epithelial injury which can be caused due to release of oxygen radicals and vasoconstrictors. Furthermore, endothelin release and decrease in NO contributes to enhance endothelial injury during inflammation (Akcay *et al*., 2009).

There is evidence that suggests that the vascular endothelium is involved in the recruitment and migration of circulating inflammatory cells into the sites of inflammation (Umehara *et al*., 2001). Furthermore, anti-inflammatory therapies such as mycophenolate and alpha-melanocyte stimulating hormone (*α*-MSH, a known inhibitor of IL-8 and ICAM-1 induction) have protective effects that target the pro-inflammatory pathways that participate in the pathogenesis of AKI (Yalavarthy and Edelstein, 2008).

During AKI, the renal tubular epithelium is a major site of cell injury and death. Many studies have demonstrated that renal tubular epithelial cells play a pro-inflammatory role in AKI. Interferon regulatory factor-1 (IRF-1) is a transcription factor which can activate pro-inflammatory genes such as interferons and chemokines. It is stimulated by ROS during ischaemic injury both *in vitro* and *in vivo* and is produced within the S3 region of proximal tubule cells (Akcay *et al*., 2009). Research conducted on transgenic knockouts of IRF-1 shows that the animals can attenuate the renal dysfunction, injury, and inflammation after acute ischaemia (Wang *et al*., 2009). Furthermore, the Rho kinase pathway can play a crucial role in the dedifferentiation of epithelial cells and the infiltration of inflammatory cells. Further experimental data provide evidence that

inhibition of Rho kinase with a renally targeted Y27632-lysozyme conjugate attenuates fibrogenesis, tubular damage and inflammation caused by IRI by attenuating KIM-1 and E- cathrin (Prakash *et al*., 2008, Akcay *et al*., 2009).

It is recognized that cytokines are released by leukocytes and renal tubular cells into the injured kidney and play an important role in the initiation and extension of inflammation in AKI (Akcay *et al*., 2009). The pro-inflammatory cytokines/chemokines interferon- *γ* (IFN-*γ)*, interleukin-2, -6, -10 (IL-2, -6, -10), granulocyte-macrophage colony-stimulating factor (GM-CSF), transforming growth factor- β (TGF-β), chemokine ligand-1 (CXCL1), monocyte chemotactic protein-1 (MCP-1) and macrophage inflammatory protein-2 (MIP-2) are increased in the kidney in ischaemic AKI (Rice et al., 2002, Albelda et al., 1994, Goes et al., 1995, Akcay et al., 2009). During inflammation, adhesion molecules are required for leukocyte adhesion to endothelial cells and leads to inflammation and an extension of the cellular injury (Fuggle and Koo, 1998). As mentioned above (1.2.1 Pathophysiology and basic mechanism), ICAM-1 plays an important role in the pathophysiology of AKI (Molitoris and Marrs, 1999, Kelly *et al*., 1994) with both the administration of a monoclonal ICAM-1 antibody into wild-type mice and ICAM-1 deficient mice being protected against renal ischaemic injury (Kelly *et al*., 1994, Kelly *et al*., 1996).

Literature suggests that selectins and their ligands adhesion molecules that can play a significant role in the inflammatory response. P-selectin molecules are expressed as a response to the inflammatory stimulus in platelets and endothelial cells, L-selectin molecules are expressed in leukocytes and lymphocytes, and E-selectin is expressed in the endothelium (Akcay *et al*., 2009). Renal ischaemia can cause upregulation of

endothelial P-selectin but not L-selectin, with enhanced adhesion of neutrophils (Singbartl *et al.*, 2001). CD147 (also known as Basigin or Bsg) is a membrane glycoprotein that belongs to the immunoglobulin superfamily and is a ligand for E-selectin (Akcay *et al*., 2009) and is thought responsible for neutrophil recruitment in renal IRI (Kato *et al.*, 2009). Research suggests that CD147-deficient mice when compared with wild-type mice have significant suppression of neutrophil infiltration in the kidney after renal IRI (Kato *et al.*, 2009).

Toll like receptors (TLRs) are a family of transmembrane receptors that are widely expressed on leukocytes and kidney epithelial cells and regulate innate and adaptive immune responses (Akcay *et al*., 2009). Renal proximal tubular epithelial cells (PTECs) express TLR-2 and -4. Data from experimental endotoxemia, ischaemic and nephrotoxic AKI suggest that the expression of TLR-2 and TLR-4 was reported to increase in these models (Arumugam *et al*., 2009, Akcay *et al*., 2009). Furthermore, that the inhibition of TLR-2,-4 and -9 is protective against the renal dysfunction caused by renal IRI (Shigeoka *et al*., 2007, Pulskens *et al*., 2008, Cunningham *et al*., 2004, Yasuda *et al*., 2008).

During inflammation, the first step which leads to the movement of these cells from capillaries to damage and injured tissue is the neutrophils adherence to the vascular endothelium. Studies show that infiltrating neutrophils can release ROS after adherence and chemotaxis that can cause damage to the tubular cells (Linas *et al*., 1988, Akcay *et al*., 2009). Furthermore, many studies indicate that macrophages also contribute in inflammation by generating pro-inflammatory chemokines and cytokines such as IL-18

(Liew and McInnes, 2002, Kanai et al., 2001), IL-1*α* (Zaldivar *et al*., 2002), TNF-α, IL-1β, MCP-1, macrophage migration inhibitory factor (MIF) and NK-κB (Nikolic-Paterson and Atkins, 2001). Resident dendritic cells (DCs) form a contiguous network throughout the kidney but their role in AKI remains unclear. Following renal IRI, resident DCs release similar cytokines and chemokines as macrophages (Akcay *et al.*, 2009).

1.2.3: Oxidative stress during renal IRI

Oxidative stress plays a crucial role in the pathogenesis of many clinical diseases (Cross *et al*., 1987, Halliwell *et al.*, 1992) such as cardiovascular diseases (McCord, 1985, Jeroudi *et al.*, 1994), liver diseases (Comporti, 1985, Poli *et al*., 1987), lung disease (Kalra *et al.*, 1991, Ryrfeldt *et al.*, 1993), gastrointestinal disorders (Otamiri and Sjodahl, 1991, Das and Banerjee, 1993), and AKI (Aragno *et al*., 2003). Oxidative stress can be defined as the tissue damage resulting from an imbalance between an excessive generation of oxidant compounds and insufficient antioxidant defence mechanisms (Kao *et al*., 2010). The generation of oxidative compounds plays a physiologically important part of defence mechanism against invading micro-organisms and malignant cells. It also plays a crucial role in tissue repair, healing and remodelling (Locatelli *et al.*, 2003).

Oxidative compounds such as ROS and reactive nitrogen species have unpaired valence shell electrons and are therefore unstable in nature and interact readily with adjacent molecules. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in particular has a major role in generation of ROS. ROS can be also generated by vascular and non-vascular enzymes (Kao *et al*., 2010). Evidence shows that xanthine

oxidase, lipoxygenase, uncoupled NOS and an impaired mitochondrial respiratory chain can all produce oxidative stress (Stocker and Keaney, 2004, Laursen *et al*., 2001). It is thought that the generation of a single ROS leads to the generation of several others through radical chain reactions. ROS is generated through a univalent reduction of molecular oxygen (O_2)(Kao *et al*., 2010).

Polymorphonuclear neutrophils and monocyte-macrophages are activated after exposure to a stimuli and as a result, increase their O_2 consumption ('respiratory burst') (Locatelli *et al*., 2003). There is then a series of reactions. Firstly, O_2 will be reduced to superoxide anion ($O_2^{\bullet-}$) by the NADPH-oxidase enzyme system. Then $O_2^{\bullet-}$ either by dismutation of $O_2^{\bullet-}$ or by direct reduction of O_2 will be converted into H_2O_2, which is highly unstable. Both $O_2^{\bullet-}$ and H_2O_2 can play important role in the generation of more powerful oxidants. Increased production of all these oxidants inactivates nitric oxide (NO), resulting in reduced bioavailability of NO. These oxidants also lead to the formation of peroxynitrite ($ONOO^-$), which is cytotoxic and is responsible for both increased platelet aggregation and vasoconstriction. $O_2^{\bullet-}$ interacts with NO to form highly toxic nitrogen derivatives whereas H_2O_2 reacts with intracellular iron to form hydroxyl radicals (OH•) (Descamps-Latscha *et al*., 2001, Kao *et al*., 2010). These hydroxyl radicals then exert a pivotal effect in the degradation process of the cell membrane lipid, protein aggregation and DNA damage. H_2O_2 is also the substrate for myeloperoxidase (MPO) which produces chlorinated oxidants such as hypochlorous acid (HOCL), a potent oxidant, especially in molecules such as lipids, proteoglycans and other intracellular components (Kao *et al*., 2010). Recent studies have shown that oxidative stress and subclinical inflammation co-exist together. When released together

with pro-inflammatory cytokines, angiotensin II and advanced glycation end products (AGE), ROS release augments the whole ROS generation process (Davi *et al*., 2002, Kao *et al*., 2010).

As free radicals have a very short half-life it is very difficult to evaluate oxidative stress in the clinical setting. Therefore oxidative stress is often determined by studying more stable marker molecules that have longer half-lives, from hours to weeks. These markers include malonyldialdehyde, advanced oxidation protein products, 4-hydroxynonenal (HNE), advanced glycosylation end-products (AGE), 8-hydroxy-2′-deoxyguanosine and dimethylarginine (Kao *et al*., 2010).

1.2.4: Apoptosis and necrosis

During ischaemic AKI, renal tubular cells death is mediated by both apoptosis and necrosis, but the exact pathway is still unclear. Although, there is evidence of apoptosis occurs when ATP concentrations are reduced, it is known that prolonged ATP depletion leads to necrosis. Furthermore, apoptotic cells that are not removed from the site of injury may undergo a 'secondary necrosis'.

Necrosis was previously thought to be an uncontrolled cell death but evidence indicates that it can also occur via a transduction pathway and execution mechanisms. It can be morphologically characterised by cytoplasmic swelling and dilated organelle (endoplasmic reticulum, golgi apparatus and mitochondria), absence of chromatic condensation followed by the loss of cell membrane integrity and release of the cellular contents into the surrounding extracellular space (Kroemer *et al*., 2005, Vanden Berghe *et al*., 2010). This rapid metabolic collapse leads to early loss of plasma membrane integrity and the release of cytosolic contents including proteins such as lactate dehydrogenase (LDH) (Lieberthal *et al*., 1998a). LDH is an enzyme which catalyzes the conversion of lactate to pyruvate at a rate determined by positive feedback inhibition and is retained in the cytosol unless the cell membrane is compromised. Necrotic injury is often evaluated by quantifying the amount of LDH released into blood plasma (Breggia and Himmelfarb, 2008). Recent studies have focused on the fact that necrosis can occur through a mechanism called necroptosis or programmed necrosis (Degterev *et al*., 2005). This mechanism is activated upon stimulation by TNF-α, FasL, and TRAIL, the same ligands that activate apoptosis. Receptor interacting protein 1(RIP1) plays an

important role in necroptosis. RIP1 serine/threonine kinase activity is essential for necrotic death pathway but is not essential for NF-kB activation and apoptosis (Holler *et al*., 2000, Christofferson and Yuan, 2010). Another member of RIP1 family, RIP3, has been identified to play a crucial role in necroptosis (Cho *et al.*, 2009, He *et al.*, 2009). RIP3 expression sensitizes necroptosis insensitive cell lines to necroptosis. RIP1 and 3 interact with their RIP homotypic interaction motif (RHIM) domains (Sun *et al*., 2002). RIP1 and 3 are 33% similar in kinase domain but inhibitor of necroptosis called as necrostatin-1(Nec-1) inhibits RIP1 only (Cho et al., 2009, Degterev et al., 2008). During death receptor induced apoptosis, RIP1 and RIP3 are cleaved by caspase-8, which suppress their anti-apoptotic and/or pro-necrotic properties (Lin *et al*., 1999, Feng *et al.*, 2007). Pathogen recognition receptors (PRRs) such as TLRs, the cytosolic NOD- like receptors (NLRs) and the RIG-I-like receptors (RLRs) can lead to necrotic cell death (Kalai *et al*., 2002, Ma *et al*., 2005). Extensive DNA damage causes hyperactivation of poly-(ADP-ribose) polymerase-1 (PARP-1) and causes necrosis (Jagtap and Szabo, 2005). When there is moderate DNA damage PARP-1 repairs DNA but excessive PARP-1 activation causes depletion of NAD^+ by catalyzing the hydrolysis of NAD^+ into nicotinamide and poly(ADP ribose) (PAR), ultimately causing ATP depletion with permanent cellular energy failure and necrotic cell death. PARP-1 is mediated by activation of RIP1 and TNF receptor-associated factor-2 (TRAF2) (Xu *et al*., 2006). In execution of necrotic cell death many mediators are involved such as ROS, calcium (Ca^{2+}), calpains, cathepsins, phospholipases and ceramide (Vanlangenakker *et al*., 2008, Duprez *et al*., 2009).

Apoptosis can be marked by the reduction of cellular volume (pyknosis), rounding-up of

the cell, chromatin condensation, retraction of pseudopodes, nuclear fragmentation (karyorrhexis), with no or little ultrastructural modifications of cytoplasmic organelles, plasma membrane blebbing (but maintenance of its integrity until the final stages of the process) and engulfment by resident phagocytes' (Kroemer *et al*., 2005, Kroemer *et al*., 2009). In apoptosis two main protein families are involved, the Bcl-2 family of protein, which control mitochondrial integrity and the cysteinyl aspartate-specific proteases or caspases, which mediate the execution phase of apoptosis (Youle and Strasser, 2008).

At least 18 mammalian caspases have been identified and many of these can be split into apoptotic and inflammatory subfamilies of caspases. Apoptotic caspases can be further divided into initiator caspases: Caspase -2,-8,-9 and -10 and the executioner or effector caspases: caspase -3,-6 and -7 (Riedl and Shi, 2004). Caspases are cysteine proteases that can cleave after aspartate (Asp) residues in their substrates and irreversibly causes cell death (Chang and Yang, 2000). Caspases are inactive proenzymes and consist of an N-terminal prodomain of variable length, a large subunit (p20) and a small C- terminal subunit (p10). The long prodomain of initiator caspases contain protein: protein interaction motifs belonging to death domain superfamily, namely death effector domains (DEDs) and caspase activation and recruitment domains (CARDs)(Chang and Yang, 2000, Duprez *et al*., 2009).

Caspases undergo proteolytic activation by pro-apoptotic stimuli that cause the auto activation of initiator caspases. Initiator caspases then triggers a self-amplifying cascade of downstream caspase activation (Riedl and Shi, 2004, Nicholson et al., 1995, Thornberry and Lazebnik, 1998) leading to the activation of caspase-3. Activation of

caspase-3 leads to cell death by proteolytic cleavage of broad spectrum of cellular target proteins (Chang and Yang, 2000). In mammalian cells, caspases can be activated by the extrinsic or intrinsic apoptotic pathway.

The extrinsic pathway is induced by stimulation of death receptors belonging to TNFR family, such as TNFR, Fas and TRAIL-R. Death stimuli originated from outside of cell binds to an extracellular death ligand such as FasL, to its cell surface receptor Fas (Ashkenazi and Dixit, 1999). Cytosolic factors FADD and caspase-8 binds to these and form a complex known as death inducing signaling complex (DISC) which causes activation of caspase-8 (Thorburn, 2004) which leads to the activation of the executionary caspases as mentioned above and results in cell death.

The intrinsic pathway is activated by various stimuli, such as DNA damage and cytotoxic insults and is mediated through the mitochondria. Proteins like cytochrome c, SMAC/DIABLO (second mitochondrial derived activator of caspases/direct inhibitor of apoptosis binding protein with low pI) and AIF (apoptosis inducing factor) are released from the inter-membrane space of mitochondria due to specific apoptotic stimuli and they then activate the intrinsic pathway. Cytochrome c binds and activates APAF1 (apoptosis protease activating factor-1) which in turn binds to ATP/dATP leading to activation of caspase-9 (Green and Kroemer, 2004, Sanz *et al.*, 2008). Caspase-9 then activates executioner caspases as mentioned above and causes cell death.

Research shows that mitochondrial changes can be prevented in cells by anti-apoptotic members of the Bcl-2 family of proteins (Kaushal *et al*., 2004). Furthermore, there is

evidence that the Bcl-2 family may regulate activation of caspases through control of cytochrome c release from the mitochondria and by directly binding to APAF1, thus preventing the activation of pro-caspase-9 and subsequently caspase-3 (Gross *et al*., 1999).

The enzymatic activity of caspases is subject to inhibition by the conserved IAP (inhibitor of apoptosis) family of proteins (Deveraux and Reed, 1999, Hay, 2000). Literature suggest that there are eight distinct IAPs, such as XIAP, ML-IAP/Livin, c-IAP1 and c-IAP2 (Ashhab et al., 2001, Kasof and Gomes, 2001, Vucic et al., 2000) have been identified in mammals, and they can target caspase-9 (initiator caspase), and they can also target caspase-3 and caspase-7 (the effector caspases). These IAP proteins do not inhibit other caspases, such as caspase-6 or -8 (Shi, 2002). Furthermore, release of pro-apoptotic proteins from mitochondria such as Smac/Diablo antagonizes the action of IAPS such as XIAP, cIAP1 and 2 (LaCasse *et al.*, 2008).

During AKI in clinical and animal models, apoptosis is the main cause of tubular cell death (Basile et al., 1997, Burns et al., 1998, Kishino et al., 2004, Iwata et al., 1994) and evidence from ischaemic AKI studies suggest that mitochondrial fragmentation contributes to both mitochondrial damage and apoptosis (Brooks *et al*., 2009)There is an urgent need of therapies which can prevent apoptosis and necrosis in the setting of AKI.

1.3: Nitrite and its role in ischaemic conditions:

Nitrite (NO_2^-) is naturally occurring component and is an oxidation product that has been shown to serve as an acute marker of NO flux/formation. Initially NO_2^- was considered as being physiologically inert but it is now accepted that NO_2^- represents a major storage form of NO in the blood and tissues which can be converted to NO and other reactive nitrogen species during hypoxia to maintain physiological signalling. Most of NO_2^- is derived from the oxidation of NO Synthase (NOS)-generated NO. This is a one electron auto-oxidation of NO and is formed relatively slowly from NO_2^- when compared to the two electron oxidation of NO to nitrate (NO_3^-) by haem proteins in the blood and tissue. NO_2^- formation is catalyzed by the multicopper oxidase ceruloplasmin in the plasma or by cytochrome c oxidase (COX) in tissues (Shiva, 2013).. NO_2^- is also derived from reduction of salivary nitrate (NO_3^-) by commensal bacteria in the mouth and gastrointestinal tract and 30 % NO_2^- is derived from dietary sources like meat, vegetables and drinking water (Bryan *et al.*, 2007, Shiva, 2013). NO_2^- concentration in the plasma is very low at around 300-330 nmol/L in humans, 150-250 nmol/L in rats and 400-500 nmol/L in mice (Kleinbongard *et al*., 2003). In tissues, NO_2^- concentration is around 0.5 μmol/L to 20 μmol/L in rats (Bryan *et al*., 2004).

In ischaemic conditions there is a lack of NO because NOS is unable to produce NO without oxygen being present and oxygen is deficient in ischaemia (Samouilov *et al*., 1998). However, NO can be generated in ischaemic conditions but NOS inhibition does not affect it thereby indicating that there is an enzyme independent pathway of NO generation. NO might be formed by reduction of NO_2^- to NO during ischaemia or

hypoxia by acidic disproportion, xanthine oxidoreductase (XOR) and deoxyhaemoglobulin (Zweier *et al*., 1995, Tripatara *et al*., 2007). Thus, NO_2^- is an oxidative metabolite derived from NO and it can also be a source of NO generation in ischaemic conditions. NO has one unpaired electron and is a free radical species and can react with other free radicals like $O_2^{\bullet-}$. Reaction with $O_2^{\bullet-}$ can form $ONOO^-$, which is cytotoxic (Beckman *et al.*, 1990). NO rapidly forms nitrogen dioxide (NO_2) in air. NO_2 is a brown gas that can cause tissue damage (Moncada and Higgs, 1993) and reacts to forms dinitrogen, trinitrogen or tetraoxide, these react with water to give NO_2^- and NO_3^- (Ignarro *et al*., 1998).

Research now shows that NO_2^- can attenuate the formation of ROS by inhibiting mitochondrial complex-1 by S nitrosation during IRI and reduces damage associated with it. In IRI there is depletion of ATP formation, reduction in energy stores and the influx of oxygen during reperfusion causes ROS generation at complex I and III. Furthermore there is oxidation of proteins causing opening of mitochondrial permeablility transition pore and the release of cytochrome c to cause apoptosis. These events can be attenuated due to NO_2^- dependent S nitrosation of complex 1 which decreases cytochrome c release and inhibits opening of the permeability transition pore (PTP) ultimately reducing apoptosis and IRI (Shiva, 2013).

As NO_2^- mediated beneficial effects are dependent on NO it is necessary to understand how NO is functioning in physiological and pathological conditions. Literature suggest that NO is lipid soluble and there is a high concentration of NO at the cellular level (Ignarro *et al*. 1998). NO plays important physiological role in neurotransmission,

hormone secretion, immunity and inflammation (Kone, 1997, Bentz e*t al*., 2000). It is also involved in pathological conditions like septic shock, atherosclerosis, hypertension, carcinogenesis and IRI (Bentz *et al.*, 2000). NO plays an important role in kidney functions such as the regulation of glomerular and medullary haemodynamics, TGF response and Renin release by taking control of the extracellular fluid volume status (Kone, 1997). On the other hand, it can contribute in several common renal diseases like immune mediated glomerulo-nephritis, radio-contrast nephropathy, obstructive nephropathy, acute and chronic renal allograft and post-ischaemic renal failure (Kone, 1997).

NOS are enzymes which catalyse the oxidation in the L-arginine (L-arg) NO pathway (Beckman *et al*., 1990). There are three isoforms of NOS expressed in the kidney (a) neuronal NOS (nNOS or NOS-I) is found in epithelial cells of the macula densa (Mundel *et al*., 1992, Terada *et al*., 1992) and principal cells of collecting ducts (Wang *et al.*, 1998) (b) inducible NOS (iNOS or NOSII) is found in tubule epithelia including proximal tubule, TAL and distal convoluted tubule (Morrissey *et al*., 1994) and (c) endothelial NOS (eNOS or NOS-III) is expressed in renal vascular endothelial cells (Terada *et al*., 1992) whilst nNOS and eNOS are regulated by calcium and calmodulin (CaM) and iNOS is controlled by inflammatory stimuli such as lipo-polysacchride and cytokines but is not regulated by calcium (Xie *et al*., 1992, Forstermann *et al.*, 1994). Data suggest that human neutrophils also express iNOS (Cedergren *et al.*, 2003) whilst eNOS derived NO plays an important role in determining and maintaining normal renal function, such as proximal tubule function (Liang and Knox, 2000, Ortiz and Garvin, 2002) but that elevated levels cause proximal tubular injury (Liang and Knox, 2000).

During ischaemia eNOS activity is diminished because of its dependence on oxygen and the increasing acidosis (Giraldez *et al*., 1997). However, studies have recognised that there is NO generation from the endogenous NO_2^- pool during ischaemia in heart, liver and kidney, which is NOS independent (Zweier *et al.*, 1995, Webb *et al.*, 2004, Duranski *et al.*, 2005, Okamoto *et al.*, 2005). Recently, NO_2^- derived NO has been shown to play an important role in preventing myocardial infarction (MI) (Bryan *et al*., 2007) and renal ischaemia by XOR (Tripatara *et al*., 2007). However, as far as we are aware, no study has looked at the role of oral/dietary high NO_2^- in the setting of renal IRI.

1.4: Thiamine and benfotiamine:

Thiamine, also known as vitamin B_1 is a vitamin of the B complex which is considered to play an important role in energy metabolism. It is a colourless compound and its structure contains an aminopyrimidine and thiazole ring with methyl and hydroxyethyl side chains linked by a methylene bridge (Lonsdale, 2006). Thiamine is soluble in water, methanol, and glycerol but it is insoluble in ether, acetone, benzene and chloroform. It is stable in acidic pH, but unstable in alkaline solutions (Tanphaichitr V, 1999, Mahan LK, 2000). It has been suggested that the water soluble vitamins such as thiamine and folate are unrelated structurally and functionally but they may share properties that make them essential for normal cellular function, growth and development (Bukhari *et al*., 2010).

Thiamine is found in many foods such as cereal grain, oatmeal, brown rice, cauliflower

and potatoes. Daily requirement of thiamine as described by the recommended daily allowance (RDA) is 1 to 1.5 mg (Lonsdale, 2004, Lonsdale, 2006). Thiamine is stored in the body in the liver, skeletal muscle, kidney, brain and heart and the human body can store 25 to 30 mg of thiamine. Furthermore, phosphatase and pyrophosphatase enzymes can release thiamine in the upper portion of small intestine. This process is carrier mediated at lower concentrations whilst at higher concentrations absorption of thiamine occurs via passive diffusion. In the jejunum and ileum thiamine is absorbed by active transport which is reduced by folic acid deficiency and alcohol consumption (Mahan LK, 2000). A decline in thiamine absorption occurs at intakes above 5 mg. Intestinal mucosal cells have thiamine pyrophosphokinase activity, but it remains uncertain whether this enzyme is linked to active absorption.

Vitamin B1 is transported mainly in thiamine form and it turns into the phosphorylated active form after phosphorylation. There are 5 main phosphorylated forms of thiamine i.e. thiamine monophosphate, thiamine diphosphate (thiamine pyrophosphate, TPP), thiamine triphosphate, adenosine thiamine di and tri phosphates (Lonsdale, 2006). Thiamine present in the intestine is mostly in the pyrophosphorylated form TPP, but when thiamine arrives on the serosal side of the intestine it is often in the free form.

TPP is a coenzyme (a cofactor loosely bound to enzyme) for many cellular processes such as transketolase (TK), pyruvate dehydrogase (PDH) and α-ketoglutarate dehydogenase (α-KGDH). All of these enzymes play crucial role in energy metabolism. TK is a cytosolic enzyme which is associated with the biosynthesis of deoxyribose and oxyribose (sugars) in the pentose phosphate pathway. Furthermore, mitochondrial PDH

and α-KGDH enzymes play an important role in generating ATP. PDH links glycolysis to the citric acid cycle and α-KGDH is the rate limiting enzyme step. In the nervous system, PDH is involved in production of acetylcholine, which is a neurotransmitter and leads to formation of myelin (Butterworth, 1999, Bettendorff and Wins, 2009).

Thiamine in its active form of TPP is involved in many cellular processes and its deficiency causes fatal disease such as beri beri (polyneuritis) and cardiovascular diseases in humans (Lonsdale, 2006). One study has demonstrated that the treatment of diabetic rats with high doses of thiamine prevents diabetic retinopathy through the inhibition of AGE formation and other signalling or metabolic pathways (Hammes *et al*., 2003) and another that it can ameliorate cardiomyocyte contractile dysfunction (Ceylan-Isik *et al*., 2006). These effects of thiamine are due to its antioxidant properties (Hammes *et al.*, 2003). Furthermore, thiamine has an important role in preventing hypoxic cardiac cell death (Shin *et al*., 2004). As mentioned earlier (Bonventre, 1993) there is a massive depletion of ATP in IRI and thiamine is known to play an important role in generation of ATP (Nozaki *et al*., 2009). As inflammation, oxidative stress and ROS play important role in the pathogenesis of renal IRI and thiamine can attenuate these, there is an obvious potential for thiamine to play a very important role in the renal IRI settings.

Other thiamine derivatives have been identified such as allithiamine, sulbutiamine, prosultiamine, frusultiamine and the thiamine analog benfotiamine. These may possess better bioavailability as they are lipid soluble and therefore may cross cell membranes

easily in contrast to thiamine (Lonsdale, 2004, Volvert *et al*., 2008, Lonsdale, 2006). Benfotiamine (*S*-benzoylthiamine O-monophosphate) is a synthetic S-acyl derivative of thiamine and has an open thiazole ring compared to thiamine and it is this open thiazole ring that closes once the compound is absorbed, resulting in biological active thiamine. Furthermore, after oral administration benfotiamine is dephosphorylated to S-benzoylthiamine by the ecto-alkaline phosphatase present in the brush borders of intestinal mucosal cells. The lipid soluble S-benzoylthiamine is absorbed and then diffuses by passively through the membranes of intestinal and endothelial cells and subsequently appears in the circulation. Most of the S-benzoylthiamine is captured by erythrocytes and is converted to free thiamine. Thioesterases hydrolyse the remaining to thiamine and benzoic acid in the liver. Benfotiamine is supposed to have 5-10 times better bioavailability than thiamine at similar dose (Balakumar *et al*., 2010)

Benfotiamine should not be confused with a natural thiamine disulfide derivative called allithiamine which has a distinct pharmacological profile (Volvert *et al*., 2008). The primary use of this antioxidant is as an "anti-AGE" supplement. Research conducted with benfotiamine was found to lower AGE concentrations by 40% (Lin *et al*., 2000). At high doses, benfotiamine was shown to be an effective treatment for diabetic retinopathy, neuropathy and nephropathy. Supplementation of benfotiamine increases intracellular TPP levels (Balakumar *et al*., 2010), a cofactor of TK. This enzyme directs advanced glycation and lipoxidation end products (AGE's, ALE's) substrates to the pentose phosphate pathway, thus reducing tissue AGEs. Published data indicate that treatment with benfotiamine blocks three major pathways (the hexosamine pathway, the AGE product formation pathway and the diacylglycerol-protein kinase pathway) of

hyperglycaemic damage, probably by removal of glyceraldehyde 3-phosphate and fructose 6-phosphate through activation of the pentose phosphate enzyme transketolase (Hammes *et al*., 2003, Balakumar *et al*., 2010).

Benfotiamine has been shown to reduce glucose toxicity (Berrone *et al*., 2006, Marchetti *et al*., 2006) and it can attenuate diabetes-induced cerebral oxidative damage (Wu and Ren, 2006). It has been suggested that benfotimaine can be protective against injury caused by ischaemia in the diabetic limbs of mice (Gadau *et al*., 2006) and can also alleviate cardiomyocyte contractile dysfunction in experimental diabetes mellitus (Ceylan-Isik *et al.*, 2006). Several studies show that the administration of benfotiamine leads to higher thiamine blood levels than those achieved by the administration of water-soluble thiamine (Bitsch *et al*., 1991, Greb and Bitsch, 1998, Netzel *et al*., 2000).

As thiamine and benfotiamine have shown to have anti-oxidant, anti-inflammatory and anti apoptotic properties and as these mechanisms play an important role in pathogenesis of renal IR we herein investigated the role of thiamine and benfotiamine in the setting of renal IRI *in vivo* and PTECs apoptosis *in vitro.*

1.5: Bone morphogenic protein-7:

Bone morphogenic protein-7 (BMP-7) was previously known as osteogenic protein-1 (OP-1) and is a member of the transforming growth factor-β (TGF-β) superfamily that is expressed widely in embryonic life and plays a critical role in renal development (Godin *et al*., 1999). BMP-7 null mutation mice lack normal kidneys and the administration of BMP-7 can promote nephrogenesis in isolated metanephric mesenchyme (Vukicevic *et al.*, 1996). In the adult animal, BMP-7 expression subsides in other organs but remains high in the kidney BMP-7 RNA in the normal kidney is largely confined to the medullary tubules (tentatively identified as collecting tubules), peripheral glomerular cells (presumptive podocytes), and renal artery adventitial cells (Vukicevic *et al.*, 1998, Simon *et al*., 1999). Furthermore, BMP-7 physiological functions in adult kidney are not known but its role in renal pathophysiology has been studied in detail. In a renal IRI model in the rat, BMP-7 expression falls in the medulla and cortex (Simon *et al.*, 1999, Almanzar *et al.*, 1998). Studies have shown that BMP-7 exogenous systemic administration in conditions like ureteral ligation (Hruska *et al.*, 2000) and renal IRI (Vukicevic *et al.*, 1998) has been shown to ameliorate renal injury.

The TGF-β superfamily of proteins is the primary regulator of fibrosis whilst BMP-7 is thought to counteract its action. The TGF-β superfamily plays a role in a wide range of cellular processes. The ligands of it are regulated both outside and inside the cell. The list of ligands of TGF-β receptor include TGF-β1, β2, β3, activins, inhibins, BMPs and growth and differentiation factors (Shi and Massague, 2003, Miyazono *et al*., 2001, Patel and Dressler, 2005). These bind to receptor I and II on the cell surface. Type one

receptors are known as activin receptor like kinase or Alk of which there are 7 types. There are 5 type II receptors which are ligand specific (Samad *et al*., 2005). Upon binding of ligand, active type II activates the type I receptor by phophorylation and this causes phosphorylation of downstream signalling effectors called contraction of Sma and Mad (Mothers against decapentaplegic) known as Smad proteins (Patel and Dressler, 2005).

BMPs activate Smad1, 5 and 8 whereas activins and TGF-β activates Smad2 and 3. These Smads interact with common Smad4 and this ensuing complex translocates to the nucleus in order to regulate target gene transcription. Smad6 and 7 negatively regulate BMPs and TGF- β mediated signalling. Smad signalling is terminated by ubiquitin mediated degradation (Wrighton *et al.*, 2009, Patel and Dressler, 2005). Smad ubiquitin regulatory factor 1 (Smurf1) targets Smad1 and Smad5 for destruction (Zhu *et al.*, 1999). Smurf2 targets Smad2 (Lo and Massague, 1999) and SCF-Roc1 degrades Smad3 (Fukuchi *et al.*, 2001). Smurf1 with Smad7 targets TGF- β type 1 receptor for degradation (Ebisawa *et al.*, 2001) and Smurf1 and Smad6 targets BMP type 1 receptor for degradation (Murakami *et al.*, 2003). Inhibitors of BMP signalling include chordin, noggin, DAN (cerebrus), ectodin and gremlin (Garcia Abreu *et al.*, 2002, Wilkinson *et al.*, 2003, Balemans and Van Hul, 2002, Laurikkala *et al.,* 2003). BMP type 1 receptors Alk3 and 6 are expressed on the developing nephron, including the tip and branching ureter, mesenchymal cells and early epithelial derivatives of mesenchyme (Martinez et al., 2001, Patel and Dressler, 2005). Smad1 and 5 are important mediators of BMP-7 signalling in the adult kidney (Lin *et al.*, 2005, Wang and Hirschberg, 2004).

BMP-7 has anti-inflammatory and cytoprotective effects on PTECs (Gould *et al.*, 2002) and it is also able to prevent renal IRI (Vukicevic *et al.*, 1998). Studies with gene therapy techniques using electroporation (EP) are being considered for tissue regeneration and repair (Ferber, 2001, Nishikawa and Huang, 2001) and are easy to handle, safe and cost efficient. An examination of the use of non-viral gene therapy (EP) with BMP-7 and its role in renal IRI is presented in this thesis.

1.6: Aim and hypothesis:

The aim of this thesis was to prevent AKI using three distinct putative agents. Each treatment was to be assessed *in vitro* using a chemical model of ischaemia and *in vitro* using rodent renal IRI models.

1.6.1 Nitrite study

To investigate the possible beneficial role of oral NO_2^- supplementation on the degree of rat kidney injury found secondary to renal IRI and to assess whether NO_2^- can reduce apoptosis in PTECs.

1.6.2 Thiamine study

To investigate the possible beneficial role of oral thiamine supplementation upon rat kidney injury secondary to renal IRI, on PTECs and to see if thiamine and its lipid soluble analogue benfotiamine has anti-apoptotic, anti-oxidant and anti-inflammatory properties that can protect the rat kidney against renal IRI.

1.6.3 BMP-7 in renal IRI study

To investigate the possible role of non-viral gene therapy using hBMP-7 in the setting of renal IRI and to test if BMP-7 has anti-apoptotic properties that can prevent renal IRI and to further assess whether BMP-7 protein can reduce the degree of apoptosis in PTECs subjected to an *in vitro* model of IRI.

CHAPTER 2

EFFECTS OF LOW AND HIGH DIETARY NITRITE ON AKI

2.1: Introduction

Renal IRI is one of the principal causes of AKI (Liano and Pascual, 1998) and AKI remains a major cause of morbidity and mortality in hospitalized patients (Singri *et al.*, 2003). In addition, renal dysfunction develops in 5% of all general surgical patients and complicates the course of recovery in 15–25% of critically ill patients (Nash *et al*., 2002). Currently, the treatment of AKI is either symptomatic or dependent on RRT, so there is urgent need of new therapies to prevent AKI.

Evidence suggests that the topical application of NO_2^- directly onto the kidneys can prevent the damage and dysfunction caused by renal IRI (Tripatara *et al.*, 2007). Furthermore, NO_2^- has been shown to protect the liver and heart from damage caused by IRI (Duranski *et al*., 2005). Moreover, these studies demonstrate that the beneficial effects of NO_2^- are related to its reduction to NO under ischaemic conditions.

NOS enzyme play an important role in the generation of NO with three known isoforms with eNOS derived NO playing an important role in determining and maintaining aspects of normal renal function such as proximal tubule sodium reabsorption. However, elevated concentrations of NO can cause proximal tubule ischaemic injury (Liang and Knox, 2000). NO can therefore either attenuate or aggravate renal injury and this dual nature of NO depends on the site and the rate of NO production and the chemical fate of NO (Tripatara *et al*., 2007). Furthermore, there are multiple studies which indicate that iNOS inhibition or iNOS knockout mice can attenuate the proximal tubular injury caused by ischaemia, indicating that NO from iNOS contributes to renal injury (Chatterjee *et al.*, 2002, Chatterjee *et al.*, 2003b). Evidence also suggests that during

ischaemia or hypoxia, NO generated from sources other than NOS can play a vital role in mediating protective responses. Inorganic NO_2^- which can be produced biologically via oxidation of NO or by consumption via diet, either directly or indirectly after reduction of NO_3^- by the NO_3^- reductases present in oral bacteria, with this process being one of the key sources of NO(Shiva *et al*., 2007).

NO_2^- can be found circulating and as the tissue storage form of NO. NO_2^- is reduced to NO during hypoxia and acidosis by the enzymatic action of XOR and by haem proteins such as deoxyhaemoglobin, myoglobin, and tissue haem proteins (Basireddy *et al*., 2006, Tripatara *et al*., 2007).

As mentioned earlier, in an AKI setting the topical administration of NO_2^- upon the kidneys has been shown to be beneficial (Tripatara *et al*., 2007). Conversely the systemic i.v. administration of NO_2^- and NO_3^- has been shown to be detrimental (Basireddy *et al*., 2006) but there is evidence to suggest that oral NO_2^- (50 mg/L) given in the drinking water to mice for 7 days is able to prevent the myocardial damage caused by IRI (Bryan *et al.*, 2007) indicating that dietary high NO_2^- might play a crucial role in preventing renal IRI. As there is known to be a deficiency of NO during renal ischaemia we herein hypothesise that oral NO_2^- may prevent the damage and dysfunction caused by renal IRI in rats through NO and may also prevent apoptosis in PTECs subjected to an *in vitro* model of IRI (using immortalised human kidney cells, HK-2).

2.2. Materials and methods

2.2.1. Rat model of renal IRI

This procedure was done as described previously by workers in our lab (Kumar *et al*., 2009). Briefly, these studies were carried out on adult male Wistar rats (Charles River Ltd., Margate, UK) that weighed 250-400 g and received a standard diet and water *ad libitum*. The animals were cared for in accordance with the Home Office *Guidance on the Operation of the Animals (Scientific Procedures) Act 1986,* published by HMSO, London, UK. All rats were anesthetised with an i.p. injection of an anaesthetic cocktail consisting of ketamine and xylazine at a 2:1 ratio. Surgery was performed on a preheated mat to maintain body temperature at 37°C. Right and left kidneys were approached via lateral incisions on either flank respectively, and the renal pedicles were bilaterally clamped for 45 min with microaneurysm clamps followed by reperfusion for 24 h.

The time of ischaemia was chosen (45 min ischaemic phase) to obtain a reversible model of ischaemic AKI and to avoid animal mortality. The kidneys were observed for restoration of blood flow, as confirmed by returning to their original colour. Sham surgery consisted of the same surgical procedure except that clamps were not applied. Directly after closure of the abdomen all the rats were administered with 2 mL of normal saline (preheated to 37°C) i.p. At 24 h animals were sacrificed, blood samples were collected via a cardiac puncture and the kidneys were harvested. The kidneys were cut longitudinally into two halves and each half from respective kidneys were stored in 10% formalin for histological analysis or were immediately snap frozen in liquid N_2 for further experiments.

2.2.2 Assessment of renal function

Blood was collected into plain tubes and was allowed to clot. Centrifugation for 3 min at 9000 *g* yielded the serum. The veterinary clinical pathologists, based at VETLAB (www.vetlab.co.uk), a division of IDEXX Laboratories, Sussex, UK; blinded to the treatment given, performed serum creatinine measurements which was used as a biochemical measure of kidney injury (Kumar *et al*., 2009).

2.2.3 Experimental design

The rats were divided into the following groups: *IRI group:* (i) Control rats were given standard diet and normal drinking water for 7 days prior to ischaemia. *IRI high NO_2^- group* (ii) were given standard diet and $NaNO_2$ 50 mg/L in drinking water for 7 days prior to ischaemia. *IRI low dietary NO_2^- group* (iii) were given a low dietary NO_2^- chow for 7 days with normal drinking water. *Sham group* (iv) were given normal water and standard diet. All rats underwent the same surgical procedures except arterial clamps were not applied in the sham group.

2.2.4 Plasma NO_2^-/NO_3^- measurement by Griess assay

Plasma NO_2^- and NO_3^- concentrations were measured as an indicator of NO levels. NO_3^- in plasma was enzymatically converted to NO_2^- using a protocol similar to that described previously (Chatterjee *et al*., 2002). Briefly, NO_3^- was stoichiometrically reduced to NO_2^- by incubation of plasma (50 μL) for 15 min at 37°C in the presence of NO_3^- reductase (1 U/mL), β-NADPH (50 μmol/L) and FAD (50 μmol/L) in a final volume of 80 μL. When NO_3^- reduction was complete, the unused β-NADPH, which interferes with the

subsequent NO_2^- determination, was oxidised by L-lactate dehydrogenase (100 U/mL) and sodium pyruvate (100 mmol/L) to a final reduction volume of 100 μL and incubated for 5 min at 37°C. Subsequently, total NO_2^- in the medium was assayed by adding 100 μL of Griess reagent (Reagent A+B 1:1) to each sample. Optical density at 550 nm was measured using a spectrophotometer (Dynex Jencons PLS Technologies Microplate Reader). Total NO_2^- / NO_3^- concentration for each sample was calculated by comparison of a standard solution of sodium nitrite ($NaNO_2$) prepared in ultra pure water.

2.2.5 HK-2 Cells

The immortalised HK-2 cell line was originally established by transduction of a primary adult human PTEC line obtained from normal adult human renal cortex with human papilloma virus (HPV 16) E6/E7 genes (Ryan *et al.*, 1994). At the molecular level, the E6 and E7 gene products bind to DNA regulatory proteins, the result of which is to facilitate cell proliferation. However, these changes do not equate to malignant transformation because the features of differentiation of the host cells remain preserved (positive for alkaline phosphatase, gamma glutamyltranspeptidase, leucine aminopaptidase, acid phosphatase, cystokeratin, $\alpha_3\beta_1$ integrin and fibronectin) and their growth remains anchorage dependent and under the control of normal regulatory processes. They also retain functional characteristics of proximal tubular epithelium including sodium dependent/phlorizin sensitive sugar transport, adenylate cyclase responsiveness to parathyroid but not to anti-diuretic hormone. The cells are widely employed as a tool for studying injury and repair of the proximal tubular cell (Zager, 1999, Handa, 2001, Iwata *et al.*, 1995, Lee and Emala, 2002). Ham's nutrient mixtures were originally developed to support growth of several clones of Chinese hamster ovary

(CHO) cells, as well as clones of HeLa and mouse L-cells. Ham's F-12 DMEM has been used for the growth of primary rat hepatocytes and rat prostate epithelial cells. Ham's F-12 DMEM media (1:1) was used to grow and maintain HK-2 cell with 10 % fetal calf serum (FCS) and 1 % antibiotic in most of the work presented here unless specified. HK-2 cell cultures were re-constituted with fresh media every second day. Most experiments were performed on confluent HK-2 cells at passages 2 to 19 (after thawing frozen cell stocks of maximum 3-5 passage) that were cultured for 2 weeks.

2.2.6 In vitro ischaemia reperfusion injury model

A chemical model of IRI was induced *in vitro* according to the well-studied and extensively characterized model using a combination of antimycin A (a complex III inhibitor of mitochondrial electron transport) and 2-deoxyglucose (a non-metabolizable isomer of L-glucose) as described earlier (Kumar *et al*., 2009). Briefly, this is an *in vitro* model that chemically mimics renal IRI. HK-2 cells were seeded in a 6-well tissue culture plate and allowed to adhere for 20-24 h in an incubator at 37°C with 5% CO_2 in humidified air. After the above incubation period, cells were 70 – 80% confluent and the culture medium was replaced with Hank's Balanced Salt Solution (HBSS) (containing 1.3 mmol/L Ca^{2+} and 0.8 mmol/L Mg^{2+}) and were incubated with or without 50 μmol/L antimycin A plus 10 mmol/L 2-deoxyglucose for 1 h to induce ischaemic injury. The *in vitro* reperfusion was achieved by incubating cells in Ham's F-12 DMEM growth medium for 24 h. After 24 h cells were lifted by scrapping and centrifuged at 3000 *g* for 5 min. The supernatant was removed from the cells and the pellets were frozen at -20^0C. Pellets were then lysed with radio-immunoprecipitation assay (RIPA) buffer and centrifuged at 16000 *g.* Cell debris was then removed and further assays were

performed on the resulting cell lysates.

2.2.7 Cell viability assay

Cell viability was determined by using the Celltiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Southampton, UK) according to the manufacturer's instructions. This cellular reduction involves the MTS tetrazolium compound being converted to formazan by the NADH and NADPH generated by the metabolically active cells. Hence, the amount of formazan formed, as monitored by absorbance, directly correlates with the metabolic activity of the cells.

Briefly, 10,000 cells (in 100 μl) were seeded in a 96 well tissue culture plate and incubated at 37^{o}C in the cell culture incubator for 20-24 h and allowed to adhere and proliferate. The cells were treated according to the experiment protocol and 24 h later 20 μl of the yellow MTS solution was added into each well. The plate was incubated under the same conditions for up to 4 h and the absorbance was measured at 490 nm.

2.2.8 Serum starvation model

Serum Starvation was done as described previously (Kumar *et al*., 2009). Serum starvation can be used as an *in vitro* model to mimic ischaemia. In this study, HK-2 cells were seeded in a 6-well tissue culture plate and allowed to adhere for 20-24 h in an incubator at 37°C with 5% CO_2 in humidified air. After the above incubation period, when cells were 50 – 60% confluent the culture medium was replaced with fresh culture medium with 10% FCS for controls whilst for serum starved and treatment groups, no

FCS (with or without treatment) was provided for 24 h. After 24 h, cells were lifted by scrapping and centrifuged at 3000 *g* for 5 min. The supernatant was removed from the cells and the pellets were frozen at -20^{0}C. Pellets were then lysed with RIPA buffer, centrifuged at 16000 *g.* Cell debris was then removed and further assays were performed on the resulting cell lysates.

2.2.9 Assessment of apoptosis

2.2.9.1 Caspase activity

The activity of caspase-3 was measured using the fluorometric substrate N-Acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) and N-Acetyl-Asp-Glu-Val-Asp-al (Ac-DEVD-CHO) as inhibitor, as described previously by our laboratory (Allen *et al*., 2003, Kumar *et al*., 2009). The assay is based on the hydrolysis of Ac-DEVD-AMC by caspase-3, resulting in the release of the fluorescent 7-amino-4-methylcoumarin (AMC) moiety. The excitation and emission wavelengths used to determine AMC concentrations were 360 nm and 460 nm respectively.

Briefly, the assay protocol is as follows. 10μl of the samples (extracted protein for HK-2 cells or kidney homogenates prepared in RIPA lysis buffer 50 mmol/L Tris HCl, pH 7.5, 150 mmol/L NaCl, 50 mmol/L NaF, 0.5% deoxycholic acid, 1% NP-40, 1 mmol/L sodium orthovanadate, 0.1% SDS devoid of protease inhibitor cocktail) in duplicate were added to a black 96 well plate. 90 μl of the prepared assay buffer (caspase assay buffer contains,100 mmol/L HEPES buffer pH 7.5 containing 325 mmol/L sucrose,1.8 mmol/L CHAPS, dithiothreitol 11.1 mmol/L, and purified water in appropriate concentration) was

added to the above samples. 2 μl (50 μmol/L) of the diluted caspase inhibitor was added to half of the wells with vehicle added to paired samples followed by incubation at 37°C in the dark for 30 min. Subsequently, 2μl (50 μmol/L) of diluted substrate was added to all the wells followed by mixing on the shaker and incubating under the same conditions except for an hour instead of 30 min. The concentration of the AMC released can be calculated from the standard curve determined with defined AMC solutions from stock AMC. Fluorescence readings, obtained using a BMG Fluostar Galaxy plate reader (BMG Labtech Ltd, Aylesbury, Bucks, UK) from wells that contained inhibitor were subtracted from total fluorescence, and results were calculated as nmol AMC/min per mg protein. The activity of caspase-9 was determined in a similar manner using N-Acetyl-Val-Glu-His-Asp-7-amido-4-methylcoumarin (Ac-VEHD-AMC) as the substrate and Ac-VEHD-CHO as the inhibitor. The activity of caspase-8 was determined by using the Acetyl-Ile-Glu-Thr-Asp-7-amido-4-methylcoumarin (Ac-IETD-AMC) as a substrate and Acetyl-Ile-Glu-Thr-Asp-Aldehyde Inhibitor (Ac-IETD-CHO) as inhibitor.

2.2.10 In vitro reperfusion model

The H_2O_2 was utilised to induce injury as an *in vitro* model that could mimic reperfusion injury in animals. H_2O_2 was given to cells when confluent at a dose of (500 μmol/L) for 2-4 h. After 2-4 h cells were lifted by scrapping and centrifuged at 3000 *g* for 5 min. Supernatants were collected and used for cytotoxicity assay. Cytotoxicity was then assessed by a cytotoxicity assay (LDH assay).

2.2.11 LDH cytotoxicity assay

Lactate dehydrogenase (LDH) release into the supernatant was assayed using a

commercial colourimetric method (Cytotoxicity Detection Kit; Roche Diagnostics). Results were expressed as a percentage of the total cellular LDH measured from cells lysed in 1% Triton-X100 and corrected with appropriate media controls and total lysate protein.

2.2.12. Bicinchoninic (BCA) assay

The protein concentrations were measured using a BCA assay (Perbio Science, Cramlington, UK) following the manufacturer's instructions. The BCA Protein Assay combines the well-known reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{1+}) by bicinchoninic acid. In the first step, peptides containing three or more amino acid residues form a coloured chelate complex with cupric ions in an alkaline environment containing sodium potassium tartrate. In the second step of the colour development reaction, bicinchoninic acid (BCA) reacts with the reduced (cuprous) cation that was formed in step one. The intense purple-coloured reaction product results from the chelation of two molecules of BCA with one cuprous ion. The BCA/copper complex is water-soluble and exhibits a strong linear absorbance response at 562 nm with increasing protein concentrations.

Cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in ice-cold modified RIPA lysis buffer without protease inhibitor. Insoluble material was removed by centrifugation at 12,000 *g* for 15 min at 4^0C. Clear 96 well plates were used. 25 µL of cell lysates and standards were added (standard range 25-2000 µg/mL). BCA reagents A and B were mixed together at a ratio of 50:1 before use and 200 µL was added to every well. The plate was then sealed and incubated for 30 min in an oven at 37^0C.

Plates were read at 550 nm in a plate spectrophotometer (Dynex Jencons PLS Technologies Microplate Reader)

2.2.13 Materials

Unless stated otherwise, all compounds used in this study were purchased from Sigma Aldrich-Company Ltd (Poole, Dorset, UK). All solutions used were prepared using genetic grade water from ELGA process water, Marlow International, Marlow, UK. Thiopentone sodium (Intraval Sodium) was obtained from Rhone Merieux (Harlow, Essex, UK).

2.2.14: Statistical analysis

All values are reported as mean ± SDs. LDH assay, caspase assays, and MTS assays were performed. Each experiment was repeated 3 times in total. All multiple group comparisons were performed using ANOVA with a post-test according to Bonferroni. We have used an unpaired Student's t test to compare the means of two different groups. All statistical analysis was performed using GraphPad Prism version 5 (GraphPad Software, San Diego, CA). A *P* value of < 0.05 was considered significant.

2.3 Results

2.3.1: Sodium nitrite ($NaNO_2$) protects HK-2 cells against apoptosis caused by serum starvation (SS)

Caspase-3 activity was significantly increased in SS treated HK-2 cells after 24 h when compared with control cells treated with 10% FCS whereas $NaNO_2$ (50,100 & 500 nmol/L) attenuated this increase (see Figure 1). Although 100 nmol/L $NaNO_2$ was found to be optimal for this effect there was also a significant reduction in caspase-3 activity when using 50 nmol/L (Figure 1 and 2). Similar effects were seen when caspase-9 was measured whilst no caspase-8 activity was seen (Figure 3). Furthermore, this anti-apoptotic effect translated into preserved cell viability at several concentrations (Figure 4)

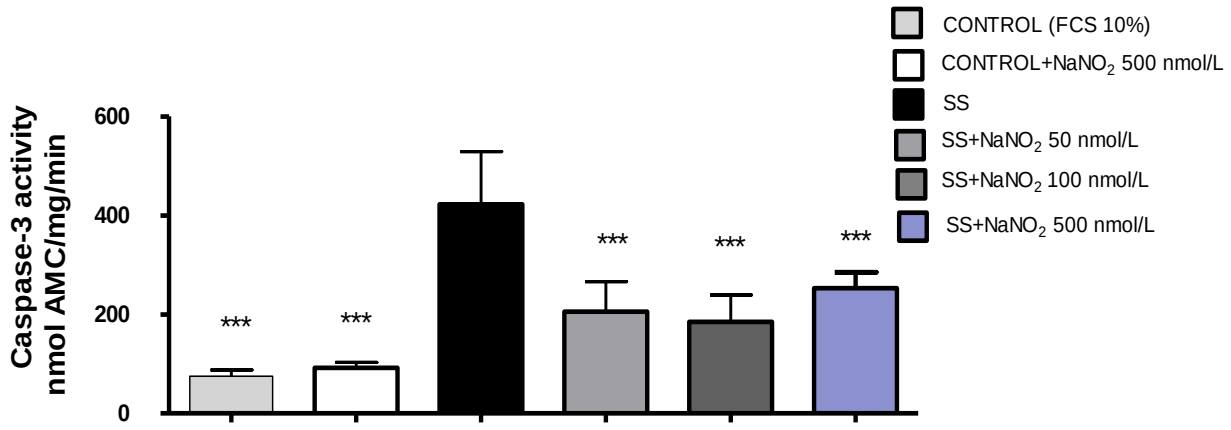


Figure 1: $NaNO_2$ (50 nmol/L, 100 nmol/L and 500 nmol/L) protected HK-2 cells against apoptosis caused by SS.

SS caused significant increase in apoptosis when compared to control FCS treated cultures. This increase was attenuated by $NaNO_2$ at several concentrations. Apoptosis was measured by caspase-3 activity (nmol AMC/mg/min), n=3, ***= *P*<0.001.

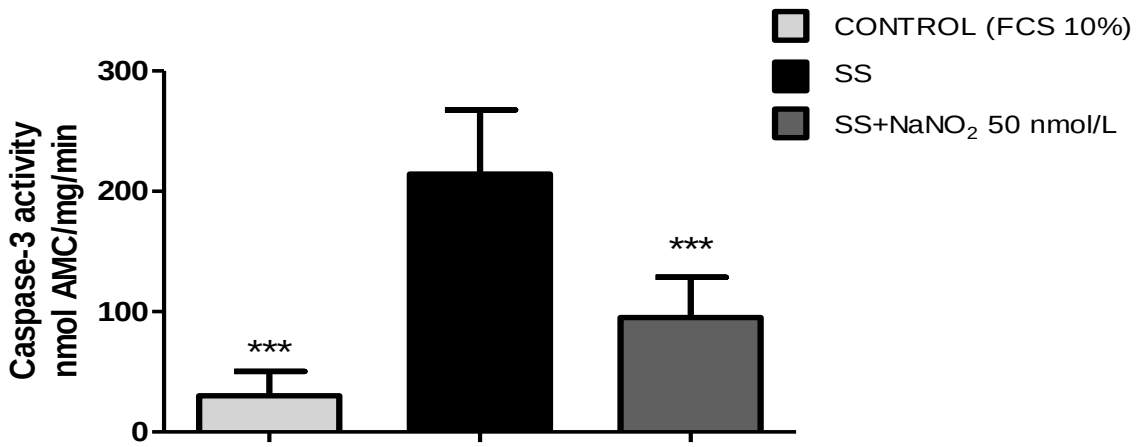


Figure 2: $NaNO_2$ protected HK-2 cells against SS induced apoptosis.

SS of HK-2 cells for 24 h caused significant activation of caspase-3 activity compared to control (with serum) and this increase was attenuated by $NaNO_2$ treatment (50 nmol/L), n=3, ***= *P*<0.001

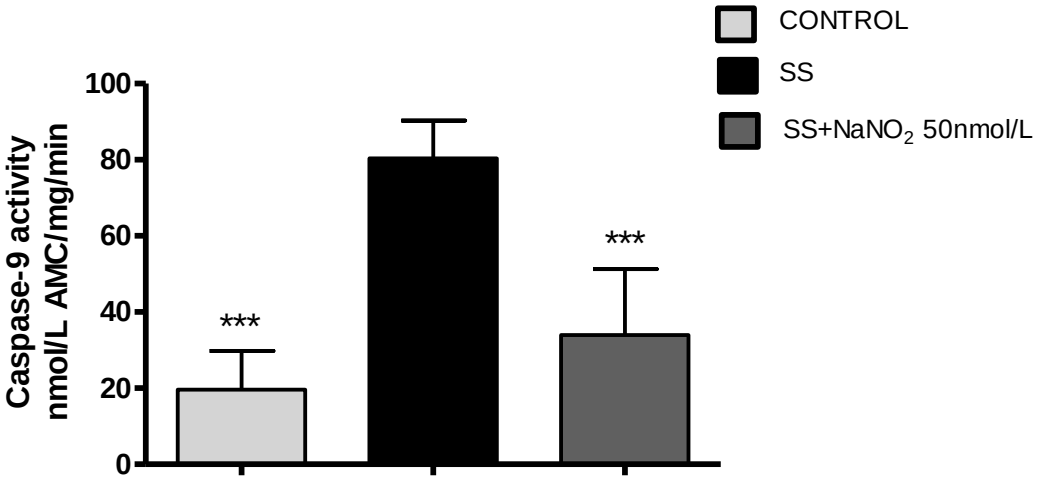


Figure 3: $NaNO_2$ protection against apoptosis was mediated via caspase-9 (intrinsic mitochondrial pathway).

HK-2 cells were incubated with $NaNO_2$ at 50 nmol/L simultaneously with SS for 24 h. Caspase-9 activity was significantly increased in the SS group which was attenuated by $NaNO_2$, n=3, ***= *P*<0.001. No caspase-8 activity was detected.

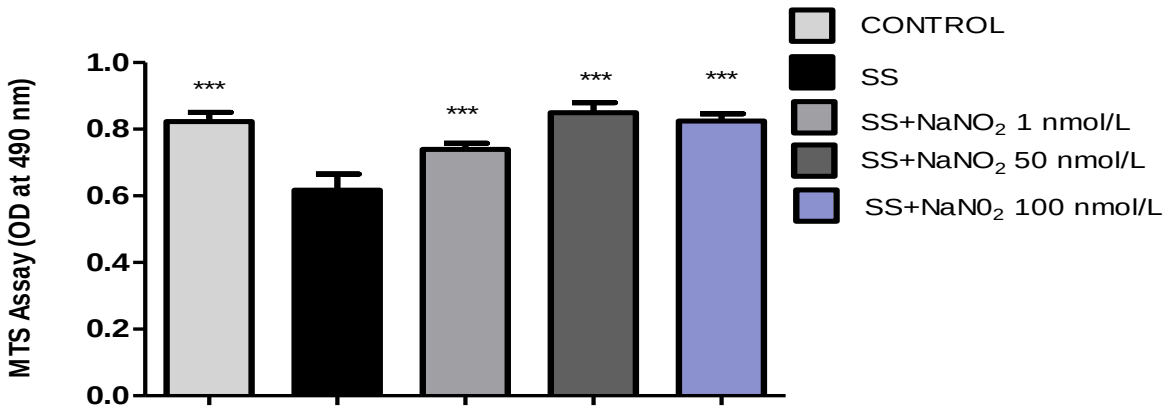


Figure 4: $NaNO_2$ improves HK-2 cell viability caused by 24 h SS.

Cell viability was significantly reduced in SS treated cells compared to control treated cells. $NaNO_2$ significantly improves viability of HK-2 cells after SS for 24 h at several concentrations. The cell viability was determined by MTS assay and the absorbance, proportional to the number of viable cells was measured at 490 nm. n=8, ***= *P*<0.001.

2.3.2 $NaNO_2$ protects HK-2 cells against in vitro IRI induced apoptosis

Caspase-3 activity was significantly increased in HK-2 cells treated with antimycin A (50 µmol/L) and 2-deoxyglucose (10 mmol/L) with HBSS (antimycin A model, AA) when compared with control treated cells whereas, $NaNO_2$ (100 nmol/L) attenuated this increase when given with or after *in vitro* ischaemia injury, but high $NaNO_2$ (10 µmol/L) given with or after *in vitro* ischaemia did not protect the cells from injury. This suggests that NO_2^- can play a significant protective role in preventing apoptosis caused by our *in vitro* model of renal IRI (Figure 5).

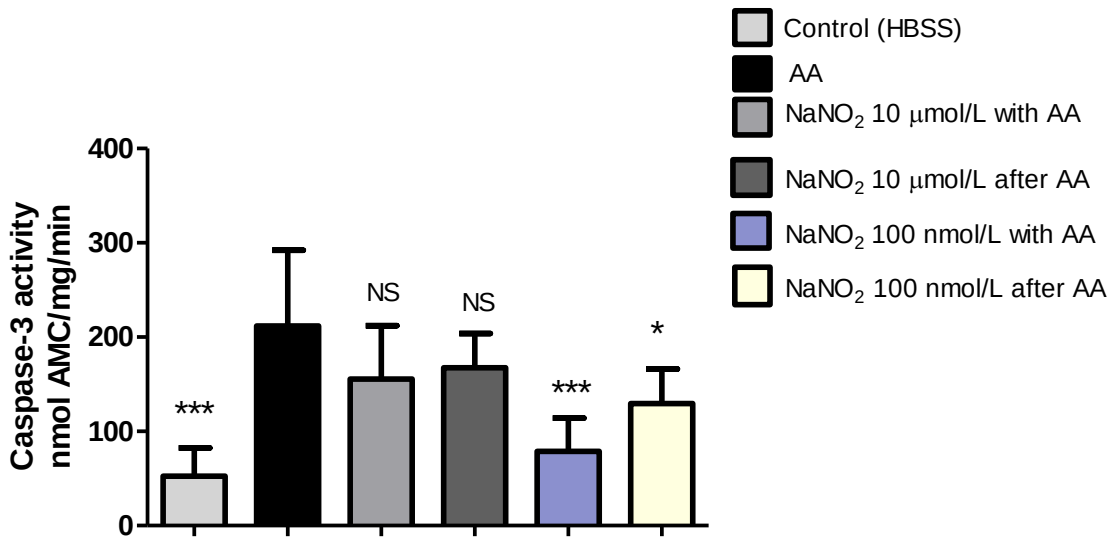


Figure 5: $NaNO_2$ protects HK-2 cells against cellular IRI-induced apoptosis.

HK-2 cells were incubated with (50 μmol/L) antimycin A plus (10 mmol/L) 2-deoxyglucose in HBSS (AA) for 1 h to induce ischaemic injury *in vitro*. The *in vitro* reperfusion was achieved by incubating cells in DMEM growth medium with FCS 10% and antibiotic 1%. Cells were then left to incubate for 24 h in an incubator. Caspase-3 activity was significantly increased in the AA group compared to control treated cells, this increase was significantly reduced by $NaNO_2$ at 100 nmol/L when given with or after *in vitro* ischaemia. Although a 10 μmol/L dose did afford some protection it was found not to be significant. n=3, ***= $P<0.001$, *= $P<0.05$, NS= not significant.

2.3.3 Effects of $NaNO_2$ on HK-2 cell necrosis

The LDH cytotoxicity assay showed a significant increase in LDH release in the H_2O_2 treated group (500 μmol/L in DMEM with 10% FCS and 1% antibiotic) when compared to the untreated control HK-2 cells (given H_2O as a vehicle). However, this increase could not be abolished by $NaNO_2$ supplementation (Figure 6).

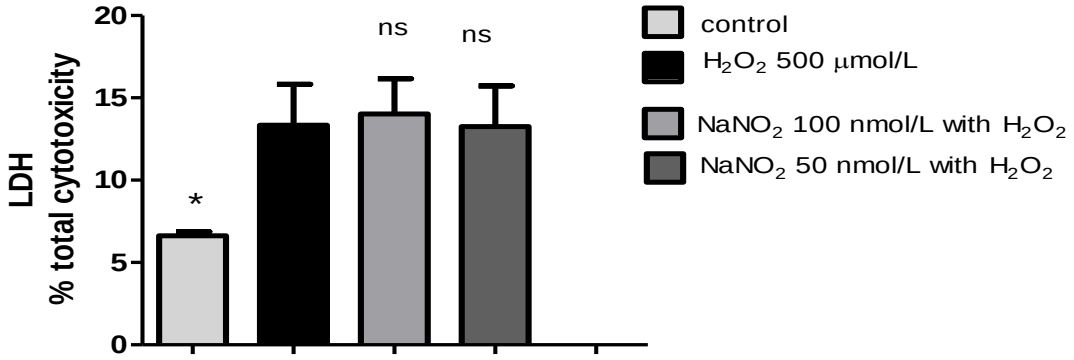


Figure 6: $NaNO_2$ does not attenuate necrosis caused by *in vitro* reperfusion model.

HK-2 cells were given H_2O_2 (500 μmol/L) for 2 h with or without $NaNO_2$. LDH cytotoxicity assay was performed to assess necrosis. There was significant necrosis in the H_2O_2 group when compared to control HK-2 cells P <0.05. $NaNO_2$ could not attenuate this significant increase. n=3, NS= not significant.

2.2.4: Pre-treatment with $NaNO_2$ does not prevent apoptosis at several doses

Caspase-3 activity was significantly increased in SS treated HK-2 cells after 24 h when compared with control cells treated with 10% FCS (SS=348.2±73.5; control=199±60.3, P<0.05) whereas pre-treatment for 4 h prior to SS with several doses of $NaNO_2$ did not attenuated this increase (Figure 7).

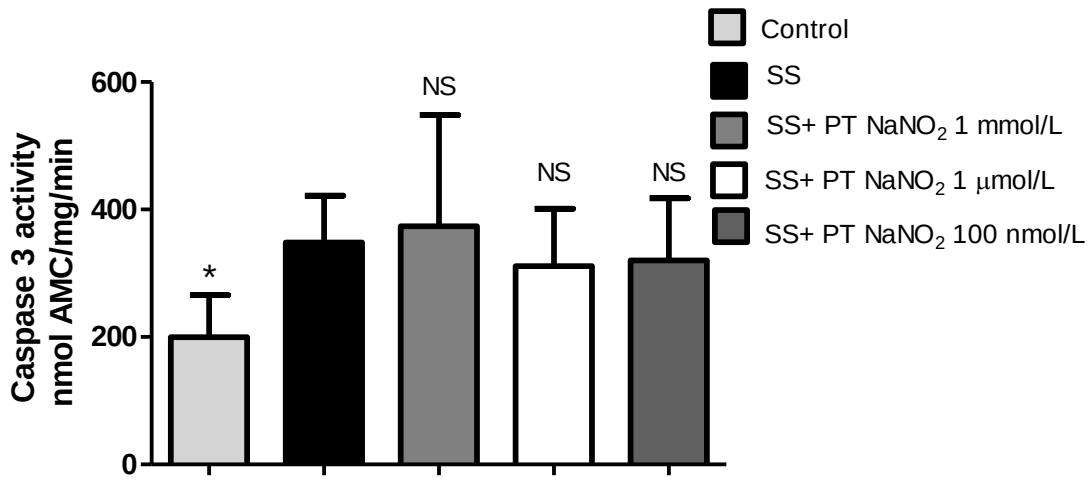


Figure 7: Pre-treatment with $NaNO_2$ does not attenuate apoptosis caused by SS.

HK-2 cells were cultured in 6 well plates for 24 h in incubator with DMEM , 1 % Antibiotic and 10 % FCS. When cells were confluent media in SS + pretreatment group (SS+ PT $NaNO_2$) was added $NaNO_2$ at several different doses for 4 h. After 4 h, media was removed and SS injury was induced by removing FCS from the media and cells were placed in incubator for 24 h. After 24 h, cells were scraped and caspase-3 assay was performed. The result provide evidence that there is significant apoptosis in SS group when compared to control HK-2 cells and pre-treatment with $NaNO_2$ at several different doses cannot attenuate apoptosis caused by SS. n=3, *= P<0.05, NS= not significant.

2.2.5: Pre-treatment with $NaNO_2$ does not preserve cell viability at several doses

Cell viability was significantly decreased in antimycin treated HK-2 cells after 24 h when compared with control cells treated whereas pretreatment for 4 h before AA with several doses of $NaNO_2$ did not preserve HK-2 cell viability (Figure 8).

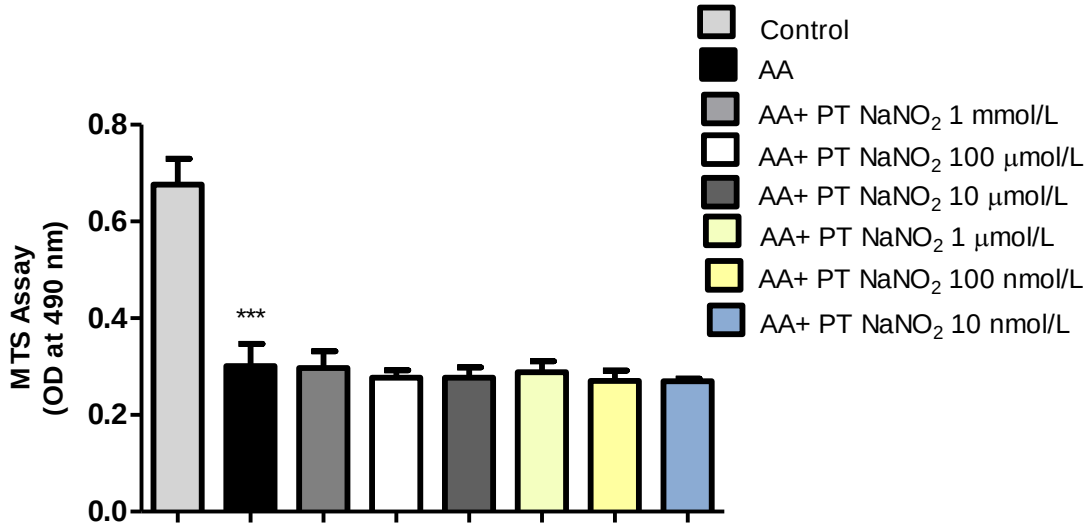


Figure 8: Pre-treatment with NaNO2 did not preserve the HK-2 cell viability.

HK-2 cells were cultured for 24 h. When cells were confluent HK-2 cells were pretreated with $NaNO_2$ at several different doses for 4 h. Then media was removed and HK-2 cells were incubated with (50 μmol/L) antimycin A plus (10 mmol/L) 2-deoxyglucose in HBSS (AA) for 1 h to induce ischaemic injury *in vitro*. The *in vitro* reperfusion was achieved by incubating cells in DMEM growth medium with FCS 10% and antibiotic 1%. Cells were then left to incubate for 24 h in an incubator. Cell viability was significantly decreased in the AA group compared to control treated cells, but pre-treatment with $NaNO_2$ at several doses did not protect cell viability. n=8, ***= P<0.001.

2.3.6: Plasma NO_2^-/NO_3^- levels

Plasma NO_2^-/NO_3^-concentrations were as expected significantly higher in the IRI+high $NaNO_2$ treatment (50 mg/L) group when compared to IRI group (Figure 9). IRI group were fed upon a normal NO_2^- diet. Although plasma NO_2^-/NO_3^- concentrations were increased in the high $NaNO_2$ group there was no protection from injury seen against renal IRI.

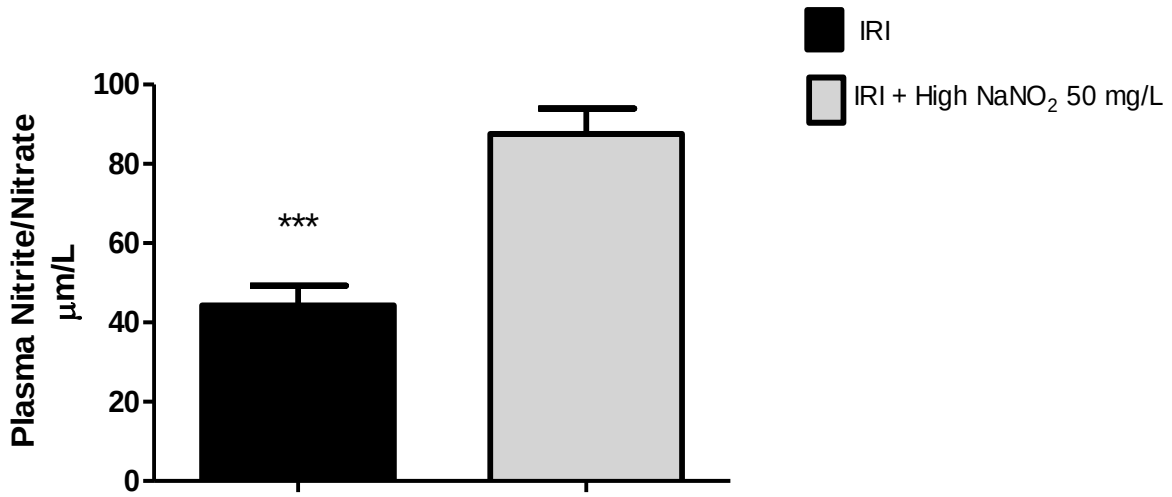


Figure 9: Plasma NO_2^-/NO_3^- levels in high $NaNO_2$ vs IRI group before renal ischaemia.

IRI rats were drinking normal water and IRI+ high $NaNO_2$ were given $NaNO_2$ at 50 mg/L in drinking water. Blood was collected prior to IRI to assess NO_2^-/NO_3^- levels in serum. The Griess assay was performed as shown previously (Chatterjee *et al*., 2002). The result of the Griess assay showed that plasma NO_2^-/NO_3^- levels were significantly higher in the high oral $NaNO_2$ group when compared to the IRI group, n=4, ***= $P<0.001$

2.3.7: Effect of $NaNO_2$ on renal IRI in rats

In comparison with sham animals, control animals (normal NO_2^- diet) that were subjected to renal IRI (IRI group) had significant renal dysfunction at 24 h as suggested by a significant increase in serum creatinine concentration (Figure 10). However, pre-treatment for 7 days with $NaNO_2$ (50 mg/L) in the drinking water (IRI+high NO_2^- treatment) with normal NO_2^- chow or low NO_2^- diet chow group (IRI+low NO_2^- diet 1.38 µg/L) with normal water prior to renal IRI neither attenuated nor worsened the deleterious effects of IRI.

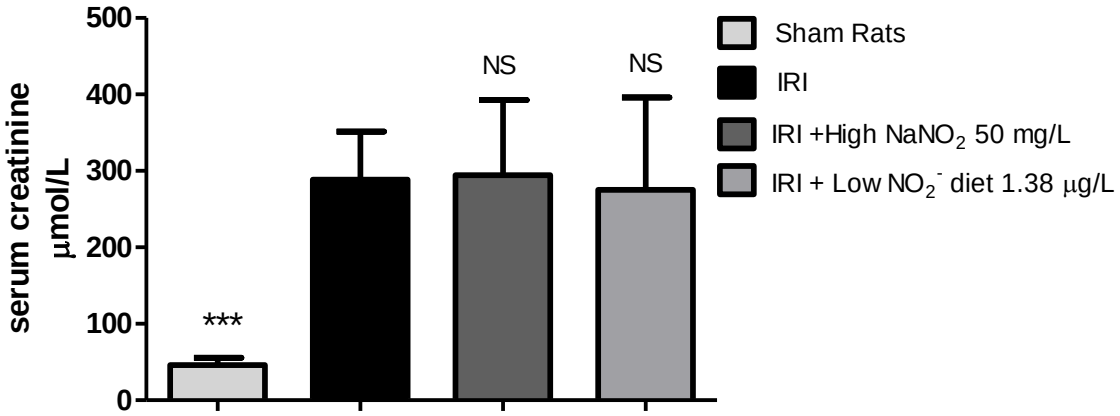


Figure 10: Showing a significant difference in serum creatinine concentration between sham operated animals vs IRI+V, *P* <0.001.

Sham rats and IRI received normal water and standard diet for 7 days. IRI+ high $NaNO_2$ received normal standard diet and $NaNO_2$ was given in drinking water at a concentration of 50 mg/kg for 7 days. IRI+ low NO_2^- diet group were given normal water and standard diet was replaced with special low NO_2^- diet (1.38 µg/L). IRI was performed on all rats leaving sham in which renal pedicle was not clamped. The results provide evidence that pre-treatment with high $NaNO_2^-$ or low NO_2^- diet did not attenuate the increase in serum creatinine concentration when compared to IRI group, ***= *P* <0.001, n=9, NS = not significant.

2.4 Discussion

The main findings of these experiments are that (a) NO_2^- cannot abolish the damage and dysfunction caused by renal IRI at lower and higher dose in rats (b) NO_2^- can protect human renal PTECs against apoptosis caused by *in vitro* IRI model and can preserve cell viability (c) pretreatment with NO_2^- both *in vivo* and *in vitro* does not ameliorate the damage and dysfunction associated with renal IRI. Taken together, these observations suggest that oral NO_2^- therapy is not protective against renal IRI. However, nitrite may prevent apoptosis and preserves cell viability if given at time of injury or post injury in PTECs.

Investigators in our laboratory have previously demonstrated the beneficial effects of the topical application of NO_2^- on rat kidneys when applied just before reperfusion in a renal IRI model through the XOR mechanisms (Tripatara *et al.*, 2007). However, i.v. NO_2^- has also been shown to be detrimental in renal IRI (Basireddy *et al.*, 2006).The study presented here explores whether altering the oral NO_2^- diet in rats (low and high) can prevent the damage and dysfunction caused by renal IRI. The results demonstrated that both the low and high NO_2^- diets were unable to protect against renal IRI, this being in contrast to the beneficial effects seen in both cardiac and hepatic IRI (Duranski *et al.*, 2005). In a renal IRI model we have previously demonstrated beneficial effects of NO_2^- but the mode of administration was topical upon the kidneys, the dosage was lower (60 μmol/L) and the ischaemia duration was different (60 min) (Tripatara *et al.*, 2007). Previously, NO donors have been shown to be beneficial if given prior to the onset of ischaemia in IRI (Schulz *et al*., 2004, Lefer *et al.*, 1993) but at high doses NO donors have been found to be harmful. Higher sustained generation of NO at concentrations

>300 nmol/L, which causes iNOS activation is known to be pro-apoptotic and leads to increased lipid peroxidation (Goligorsky *et al.*, 2002).

One of the likely reasons why NO_2^- treatment has been seen to be detrimental can be that a too high a dose has been used, as most of the previous studies have demonstrated that nanomolar doses of NO_2^- are beneficial in a number of organs and species (Lefer *et al*., 1993, Bolli, 2001, Lefer, 1995). However, in contrast to above mentioned studies our low dietary NO_2^- group was not found to be protective against renal IRI. Furthermore, the NO_2^- beneficial effects are dependent on its conversion to NO by XOR (Tripatara *et al.*, 2007) and there is also evidence suggesting that XOR is associated with the generation of ROS and is detrimental to tissues (Tiravanti *et al*., 2004). The NO protective effects are due to eNOS derived NO, which causes vasodilation and inhibits leukocyte adhesion and platelet aggregation (Raat *et al*., 2009, Tripatara *et al*., 2007). Damaging inflammatory effects are due to iNOS derived NO which forms $ONOO^-$ from the interaction of NO and $O_2^{\bullet -}$ (Raat *et al.*, 2009).

As expected our results showed that plasma NO_2^-/NO_3^- concentrations were elevated before renal IRI in the high NO_2^- treatment group and our results are in accordance with others work which shows that plasma NO_2^-/NO_3^- levels were significantly higher in rats at 30 min after i.v administration of NO_2^- compared to control group (Basireddy *et al*., 2006). Although, NO_2^-/NO_3^- levels were higher they were unable to see any beneficial effects of NO_2^- via systemic administration. Their finding mimic our findings as we also did not find any beneficial effects of NO_2^- via oral administration (Basireddy *et al*., 2006). However, contrary to our study, previous works demonstrates that plasma NO_2^-/NO_3^-

levels were surprisingly higher in renal IRI group after 6 h of reperfusion when compared to NO_2^- treated group (Tripatara *et al*., 2007). Triptara further measured the levels of NO_2^- derived NO in kidney homogenates under anaerobic conditions which showed that there was significant NO generated from NO_2^- in NO_2^- treated group during anaerobic conditions compared to IRI group (Tripatara *et al*., 2007). Further research is needed to understand whether pre-treatment of NO_2^- contributes towards the generation of $ONOO^-$ as a by-product of $O_2^{\bullet-}$ and NO which is normally seen where iNOS is activated (Raat *et al.*, 2009). It will also be worthwhile to investigate the levels of NO_2^- and NO during ischaemia with pretreatment of NO_2^-. Furthermore, it might be of interest to perform nitrotyrosine staining on the kidney homogenates to assess whether pretreatment of NO_2^- is contributing towards increased $ONOO^-$ formation and to perform western blot analysis to look at iNOS, eNOS and XOR expression in order to identify the possible mechanism. The findings of these *in vivo* experiments may be clinically relevant as pre-treatment of oral NO_2^- by patients undergoing high risk procedures such aortic aneurysm surgery or coronary artery bypass graft would have been the natural strategy if pre-clinical data had shown any benefit.

To summarise, the oral route of administration for NO_2^- is unable to prevent renal IRI and the damage and dysfunction associated with it. To date, the effect of NO_2^- upon apoptosis in PTECs have not been studied. The main finding of these *in vitro* experiments are that a direct cytoprotective effects of NO_2^- upon PTECs occurred in both SS and simulated IRI models. Furthermore, this anti-apoptotic protection is mediated via the intrinsic pathway in the SS *in vitro* model.

NO_2^- was also able to preserve cell viability in the SS model, but was not able to ameliorate the degree of necrosis caused by H_2O_2, in an *in vitro* reperfusion model. Although the relevance of SS induced apoptosis as an *in vitro* mimic of renal IRI remains controversial, our findings that NO_2^- exerts a cytoprotective effects against the well established *in vitro* (simulated) cellular IRI model confirms its pro-survival role against IRI.

Research indicates that intracellular ATP depletion and a decrease in tissue oxygen content with increase in intracellular calcium are the main features of renal IRI. In the above experiments, we produced ATP depletion mediated apoptotic cell death as an *in vitro* model of ischaemic or ATP depletion injury. This injury was achieved by a combination of metabolic inhibitors antimycin A and 2-deoxyglucose (Iwata *et al*., 1994). It has been demonstrated that this treatment produces approximately 90% ATP depletion compared to untreated cultures (Iwata *et al*., 1994). Our data suggest that NO_2^- prevents apoptosis through the intrinsic pathway by reducing the caspase-9 activity and preserves cell viability if given at lower doses in SS and in *in vitro* IRI model but pre-treatment with NO_2^- seems to be of little or no therapeutic effect which confirms our *in vivo* data. It is possible that preloading with NO_2^- both *in vivo* and *in vitro* is ineffective.

In conclusion, our *in vivo* study demonstrates that pre-treatment with dietary NO_2^- is not an effective treatment for AKI. Although *in vitro* it was found to reduce apoptosis and preserve cell viability if given at time of ischaemic injury. Further research is needed to understand the NO_2^- mechanism of action and role in AKI and renal tubular apoptosis.

CHAPTER 3
ROLE OF THIAMINE AND BENFOTIAMINE IN AKI

3.1: Introduction

Renal IRI is a major cause of AKI (Liano and Pascual, 1998) and is a consequence of acute tubular injury initiated by a combination of ROS generation and ATP depletion (Bonventre, 1993). Thiamine has been previously shown to play an essential role in the scavenging of ROS and the regeneration of ATP (Nozaki *et al*., 2009, Natera *et al*., 2011). Studies have shown that it reduces ischaemic myocardial damage and has marked cytoprotective effects against MI (Larrieu *et al*., 1987). Furthermore, thiamine has been shown to attenuate the degree of hypoxic/ischaemic cardiac cell death (Shin *et al*., 2004) and has been found to prevent incipient diabetic nephropathy at high doses (Babaei-Jadidi *et al*., 2003). Tissue thiamine deficiency is a major cause of delayed graft function during transplantation and its supplementation to the graft donor can prevent the delayed graft function (Klooster *et al*., 2007). Moreover, thiamine deficiency has been revealed to cause the oxidative stress that leads to the formation of ROS, lipid peroxidation and apoptosis in cardiac muscle and eventually leads to heart failure (Gioda *et al*., 2010). In a similar manner there is an increase in COX-2 expression in the brain of thiamine deficient rats which leads to the generation of ROS such as peroxide, $O_2^{\bullet -}$ and cause neuronal cell death *in vivo* and *in vitro* (Pepicelli *et al*., 2002).

In vitro studies using cultured neonatal rat cardiomyocytes have identified the cytoprotective role of thiamine by attenuating hypoxia-induced apoptosis with an inhibition of caspase-3 activation, PARP cleavage and DNA fragmentation. This cytoprotective effects could be abolished by anti-Hsp70 antibodies (Shin *et al*., 2004).

In many diseases, such as Wernicke's encephalopathy (Victor *et al*., 1971), diabetic nephropathy (Babaei-Jadidi e*t al*., 2003) and diabetic dyslipidaemia (Babaei-Jadidi *et al.*, 2004) a high dose of thiamine is able to prevent known symptoms. Thiamine is transported across plasma membranes by high affinity carriers (Bettendorff and Wins, 1994) but the rate of transport is generally very slow. Many of lipophilic thiamine derivatives have been synthesized which can diffuse easily through the plasma membrane with better bioavailability (Greb and Bitsch, 1998, Lonsdale, 2006). These lipophilic derivatives bypass the rate limiting transport system required by thiamine. These lipophilic derivatives can be converted to thiamine inside the cell by enzymatic or non-enzymatic processes (Volvert *et al*., 2008). Currently, two lipophilic disulphide derivatives are used as therapeutic agents: thiamine tetrahydrofurfuryl disulphide (fursultiamine) (Lonsdale, 2004, Volvert *et al*., 2008) and O-isobutyrylthiamine disulphide (sulbutiamine) (Van Reeth, 1999). Sulbutiamine is used as a symptomatic drug in treating asthenias (Van Reeth, 1999). Chronic treatment with sulbutiamine increases thiamine, ThMP, ThDP and ThTP levels in brain and peripheral tissues (Bettendorff *et al*., 1990).

Benfotiamine is a third derivative with better bioavailability than thiamine. In contrast to other derivatives it is an S-acyl derivative and has been shown to protect against diabetic complication (Wu and Ren, 2006). Benfotiamine is able to prevent glucose toxicity (Berrone *et al*., 2006, Marchetti *et al*., 2006) and blocks three major pathways (the hexosamine pathway, the AGE formation pathway and the diacylglycerol-protein kinase pathway) of hyperglycaemic damage, by removing glyceraldehydes-3-phosphate and fructose-6-phosphate through the activation of transketolase enzyme which plays a

crucial role in pentose phosphate pathway (Hammes *et al*., 2003). Furthermore, benfotiamine has been shown to protect against ischaemia induced toe necrosis, improve hind limb perfusion and oxygenation and restore endothelium dependent vasodilation in streptozocin-induced diabetic mice models (Gadau *et al*., 2006).

Benfotiamine has also been shown to improve the recovery from MI by reducing oxidative stress, correcting the impairment of adaptive pro-survival mechanism and moderating neurohormonal activation. This study identifies that benfotiamine improves myocardial perfusion, promotes reparative neovascularization in the infarcted heart, reduces oxidative stress and apoptosis and activates pro-survival signalling mechanisms, in the infarcted heart (Katare *et al*., 2010). Another study shows that benfotiamine protects against peritoneal and kidney damage associated with peritoneal dialysis. The study identifies that benfotiamine decreases glucose-induced tissue damage in a model of peritoneal dialysis in uraemic rats and treatment with benfotiamine decreased peritoneal fibrosis, markers of inflammation, and neovascularization, resulting in improved characteristics of peritoneal transport (Kihm *et al*., 2011). Furthermore, this study also shows that rats treated with benfotiamine exhibited a lower expression of advanced glycation end products (AGE) and their receptor in the peritoneum and the kidney, reduced glomerular and tubulointerstitial damage, and less albuminuria. Increased activity of transketolase in tissue and blood contributed to the protective effects of benfotiamine (Kihm *et al*., 2011).

Studies have shown that the administration of benfotiamine leads to higher thiamine levels in blood compared to water soluble thiamine (Greb and Bitsch, 1998, Lonsdale,

2004). Another study has identified that benfotiamine is not as potent as previously thought compared to other lipophilic thiamine derivatives (Volvert *et al*., 2008). Furthermore, recent study suggests that there is downregulation of thiamine transporters in the CKD rats and it is suggestive of severe deficiency of thiamine in these rats (Bukhari *et al*., 2010). Similarly, recently published data shows that thiamine pyrophosphate can reduce the oxidative damage in renal IRI but they did not evaluated its effects on renal IRI (Altuner *et al*., 2013). Thiamine and benfotiamine have been shown to play a crucial role in preventing ischaemic damage in different models by reducing oxidative stress, inflammation and apoptosis whilst renal IRI is a consequence of oxidative stress, inflammation and apoptosis. The possible utility of both these compounds appear obvious so that the aim of this study was to investigate the effects of thiamine and benfotiamine upon the rat kidney (functional and histological) in a renal IRI model and to investigate if thiamine has anti-oxidant, anti-inflammatory and anti-apoptotic effects.

3.2 Materials and methods

3.2.1 Rat model of renal IRI

The protocol was the same as described in chapter 2 (2.2.1 Rat model of renal IRI). Briefly, the procedure requires that the renal pedicles are bilaterally clamped for 45 min with microaneurysm clamps followed by reperfusion for 24 h with the time of ischaemia chosen to obtain a reversible model of ischaemic AKI and to avoid animal mortality. Sham surgery consisted of the same surgical procedure except that clamps were not applied.

3.2.2 Assessment of renal function

The protocol was as described earlier (2.2.2 Assessment of renal function). End-points examined include histological evaluation by both haematoxylin and eosin staining and TUNEL assay. IDEXX Laboratories, Sussex, UK, were blinded to the treatment given, performed serum creatinine measurements.

3.2.3 Experimental design

The study was divided into the following groups;

(i)*SHAM OPERATED RATS*: The renal pedicle was not clamped but the remaining procedure was the same as for the IRI group and rats were gavaged once daily with saline for 3 days.

(ii) *IRI GROUP*: Rats subjected to IRI of the kidney (IRI group) and were gavaged once

daily with saline for 3 days.

(iii)*THIAMINE GROUP*: Rats subjected to IRI plus pre-treatment with thiamine HCL (500 mg/kg) by gavage once daily for 3 days.

(iv)*THIAMINE LOW DOSE GROUP*: Rats subjected to IRI plus pre-treatment with thiamine HCL (125 mg/kg) gavaged 1 h before IRI.

3.2.4: Histological evaluation

Histological evaluation was done as described earlier (Kumar *et al*., 2009). Briefly a renal pathologist, who was blinded to the treatment given, performed the morphologic assessment. Kidney sections were stained with haematoxylin and eosin. A hundred intersections were examined for each kidney and a score from 0 to 3 was given for each tubular profile. Briefly, 0, normal histology; 1, tubular cell swelling, brush border loss, nuclear condensation, with up to one third of tubular profile showing nuclear loss; 2, as for score 1 but greater than one third and less than two thirds of tubular profile shows nuclear loss; and 3, greater than two thirds of tubular profile shows nuclear loss. The total score for each kidney was calculated by addition of all 100 scores with a maximum score of 300.

3.2.5 Terminal deoxynucleotide transferase nick end labeling (TUNEL) assay

Terminal deoxynucleotide transferase nick end labelling (TUNEL) assay done as described earlier (Kumar *et al*., 2009). Briefly TUNEL was employed to detect apoptosis on histological sections. The TUNEL method is based on direct, specific, labelling of

DNA breaks in nuclei, *in situ*. One of the characteristics of apoptosis is the degradation of DNA after the activation of Ca^{2+}/Mg^{2+} dependent endonucleases. This DNA cleavage leads to strand breaks within the DNA. The method is based on the specific binding of terminal deoxynucleotidyl transferase (TdT) to 3'-OH ends of the strand breaks of cleaved DNA ensuring the synthesis of a polydeoxynucleotide polymer. After the exposure of nuclear DNA on histological sections by proteolytic treatment, TdT is used to incorporate biotinylated deoxyuridine at sites of DNA breaks. The biotin-labelled cleavage sites are then detected by reaction with HRP conjugated streptavidin and visualized by diaminobenzidine showing as a brown colour.

The TUNEL assay was performed by using a TUNEL detection kit according to the manufacturer's instructions (Apotag, peroxidase ISOL apoptosis detection kit). Briefly, sections were incubated with 15 µg/mL proteinase K for 15 min at room temperature and then washed with PBS. Endogenous peroxidase was inactivated by 3% H_2O_2 for 5 min at room temperature and then washed with PBS. Sections were immersed in TdT buffer containing deoxynucleotidyl transferase and biotinylated dUTP in TdT buffer, incubated in a humid atmosphere at 37°C for 90 min, and then washed with PBS. The sections were incubated at room temperature for 30 min with anti-horseradish peroxidase-conjugated antibody, and the signals were visualized with diaminobenzidine. The number of TUNEL-positive cells/high power field was counted in 5 to 10 fields for each coded slide.

3.2.6 Tissue extracts

Cytosolic and nuclear extracts were prepared as previously described (Tripatara *et al.*,

2008). Briefly, kidneys were homogenized at 10% (w/v) in a Potter Elvehjem homogeniser (Fisher Scientific Ltd., Loughborough, UK) using a homogenisation buffer containing 20 mM HEPES, pH 7.9, 1 mmol/L $MgCl_2$, 0.5 mmol/L EDTA, 1% NP-40, 1 mmol/L EGTA, 1 mmol/L Dithiothreitol (DTT), 0.5 mmol/L Phenylmethyl Sulphonyl Fluoride (PMSF), 5 μg/mL aprotinin, 2.5 μg/mL leupeptin. Homogenates were centrifuged at 4,000 *g* for 5 min at 4°C. Supernatants were removed and centrifuged at 14,000 *g* at 4°C for 40 min to obtain the cytosolic fraction. The pelleted nuclei were resuspended in extraction buffer containing 20 mmol/L HEPES, pH 7.9, 1.5 mmol/L $MgCl_2$, 300 mmol/L NaCl, 0.2 mmol/L EDTA, 20% glycerol, 1 mmol/L EGTA, 1 mmol/L DTT, 0.5 mmol/L PMSF, 5 μg/mL aprotinin, 2.5 μg/mL leupeptin. The suspensions were incubated on ice for 30 min for high-salt extraction followed by centrifugation at 15,000 *g* for 20 min at 4°C. The resulting supernatants containing nuclear proteins were carefully removed and protein content was determined using a BCA protein assay following the manufacturers' directions. Samples were stored at -80°C until use.

3.2.7 ROS detection

ROS were measured in cytosolic fractions using 2',7'-dichlorofluorescein diacetate (DCFH-DA) as a probe. DCFH-DA is a stable, non-fluorescent molecule that readily crosses the cell membrane and is hydrolysed by intracellular esterases to non-fluorescent 2',7'-dichlorofluorescein (DCFH), which is rapidly oxidised, in the presence of peroxides, to highly fluorescent 2',7'-dichlorofluorescein (DCF). The DCF is then measured fluorimetrically (Ravindranath, 1994). Results are expressed as units of fluorescence (UF)/mg protein.

3.2.8 End products of lipid peroxidation

Lipid peroxidation was investigated by measuring the main end-product of peroxidation, hydroxynonenal (HNE), in the cytosolic fractions. HNE concentration was determined on fresh cytosolic fractions by Esterbauer's method (Esterbauer *et al.*, 1986). An aliquot of cytosol (100 µL) was extracted in an equal volume of a solution of acetic acid/acetonitrile (4/96, v/v). After centrifugation at 250 *g* for 20 min at 4°C, 50 µL of supernatant were injected on to an HPLC Symmetry C_{18} column (5 µm, 3.9x150 mm). The mobile phase used was acetonitrile:bi-distilled water (42%, v/v). The HNE concentration was calculated by comparison with a standard solution of HNE (Calbiochem, Merck Chemicals Ltd, Beeston, UK) of known concentration.

3.2.9 Myeloperoxidase activity

Myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocyte accumulation, was determined at 24 h reperfusion as described previously (Mullane *et al*., 1985). At the specified reperfusion time, kidneys were obtained and weighed and each piece homogenized in a solution containing 0.5% (w/v) hexadecyltrimethyl-ammonium bromide dissolved in 10 mmol/L potassium phosphate buffer (pH 7) and centrifuged for 30 min at 20,000 *g* at 4°C. An aliquot of the supernatant was then allowed to react with a solution of 1.6 mmol/L tetramethylbenzidine and 0.1 mmol/L H_2O_2. The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1 µmol of peroxide per min at 37°C and was expressed in mU per gram of wet tissue.

3.2.10. Western blot analysis

About 60 µg of protein was loaded. Proteins (COX-2 and ICAM-1) were separated on pre-cast 8% sodium dodecyl sulphate-polyacylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinyldenedifluride (PVDF) membrane, which was then incubated with blocking buffer overnight at 4^0C. Membranes were incubated with primary antibody (rabbit anti-COX-2, goat anti-ICAM-1). Blots were then incubated with secondary antibody conjugated with horseradish peroxidase for 30 min at room temperature and the immunoreactive bands were visualised by Enhance Chemiluminescence (ECL) reagent (GE Healthcare, Buckinghamshire, UK) and the density of the bands were evaluated densitometrically using NIH Image J software. The membranes were stripped and incubated with β-actin monoclonal antibody and subsequently with anti-mouse antibody, both for 30 min at room temperature, in order to assess gel-loading homogeneity.

3.2.11 Administration of benfotiamine

Benfotiamine (Sigma-Aldrich) was dissolved in a 200 mmol/L solution of hydroxypropyl-β-cyclodextrin (HP-β-CD, Sigma Aldrich Chemical Company) at a concentration of 25 mg/mL as described by others (Volvert *et al*., 2008). The solution was homogenized at 4°C. The rats were gavaged with a dose of 250 mg benfotiamine/kg once daily for 3 days Control animals were gavaged HP-β-CD solution (200 mmol/L) prepared in saline once daily for 3 days without benfotiamine.

3.2.12 Experimental design

The study was divided into the following groups;

(i) *SHAM OPERATED RATS:* The renal pedicle was not clamped but the remaining protocol was the same as for the IRI group and rats were gavaged using HP-β-CD solution (200 mmol/L) without benfotiamine once daily for 3 days.

(ii) *IRI GROUP*: Rats subjected to IRI of the kidney (IRI group) and were gavaged with HP-β-CD solution (200 mmol/L) without benfotiamine once daily for 3 days.

(iii) *BENFOTIAMINE GROUP:* Rats subjected to IRI plus pre-treatment with benfotiamine (250 mg/kg) by gavage once daily for 3 days.

3.2.13 In vitro IRI model

As described in section 2.2.6

3.2.14 LDH cytotoxicity assay

As described in section 2.2.11

3.2.15 Cell viability (MTS) assay

As described in section 2.2.7.

3.2.16 Serum starvation model

As described in section 2.2.8.

3.2.17 Materials and statistical analysis

As described in section 2.2.13 and 2.2.14.

3.3 Results

3.3.1: Thiamine attenuates apoptosis in HK-2 cultures in renal IRI (in vitro model)

Caspase-3 activity was found to be significantly increased in the lysates derived from HK-2 cells treated with antimycin A 50 μmol/L and 2-deoxyglucose (10 mmol/L) in HBSS when compared with control treated cells (HBSS only). Thiamine (10 μmol/L-100 μmol/L) attenuated this increase (Figure 11). This anti-apoptotic effect of thiamine was also translated into preserved cell viability (Figure 12).

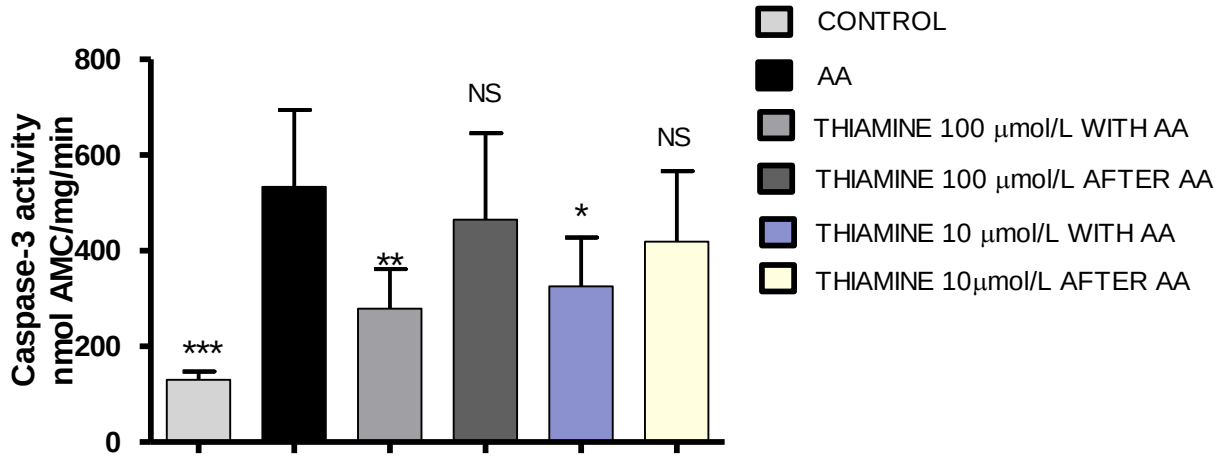


Figure 11. Thiamine protects HK-2 cells against cellular IRI induced apoptosis.

HK-2 cells were incubated with 50 μmol/L antimycin A plus 10 mmol/L 2-deoxyglucose with HBSS for 1 h (AA) to induce ischaemic injury *in vitro*. The *in vitro* reperfusion was achieved by incubating cells in DMEM with FCS 10% and antibiotic 1%. Cells were allowed to grow for 24 h in an incubator. Caspase-3 activity was significantly increased in the AA model group compared to control treated cells, P <0.05, which was largely prevented by thiamine 10-100 μmol/L if given with *in vitro* ischaemia P <0.01. There was no protection seen if thiamine was given after AA treatment, n=3, *= P<0.05,**= P<0.01, ***= P<0.001.

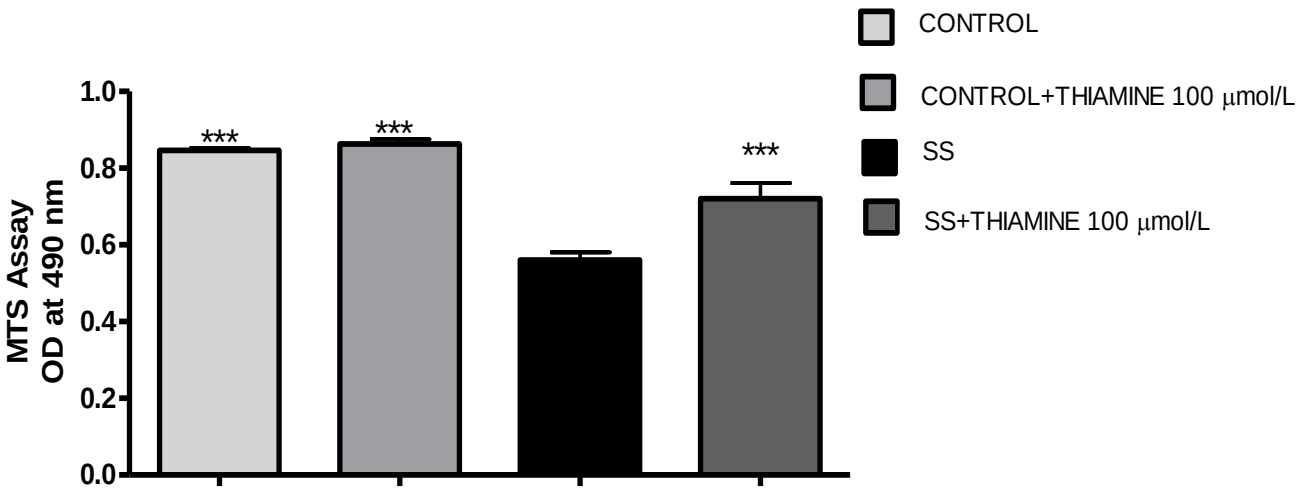


Figure 12. Thiamine preserves viability of HK-2 cells in a serum starvation model.

Cell viability was significantly attenuated by 24 h of serum starvation (SS) compared to control cells (*P* <0.001). Thiamine (100 μmol/L) significantly preserved viability of HK-2 cells upon serum starvation (SS) for 24 h *P* <0.001. Cell viability was determined by MTS assay and the absorbance, proportional to the number of viable cells was measured at 490 nm, n=3, ***= *P*<0.001.

3.3.2: Effects of thiamine on necrosis caused by in vitro reperfusion model

LDH assay shows significant increases in cytotoxicity in H_2O_2 (500 μmol/L) treated group when compared to control HK-2 cells, *P* <0.001 (Figure 13). However, this increase could not be attenuated by the addition of thiamine (100 μmol/L).

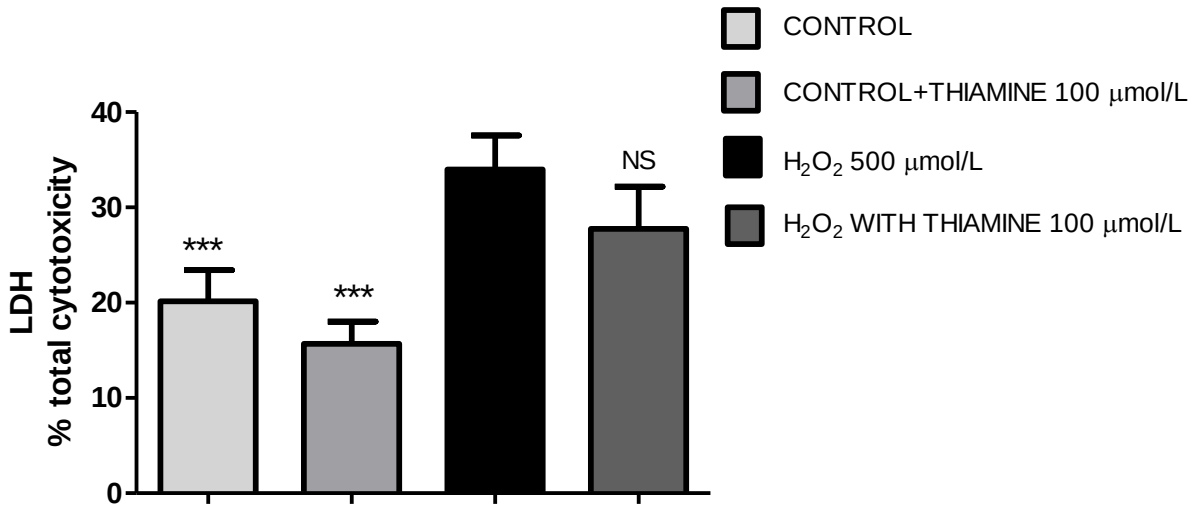


Figure 13. Thiamine does not protect HK-2 cells against necrosis caused by hydrogen peroxide.

HK-2 cells were given H_2O_2 (500 μmol/L) for 2 h with or without thiamine (100 μmol/L) and then the LDH assay was performed to assess necrosis. There was significant necrosis in the H_2O_2 group when compared to control HK-2 cells P <0.001. However, thiamine was unable to protect against H_2O_2 induced cytotoxicity, NS = not significant, ***= P<0.001.

3.3.3 Thiamine ameliorates renal dysfunction and attenuates the histological damage of rat renal IRI

An initial pilot study with thiamine at 125 mg/kg was performed to assess whether thiamine treatment can prevent renal IRI at that dose given 1 h prior to renal IRI. It showed a trend indicating that thiamine might be able to prevent renal IRI at a higher dose (data not shown). From this we chose to use a higher dose of thiamine (500 mg/kg).

In the study presented here, in comparison with sham treated animals, control animals that were subjected to renal IRI (IRI+V) had significant renal dysfunction at 24 h as shown by a significant increase in serum creatinine (Figure 14). However, the rats that were orally gavaged thiamine (500 mg/kg) for 3 days prior to ischaemia (IRI+THI) had a

marked reduction in serum creatinine by comparison (224 μmol/L ± 64 to 119 μmol/L ± 55; IRI+V versus IRI+THI, *P*< 0.012, respectively (Figure 14). Histology data indicated that the kidneys from the IRI+V group (Figure 15 A) had signs of severe tubular damage in the outer medulla, as evidenced by widespread tubular necrosis when compared with sham group (Figure 15 C). In contrast, all renal sections obtained from animals that were treated with thiamine showed significantly less tubular damage at the same time point of 24 h (Figure 15 B). Quantification of the tubular damage revealed a markedly lower mean tubular injury score from the kidneys of rats treated with thiamine (Figure 15 D).

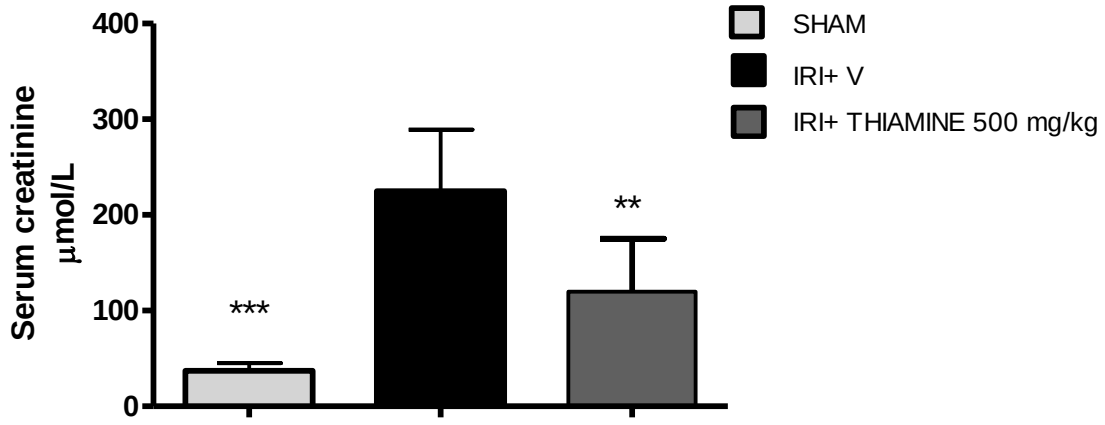


Figure 14 Thiamine attenuates the increase in serum creatinine caused by renal IRI.

There was significant increase in the serum creatinine concentrations of the IRI+V group when compared to sham rats. This increase in serum creatinine at 24 h post-reperfusion after renal IRI is abrogated by the gavaging of thiamine (500 mg/kg, orally) for 3 days prior to ischaemia, n =6, ** = *P*<0.01, ***= *P*<0.001.

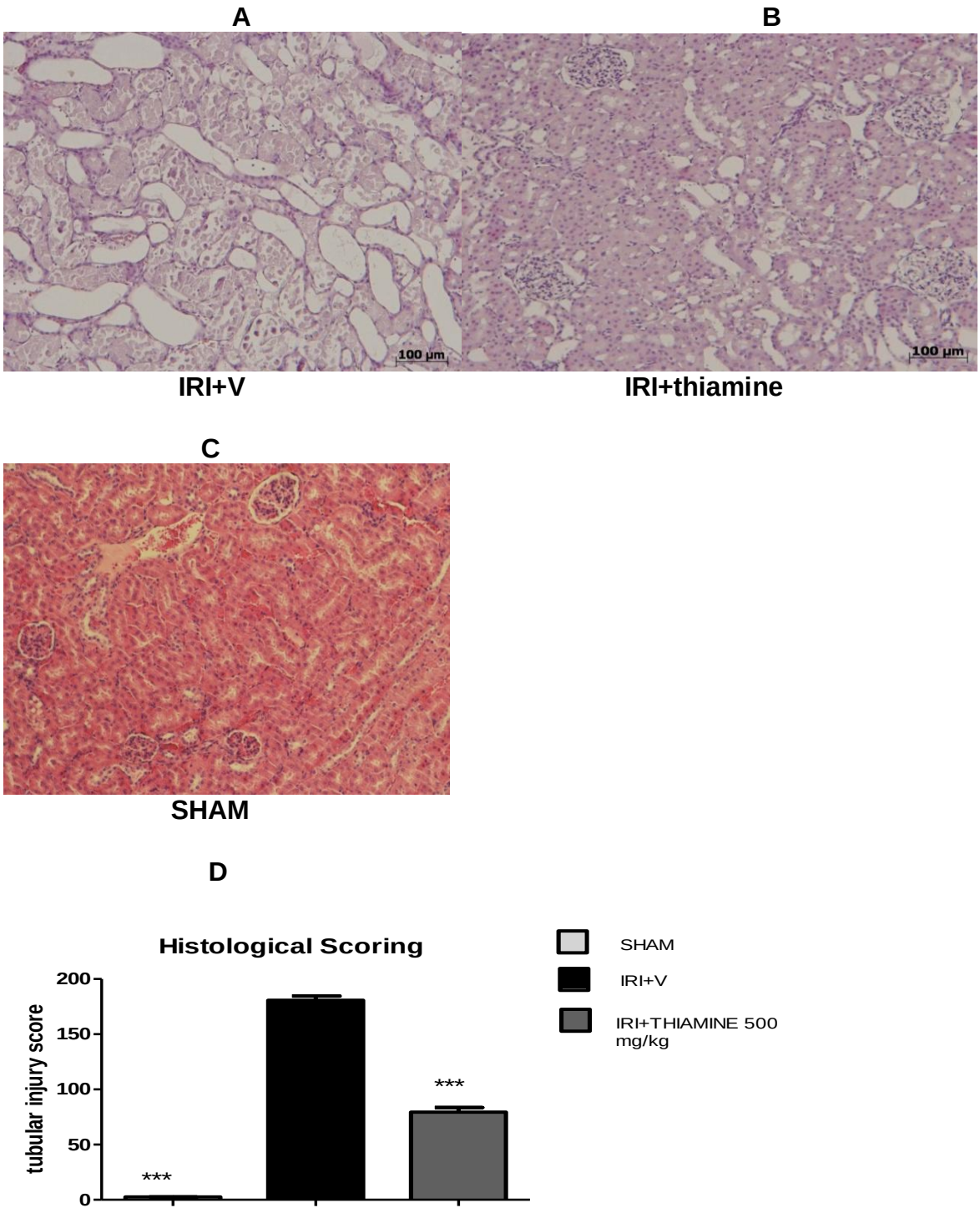


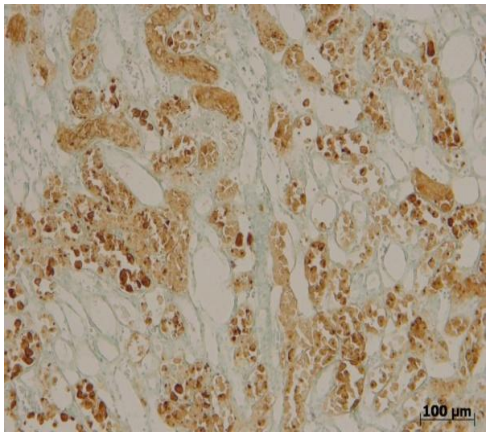
Figure 15 (A-D). Thiamine ameliorates renal dysfunction and attenuates the histological damage caused by renal IRI.

Panels A, B) Renal histopathology of hematoxylin and eosin stained sections are from kidney (representative images of at least three experiments). (D) Quantitative scores of tubular injury in the outer medulla, ***= *P*<0.001, n=6.

3.3.4 Thiamine attenuates apoptosis post renal IRI in rat kidney

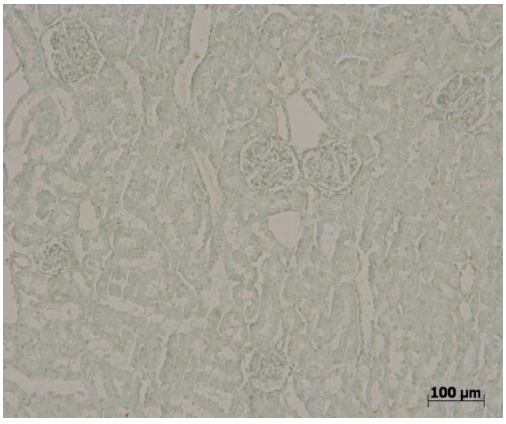
Kidneys from control animals (IRI+V) showed extensive nuclear changes consistent with apoptotic cell death as demonstrated by intense and widespread TUNEL positive staining cells (mean±SD=2.41±0.88) (16 A). Thiamine oral administration (500 mg/kg) for 3 days before ischaemia led to significant reduction in the percentage of TUNEL positive cells (mean±SD=0.31±0.7) when compared to IRI+V (mean±SD=2.41±0.88) suggesting a profound anti-apoptotic effect *in vivo* (Figure 16 B).

A



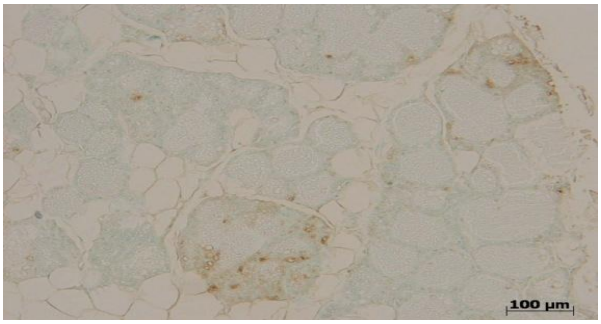
IRI+V

B



IRI+THIAMINE 500 mg/kg.

C



Kit positive control

Figure 16 (A-C). Thiamine has anti-apoptotic effects against renal IRI.

Apoptosis was investigated by TUNEL staining. TUNEL staining revealed strikingly

fewer apoptotic nuclei in rats treated with thiamine (IRI + thiamine, 500 mg/kg) (B) when compared to control (IRI+V) group (A). The number of TUNEL positive cells/high-power field were counted in 5 to 10 fields for each coded slide. There was significant apoptosis seen in IRI+ V and this was significantly reduced by thiamine supplementation, **= $P<0.01$, n=6

3.3.5 Thiamine Attenuates ICAM-1 and COX-2 expression after renal IRI:

Western blots were performed on kidney homogenate to examine the expression of ICAM-1 and COX-2. There was a significant increase in the expression of ICAM-1 and COX-2 in the IRI+V group when compared to the sham group in kidney homogenate and this increase was significantly attenuated in thiamine treated animals (Figure 17, panels A and B).

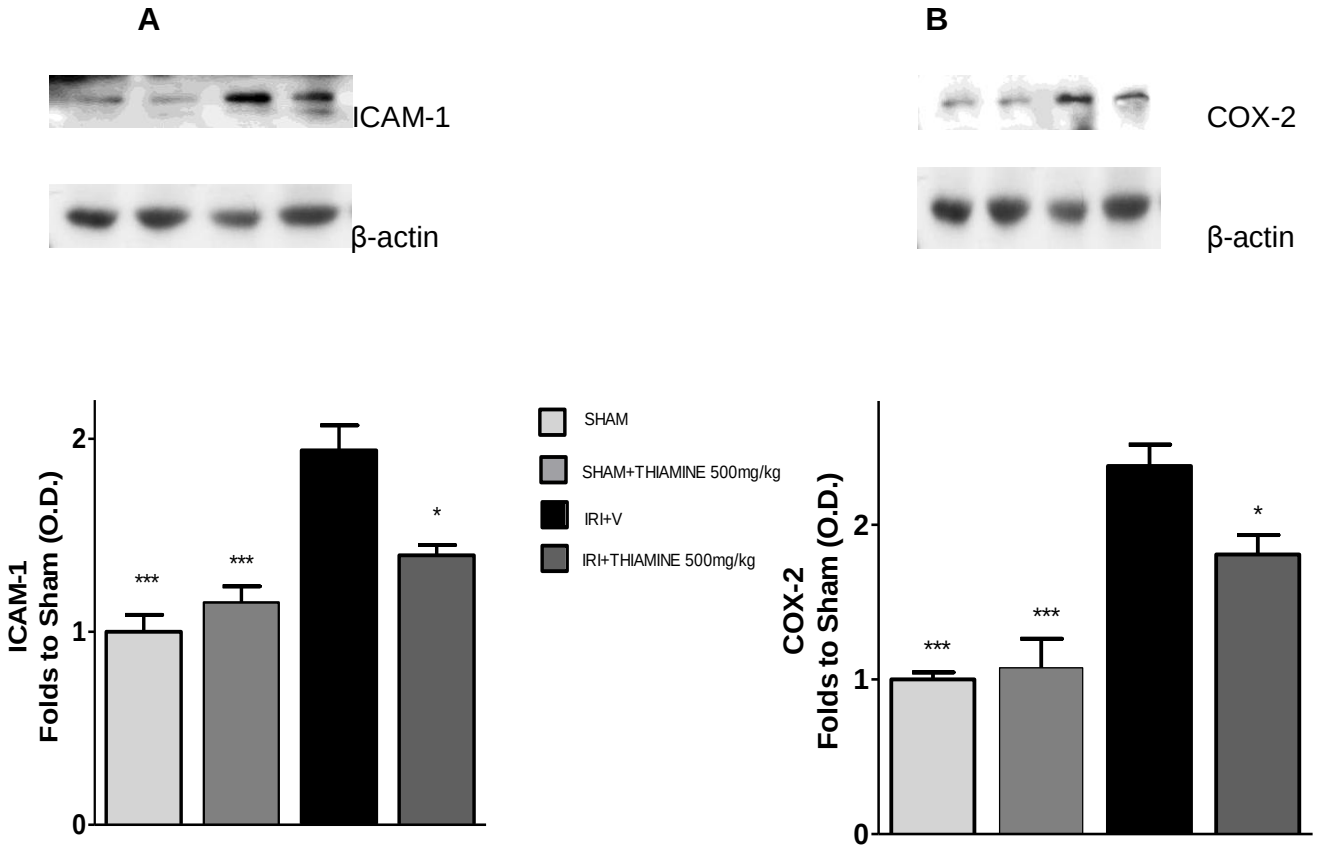


Figure 17. Thiamine has anti-inflammatory effects in renal IRi as it causes significant reduction in expression of (A) ICAM-1 and (B) COX-2.

Each immunoblot is from a single experiment and is representative of three separate experiments. Densitometry analysis of the bands is expressed as relative optical density (O.D.), corrected for the corresponding β-actin and normalised using the related sham-operated band. Data are means ± SD. of three separate experiments, n=6, *= *P*<0.05, ***= *P*<0.001.

3.3.6: Thiamine attenuates oxidative stress and inflammation in renal IRI

DCF assay demonstrated a significant increase in ROS generation in the IRI +V group when compared to sham, and this increase was significantly attenuated by treatment with thiamine (500 mg/kg) for 3 days prior to ischaemia (Figure 18A). End product of lipid peroxidation was assessed by measuring hydroxynonenal (HNE). There was a significant increase in HNE levels in IRI+V group when compared to sham group and this increase was significantly attenuated by treatment with thiamine (500 mg/kg) gavage for 3 days prior to ischaemia (Figure 18B). Furthermore the MPO assay showed an increase in levels in the IRI+V group and this increase was also attenuated by thiamine (500 mg/kg) gavaged for 3 days prior to ischaemia (Figure 18C).

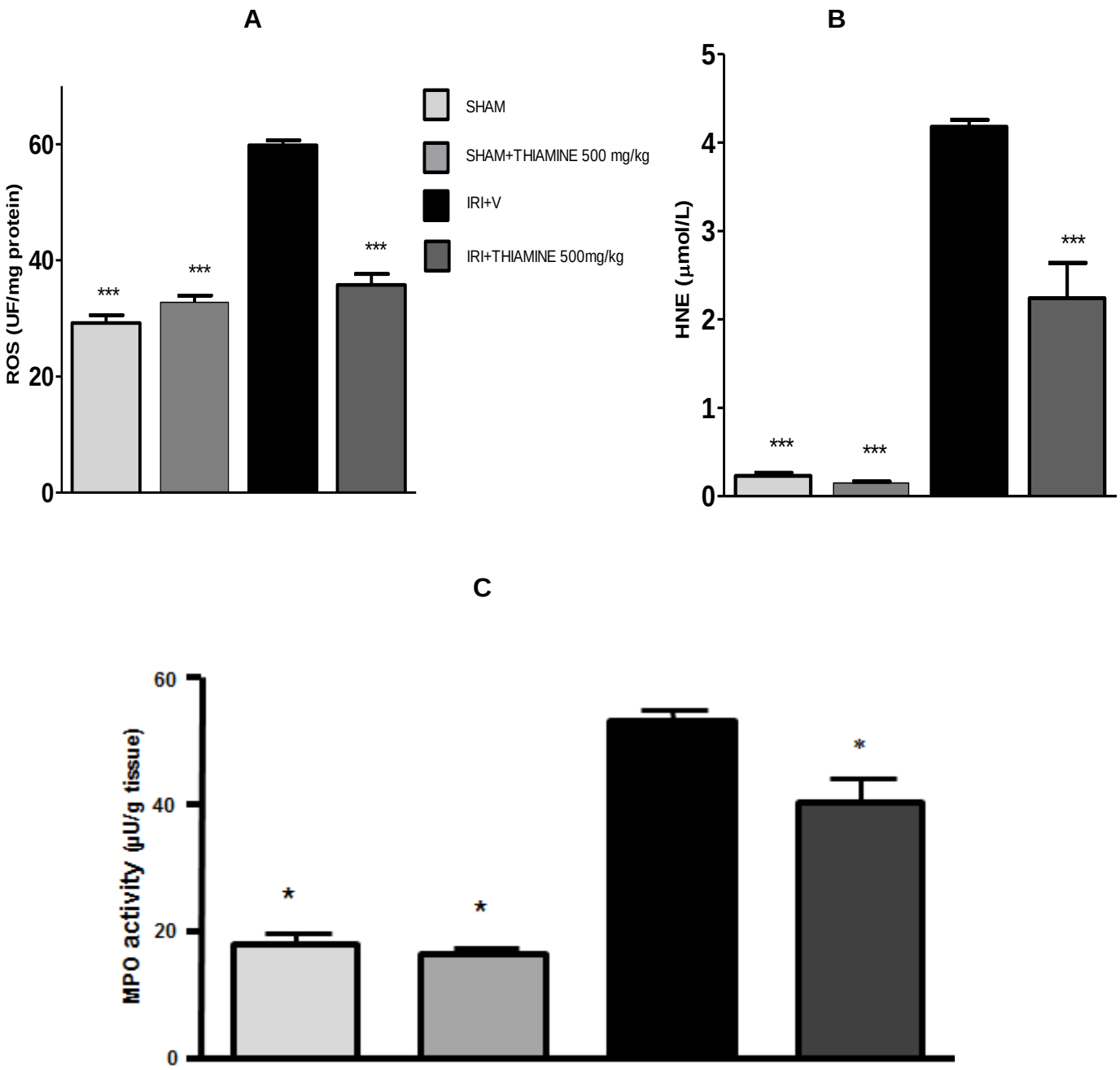


Figure 18. Thiamine has anti-oxidant and anti-inflammatory effects in renal IRI.

Panel (A) shows a significant reduction of ROS in thiamine treated animals when compared to IRI+V group. Panel (B) shows a significant reduction in HNE-4 levels in thiamine treated animals when compared to IRI+V group. Panel (C) shows a significant reduction in MPO activity in thiamine treated animals compared to IRI+ V group, n=6, *= $P<0.05$, ***= $P<0.001$.

3.3.7 Benfotiamine role in renal IRI

Benfotiamine was given to rats by gavaging once daily for 3 days prior to renal IRI at a dose of 250 mg/kg. Sham and IRI+V were gavaged with saline once daily for 3 days prior to renal IRI. At day 3 renal IRI was performed. The results demonstrate that there was a significant increase in serum creatinine in the IRI+V group when compared to sham rats (Figure 19). However, benfotiamine was not able to reduce this increase (Figure 19).

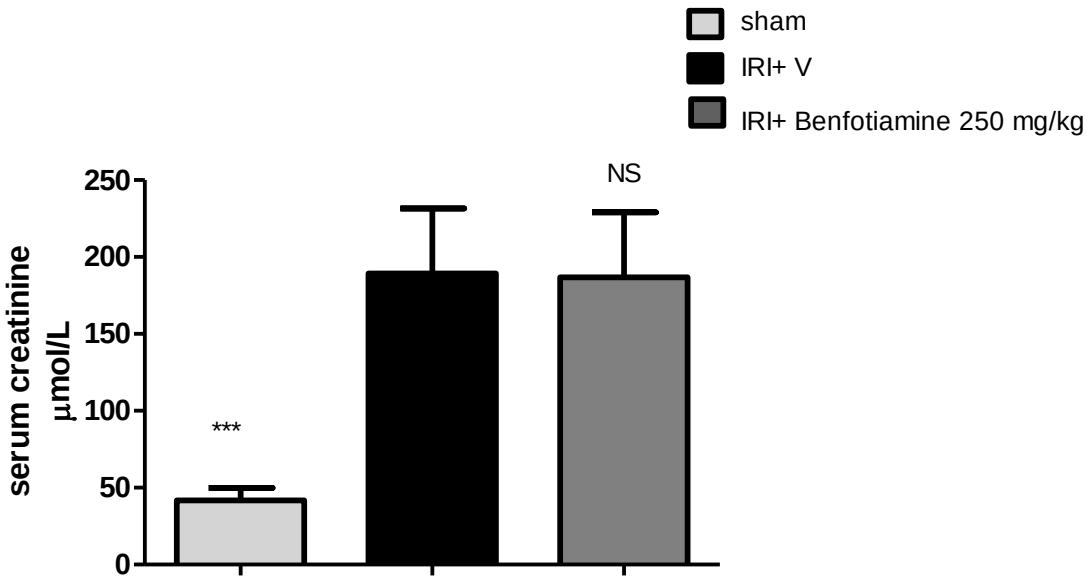


Figure 19. Benfotiamine does not prevent damage and dysfunction casued by renal IRI.

Sham rats and IRI animals received normal water and standard diet for 7 days and were gavaged saline once daily for 3 days prior to ischaemia. The IRI+ benfotiamine (250 mg/kg) group received normal standard diet and were gavaged with benfotiamine once daily for 3 days prior to ischaemia. IRI was performed on all rats except sham in which renal pedicle was not clamped. Blood was collected at 24 h after ischaemia and serum was sent for biochemical analysis. The results provide evidence that pre-treatment with benfotiamine did not attenuate the increase in serum creatinine concentration when compared to IRI group, ***= *P* <0.001, n=6, NS = not significant.

3.4: Discussion

The main findings of these experiments are that (a) thiamine is reno-protective against renal IRI and (b) the direct cytoprotective effect of thiamine on human renal PTECs might contributes to this observed protection and (c) thiamine ameliorates renal IRI by its anti-oxidant and anti-inflammatory properties (d) benfotimaine cannot attenuate the damage and dysfunction associated with renal IRI. Taken together, these observations suggest that thiamine therapy is protective against renal IRI. In comparison to thiamine, benfotiamine was not able to attenuate the damage and dysfunction associated with renal IRI.

Several observations led us to evaluate thiamine in the setting of renal IRI. Firstly, thiamine treatment significantly increased cardiomyocyte survival after a hypoxic insult, and did so by protecting the cells from hypoxia-induced apoptosis associated with caspase-3 activation, PARP cleavage and DNA fragmentation (Larrieu *et al*., 1987, Shin *et al*., 2004). The studies described here are the first to provide evidence that thiamine can prevent renal IRI by an anti-apoptotic effect both *in vivo* shown by reduction in TUNEL positive staining and *in vitro* by preventing apoptosis in HK-2 cells caused by a renal IRI model.

Much data exists to indicate that thiamine can prevent IRI in the brain and heart (Larrieu e*t al*., 1987, Sheline and Wei, 2006, Klooster *et al*., 2007). These studies suggest the possibility that thiamine can have a potential role in the IRI settings. However, a further study conducted by Klooster *et al.* indicated that in the renal IRI setting severe thiamine

deficiency and weight loss is beneficial (Klooster *et al*., 2009), but they believed that the beneficial effects were likely due to fasting rather than due to thiamine deficiency. Furthermore, there is evidence that fasting protects the heart against IRI (Schaefer and Ramasamy, 1997). Our study provides evidence that thiamine can reduce renal IRI that mimics the previous studies that have established the role of thiamine in IRI models of heart and brain(Larrieu et al., 1987, Sheline and Wei, 2006). It is notable that the dose of thiamine used in the work of Klooster *et al* (Klooster *et al*., 2009) was very low (400 μg/daily), both in the control rats and thiamine deficient rats as they received only 0.16 μg/kg of body weight. The renal IRI model was also different to the one presented here as they performed unilateral left kidney ischaemia for 45 min with partial nephrectomy of the right kidney (Klooster *et al*., 2009), whereas our model consisted of a bilateral ischaemia on both kidneys for 45 min. This might be the explanation for the differences in outcome of these studies. The profound reno-protective effects of thiamine that we found were reflected in the histological evaluation of renal sections, as marked preservation of tubular architecture and a concomitant striking reduction in the tubular injury score. Furthermore the work described here has revealed that the kidneys harvested from thiamine treated rats were noted as showing a huge reduction in the TUNEL positive staining and that this anti-apoptotic effect was also seen in an *in vitro* IRI model by the reduction in caspase-3 and preserved cell viability.

Studies demonstrate that thiamine deficiency leads to oxidative stress and that thiamine itself is purported to have anti-oxidant properties (Depeint *et al.*, 2007). During ischaemia there is significant oxidative stress which upon reperfusion leads to the formation of ROS and causes lipid peroxidation (Locatelli *et al*., 2003). This led us to

investigate the role of thiamine in preventing renal IRI through anti-oxidant effects. This hypothesis was proven correct as indicated by the reduction in ROS levels after 24 h of reperfusion in the cytosolic fractions of renal IRI kidneys treated with thiamine (500 mg/kg). An end product of lipid peroxidation, HNE-4 was also reduced in thiamine treated renal IRI kidneys, confirming that thiamine protect through an anti-oxidant mechanism.

Finally some studies have shown that thiamine deficiency is associated with inflammation and that there is an increase in COX-2 and ICAM-1 in the thiamine deficient rat brain (Calingasan *et al.*, 2000). The administration of both anti–ICAM-1 antibodies and the creation of ICAM-1–deficient mice have both been shown to be protective strategies against renal IRI (Kelly *et al.*, 1994, Kelly *et al*., 1996). However, no study has demonstrated that thiamine can ameliorate renal IRI by anti-inflammatory mechanisms. We therefore investigated whether thiamine can reduce inflammatory markers such as MPO, COX-2 and ICAM-1 and found that it could attenuate the increase in these inflammatory markers in a renal IRI setting. As far as we are aware, this study is the first to establish that thiamine can ameliorate injury and dysfunction caused by renal IRI in rats by anti-oxidant, anti-inflammatory and anti-apoptotic mechanisms. This study is also the first to show that thiamine reduces tubular injury (*in vivo*) and tubular apoptosis (both *in vivo* and *in vitro*) thereby providing evidence that thiamine has anti-apoptotic properties. We therefore believe we are the first group to demonstrate that thiamine can ameliorate AKI.

The second part of the study was to evaluate the possible role of benfotiamine in the renal IRI setting. S-acyl thiamine derivatives and lipid-soluble thiamine disulphide derivatives have been developed because they have a 5-10 fold higher bioavailability than thiamine (Volvert *et al*., 2008). Benfotiamine has been able to prevent diabetic nephropathy (Babaei-Jadidi et al., 2003) and play a crucial role in ischaemic conditions (Gadau *et al*., 2006).This led us to investigate the role of benfotiamine in renal IRI at a lower dose compared to thiamine to see if we could prove that lipid soluble benfotiamine has a better bioavailability and could reduce the renal IRI at a much lower dose compared to thiamine. The result of renal IRI study performed on rats shows a significant rise of serum creatinine in the renal IRI group when compared to the sham rats but this increase was not abolished by the supplementation of 250 mg/kg of benfotiamine. This suggests that benfotiamine cannot reduce the degree of renal IRI at a lower dose when compared to thiamine.

There is a debate that whether these S-acyl thiamine derivatives and lipid-soluble thiamine disulphide derivatives are needed when thiamine is available. One of the reason is that tissue uptake of these is highly variable across different tissues and organs (Volvert *et al.*, 2008). Secondly, oral thiamine when given over a 1-week period was able to produce blood levels that approach those obtained by intramuscular and intravenous administration (Baines *et al*., 1988, Royer-Morrot *et al*., 1992). Thirdly, *in vitro* studies that have compared thiamine to thiamine derivatives have generally found them to have similar effects (Babaei-Jadidi *et al*., 2003, Beltramo *et al*., 2004, Beltramo *et al*., 2009, Berrone *et al*., 2006, Karachalias *et al*., 2005, Karachalias *et al*., 2010).

Another study shows that benfotiamine has a different mechanism of action and has a different pharmacological profile than lipid soluble thiamine disulphide derivatives. The study showed that whilst levels of thiamine in blood and liver are increased when supplemented with benfotiamine or sulbutiamine but were low in the brain in the benfotiamine group when compared to sulbutiamine group (Volvert *et al.*, 2008). Furthermore, the same study also suggested that benfotiamine penetrates the cells only after dephophorylation by intestinal alkaline phosphatases. It then enters the blood stream as S-benzoylthiamine before being converted to thiamine in erythrocytes and liver. Moreover, it has been claimed that benfotiamine should not be treated as a true lipid soluble drug (Volvert *et al*., 2008). Although our study with benfotiamine suggests that it can not reduce renal IRI at lower dose than thiamine it still leaves open a window for future research using the newly found thiamine derivatives such as sulbutiamine and allithiamine which have better bioavailability at the tissue level when compared to thiamine and benfotiamine (Volvert *et al*., 2008).

In summary, this is the first study to identify that thiamine can prevent renal IRI and its analog benfotiamine appears ineffective in the same experimental model. It identifies a potential role for thiamine in the prevention of AKI in multiple clinical settings and calls for use of large scale randomized control trials so that this potential therapeutic could eventually be realized.

CHAPTER 4

GENE THERAPY OF HUMAN BMP-7 IN RENAL IRI

4.1 Introduction

BMP-7 belongs to the TGF-β superfamily and is associated with organ development as shown in the eye, bone and kidney nephron formation (Dudley *et al.*, 1995). In adults, BMP-7 is only expressed in a few tissues and its maximum expression is seen in the kidneys. BMP-7 is expressed in the tubules of the outer medulla and glomerular parietal epithelial cells, in podocytes and in renal artery adventitial cells (Vukicevic *et al*., 1998, Simon *et al*., 1999). During renal IRI there is a massive downregulation of BMP-7 mRNA in the outer medulla and glomeruli (Gould *et al.*, 2002). Studies have demonstrated that rhBMP-7 protein can ameliorate the injury and the dysfunction caused by renal IRI at a given dose of 250 µg/kg (Vukicevic *et al*., 1998). Furthermore, BMP-7 plays an important role in renal repair and regeneration of the tubules during ischaemia (Vukicevic *et al.*, 1998). Data suggest that BMP-7 has anti-inflammatory and cytoprotective effects on renal PTECs by suppressing TNF-α stimulated pro-inflammatory cytokines such as IL-6, IL-8 and MCP-1. It also suppresses proximal tubular expression of endothelin-2 and adenosine A_1 but increases the expression of endothelin-1 (Gould *et al.,* 2002) which leads to increase blood flow to the peritubular capillaries. Conversely, the loss of endogenous BMP-7 may lead to profibrotic effects following IRI in the kidney.

BMP-7 has also been shown to reduce interstitial inflammation, fibrogenesis, apoptosis and tubular atrophy in a renal unilateral uretheral obstruction model (Hruska *et al.*, 2000). In addition, BMP-7 has been shown to reverse chronic renal injury by counteracting the TGF-β1 mediated epithelial to mesenchymal transition (Zeisberg *et*

al., 2003). In a streptozotocin-induced type 1 diabetic nephropathy model, BMP-7 levels were found to be decreased and that the use of exogenous administration of BMP-7 was able to attenuate the severity of diabetic nephropathy by reducing tubulointerestitial fibrosis and glomerular pathology and restore GFR back to normal levels and decrease proteinuria (Wang *et al*., 2003).

Gene therapy is the use of DNA as a pharmaceutical agent to treat inherited and acquired disease (Jobs, 2011). It can also be defined as a technique to correct defective genes responsible for a disease. The technique uses DNA to supplement or alter genes within an individual's cells as a therapy to manage and treat disease (Patil P.M, 2012). Gene therapy can be performed by using DNA encoded with a functional gene to replace a mutated gene and this is the most common used form.

Gene therapy can be also be conducted by either directly correcting a mutation or using DNA encoding a therapeutic protein drug rather than a gene to provide treatment. A vector is used to carry the DNA encoding the therapeutic protein and takes the DNA inside cells. When inside the cell the DNA becomes part of the host chromosome and starts producing the desired protein which ultimately treats the disease. Gene therapy can be classified as somatic and germ line gene therapy. Furthermore, the gene delivery systems *in vivo* can be further categorised as viral and non-viral approaches (Liu *et al*., 1999, Crystal, 1995). Evidence suggests that virus gene therapy is an efficient form of transferring foreign genes *in vivo* but it has several major limitations. Construction and preparation of viral vectors is time consuming and some viral vectors are unable to infect non-proliferating cells, making it impossible for substantial

expression of the transgene in most normal and diseased tissues where cell turnover is relatively low. Furthermore, many other viral vectors have shown to induce severe host immune responses that not only result in a short duration of transgene expression but also make the repeated administration extremely difficult or even impossible (Yang *et al*., 2001). Safety in the usage of viral vectors for clinical gene therapy is not yet optimum, (Liu *et al*., 1999) whereas plasmid DNA (pDNA), which is a typical non-viral vector, has advantages in terms of safety compared with a viral vector (Hirayama *et al*., 2004).

Currently naked DNA gene therapy has been gaining increased interest in gene therapy applications as it has the advantages of simplicity of use, lack of immunogenicity and ease of DNA preparation (Walther *et al*., 2005). Many methods have been developed to deliver naked DNA into the desired cells or tissues *in vivo* and *in vitro*, such as *in vivo* electroporation (EP), jet injections, needle and syringe injections, ultrasound and hydrodynamics pressure, particle bombardment (gene gun), intraportal injection and hydrodynamic gene delivery (Lawrie *et al*., 2000, Zhang *et al*., 2000, Sikes *et al*., 1994, Yang *et al*., 1990, Lin *et al*., 2000, Aihara and Miyazaki, 1998, Walther *et al*., 2005).

Research indicates that the direct administration of plasmid vector with the desired genes can be a potential approach to deliver and express foreign genes *in vivo* (Liu *et al*., 1999, Li *et al*., 1997, Yang *et al*., 2001). This method has benefits such as the ease in the preparation of the plasmids and its established safety *in vivo* (Yang *et al*., 2001). A study has shown effective exogenous human EPO gene therapy with an *in vivo* gene transfection procedure by rapid injection of a large volume of naked plasmid DNA

solution via the tail vein (Wang *et al*., 2004). It has been proposed that the injected DNA solution is forced into the liver by hydrostatic pressure as the liver may tolerate large volume of solution. The hydrostatic pressure results in the crushing of endothelial barrier and it is by this mechanism by which highly efficient gene expression is seen in the liver (Wang *et al.*, 2004). This study further identified that the PCMV-EPO plasmid injection into the tail vein of rats when diluted in 3 mL of saline results in a high level of expression of EPO protein in the circulation and can help prevent hypoxic ischaemic encephalopathy (Wang *et al*., 2004). With this background in mind we decided to investigate the effects of BMP-7 gene therapy by injecting naked BMP-7 plasmid DNA in a renal IRI setting.

In rodents, nucleus acid gene delivery by hydrodynamic tail vein injection is a highly competent procedure(Jobs, 2011). Hydrodynamic gene delivery is commonly used due to its simplicity and high efficiency and the nucleic acid gene delivery with this technique has been successful in all major organs with the best results are found in the liver (Jobs, 2011). Research shows that this method of gene delivery in the liver is well tolerated and shows no biochemical evidence of liver injury after eight accumulative doses 15 day apart, whereas efficacy of transgene expression was increased accordingly (Crespo *et al*., 2005, Alino *et al*., 2007, Dicks *et al*., 2009, Jobs, 2011). However, data suggest that there is liver injury seen at 24 to 48 h after hydrodynamic gene delivery but it is temporary and non-damaging event. Furthermore it is a method with many clinical applications but adverse effects of its injection could cause circulatory collapse and death. These hazards of hydrodynamic gene therapy are those that are limiting the translation of this procedure to the clinical practice. With enhanced knowledge of the

mechanisms involved in high gene delivery efficiency it may help in developing a clinical procedure with mild hemodynamic effects (Jobs, 2011, Suda and Liu, 2007). Recent studies on the mechanisms involved in gene therapy via hydrodynamic gene delivery to the liver, provide evidence that improvement s in the technique and to conduct it in large animals by a catheterization procedure (Alino *et al*., 2007, Dicks *et al*. 2010) is a safe and non-invasive method for regional *in vivo* gene delivery under mild condition and may have potential clinical applications.

However clinically, hydrodynamic gene delivery is not considered feasible in humans because of heavy overload of fluid which can cause abnormality in cardiac function and can lead to heart failure. Furthermore, cardiac congestion may be well tolerated by rodents but it will be not considered safe for patients (Suda and Liu, 2007). Although at the experimental level hydrodynamic gene delivery shows promising results, clinically its utility is still unclear.

'Electroporation (EP) is another gene therapy technique available to introduce macromolecules such as nucleic acids into cells, either *in vivo* or *in vitro*, by brief electric pulses to induce transient and reversible permeabilization of the cell membrane'(Sardesai and Weiner, 2011). This method is now been used in clinical trials to deliver nucleic acid and drugs.to many target tissues and organs within the last decade (draghia-akli, 2009, Sardesai and Weiner, 2011). Multiple studies have indicated transient pores are generated due to transmembrane voltage function during EP (Cukjati *et al*., 2007, Trollet *et al*., 2006). Researchers have shown that macromolecules present in the extracellular medium surrounding the target cells gain access to the intracellular environment during the period of membrane destabilisation

within nano to milli seconds (Becker and Kuznetsov, 2007). Furthermore there is then a slow resealing of the membrane (on the s to min time scale) after the EP pulses. Although the exact mechanism of translocation of DNA across the membrane pores is debatable (electrophoretic facilitation versus passive diffusion) the end result of this process is upwards of 100–1000 fold enhancement of plasmid delivery and gene expression can be achieved relative to delivery of DNA alone without EP (Sardesai and Weiner, 2011). EP is an easy to perform non-viral gene therapy technique that requires only a plasmid and a device to perform EP (Kawai *et al*., 2006). The technique of EP can play an important role in the development for gene therapeutic approaches with the potential to treat many conditions with a single low dose of plasmid resulting in long-term benefits (Bodles-Brakhop *et al*., 2009).

A pilot study was conducted on large animals with plasmid growth hormone releasing hormone (GHRH) with EP to treat renal failure and to investigate its possible side effects. This study had shown that malignant melanoma can be treated by DNA delivery of xenogenic human tyrosinase DNA by EP successfully in a Phase I veterinary trial conducted on dogs (Bergman *et al*., 2003, Brown *et al*., 2009). A more recent study showed that this gene therapeutic approach by EP can attenuate the complications of CKD by increasing the life expectancy, wellbeing, and quality of life of animals with experimentally induced renal failure (Brown *et al*., 2009). Earlier studies using GHRH showed that plasmid therapy with EP can be a promising technique to produce and regulate the secretion of proteins in both experimental animal models and in humans (Tone *et al.*, 2004, Brown *et al*., 2004, Brown *et al.*, 2008, Bodles-Brakhop *et al*., 2008). Furthermore, it does not require viral vectors, expensive proteins and carrier matrices.

The technique appears to be of particular utility to our study because the direct BMP-7 protein administration has been shown to cause ectopic bone formation at the site of injection (Ripamonti *et al*., 2001a, Ripamonti *et al*., 2001b) Indeed data show that non-viral gene therapy with the EP of BMP-7 simultaneously with BMP-2 has been successful in gastrocnemius muscle and was not associated with ectopic bone formation (Kawai *et al*., 2006). As mentioned earlier, the exogenous administration of BMP-7 protein has been shown to be beneficial in renal IRI (Vukicevic *et al*., 1998) and as gene therapy with EP has been shown to be previously successful we hypothesise that gene therapy of BMP-7 with EP might have an important therapeutic value in preventing the injury and the dysfunction caused by renal IRI in mice.

4.2 Materials and methods

4.2.1. Naked DNA gene delivery

Control and BMP-7 plasmid DNA were delivered by direct injection. The injections were prepared by diluting DNA in 2.5 mL of 0.9% NaCl solution. Endotoxin-free plasmid for injection was prepared in 0.9% NaCl as a vehicle control 5-50 μg of naked DNA was injected into the tail vein. An ELISA for BMP-7 was performed on serum and kidney homogenate to assess the success of gene delivery.

4.2.2 Rat model of renal IRI

The protocol was the same as described in chapter 2.2.1. Briefly, the procedure requires the renal pedicles bilaterally clamped for 45 min with microaneurysm clamps followed by reperfusion for 24 h with the time of ischaemia chosen to obtain a reversible model of ischaemic AKI and to avoid animal mortality. Sham surgery consisted of the same surgical procedure without the clamps being applied.

4.2.3 Assessment of renal function

The protocol was as described in section chapter 2.2.2. Briefly, centrifugation of the collected blood for 3 min at 9000 *g* yielded the serum. The veterinary clinical pathologists, IDEXX Laboratories, Sussex, UK; blinded to the treatment given, performed serum creatinine measurements.

4.2.4 Experimental design

A total of 40 rats were used in this study and they were divided into the following 3 groups. Control rats were directly injected with control PCMV SPORT6 DNA (PCMV SPORT6 is the backbone vector) 3 days before ischaemia. IRI BMP-7 group were directly injected with human BMP-7 DNA 3days before ischaemia. The sham group were directly injected with control PCMV SPORT 6 DNA, again 3 days before ischaemia. All rats received normal water and standard diet and the same surgical procedures were performed for all animals except in the sham group where the arterial clamps were not applied.

4.2.5 Mice IRI model

Male mice C57/BL6 wild-type mice (WT, 25–30 g; Charles River Laboratories, Margate, UK) received a standard diet and water *ad libitum*, and were cared for in accordance with both the UK Home Office Guidance in the Operation of the Animals (Scientific Procedures) Act 1986, published by Her Majesty's Stationery Office, London, UK. Mice were subjected to renal IRI as previously detailed (Chatterjee and Thiemermann, 2003). First the mice were anaesthetised with ketamine (100 mg/mL) and xylazine (20 mg/mL) (2:1) at a dose of 1.5 mL/kg, i.p. and their abdominal hair was shaved and the skin cleaned with 70% alcohol (v/v). The mice were then placed upon a heated blanket set to a temperature of 37°C and a mid-line laparotomy was performed and their renal pedicles (consisting of the renal artery, vein and nerve) were isolated and clamped using non-traumatic microvascular clamps at time 0 as described before (Chatterjee and Thiemermann, 2003). After 30 min of acute bilateral ischaemia, the clamps were removed, the skin was then sutured following administration of buprenorphine (0.1

mg/kg, s.c.) for pain control, and the mice were allowed to recover from anaesthesia and returned to cages. At 24 h the animals were sacrificed, blood samples were collected via cardiac puncture and the kidneys harvested. The kidneys were cut longitudinally into two halves and each half from respective kidneys were stored in 10% formalin for histological analysis or were immediately snap frozen in liquid N_2 for further experiments. Mice subjected to IRI were electroporated with control PCMV-SPORT 6 DNA or human BMP-7 DNA (both at 25-50 μg/mL) 2 days before renal IRI. Sham operated mice underwent the same surgical procedures except ligation of renal pedicle and the experiments were performed in parallel.

4.2.6 Assessment of renal function in mice

Serum creatinine was measured as mentioned before (see chapter 2.2.2).

4.2.7 Plasmid purification

PCMV SPORT 6 control plasmid and human BMP-7 plasmid were purchased from I.M.A.G.E Clone, Source Bioscience, Nottingham, UK). Liquid culture of E.coli containing control or BMP-7 plasmids were grown in 12 mL tubes containing 5 mL of sterile autoclaved L-broth (Sigma) at a concentration of 20 g/L, with ampicillin 100 μg /mL and were left on an orbital shaker at 200 rpm at 37°C overnight. Next day, glycerol stocks were made with glycerol concentrations of 15% and 50% in control and BMP-7 plasmid containing culture and were stored at -80°C. The same procedure was performed onto a larger scale using a large sterile 1 L flask. The next day the large cultures were poured in to sterile autoclaved 500 mL tubes and were spun at 5000 *g* for

30 min and the resulting pellet was used for plasmid purification. Plasmid DNA was purified using the Plasmid Mega Kit (Qiagen Ltd, Crawley, UK), or when required for injection into mice using the EndoFree™ Plasmid Mega Kit (Qiagen Ltd). Plasmid DNA concentration was quantified using a Nanodrop spectrophotometer (Thermo Scientific, Loughborough, UK).

4.2.8 Experimental design

A total of 24 mice were used in this study and they were divided into the following groups. Control mice were electroporated with control PCMV SPORT 6 DNA 2 days before ischaemia. IRI BMP-7 group was electroporated with human BMP-7 DNA. The sham group was electroporated with control PCMV SPORT 6 DNA, again two days before ischaemia. All mice received normal water and standard diet and the same surgical procedures were performed for all animals except in the sham group where the arterial clamps were not applied.

4.2.9 Transfections

Transfections were performed using the OPTIFECT™ reagent (Invitrogen Life Science, Paisley, UK) according to the manufacturer's protocol. HK-2 and 293-T cells were seeded onto 6 well plates with DMEM, 10 % FCS, without antibiotics and were incubated for 24 h. When 30-50 % confluent, 1 μg of control DNA or BMP-7 DNA was diluted in DMEM without FCS and incubated for 5 min in an incubator. Simultaneously, 12 μL of OPTIFECT™ was diluted in 250 μL of DMEM and incubated for 5 min. Diluted DNA and diluted OPTIFECT™ were combined and incubated for 20 min. Control or

BMP-7 DNA lipid complexes were added to their cultured cells and incubated for 24 to 48 h. Supernatants were collected at the end of the study period and the ELISA described below (4.2.10 ELISA for detecting BMP-7 in serum and transfected cells) was performed to determine BMP-7 expression.

4.2.10 ELISA for detecting BMP-7 in serum and transfected cells

A BMP-7 ELISA was performed on the serum of mice and the supernatant of transfected 293 T (Human Embryonic Kidney 293 cells) and HK-2 cells in order to assess the expression of BMP-7. Blood samples were collected at different time points both at day 2 after EP and at day 4. The supernatant from 293 T cells and HK-2 cells were collected at day 2 after transfection. The ELISA was performed according to manufacturer's instructions (DuoSet for BMP-7, R&D Systems).

Briefly, a 96 well microplate was coated with capture antibody at a concentration of 2 μg/mL in PBS (pH 7.3) and incubated overnight at room temperature. The plate was then aspirated, washed 4 times with washing buffer (0.05% Tween 20 in PBS, pH 7.2 - 7.4) and was blocked by adding blocking buffer 300 μL (1% BSA in PBS, pH 7.2 - 7.4, 0.2 μm filtered) and incubated at room temperature for 1 h. The plate was again washed 4 times with washing buffer and standards and sample were added at 50 μL per well and were incubated for 3 h at room temperature. The plate was then washed 4 times and a detection antibody was added at a concentration of 0.5 μg/mL and left to incubate at room temperature for a further 2 h. The plate was again washed 4 times and streptavidin-HRP was added at a concentration of 1:200 in reagent diluent to each well

and incubated for 30 min in the dark. Washing was repeated as mentioned above and substrate solution (100 μL) was added to the wells and left in the dark for 30 min. After 30 min, H_2SO_4 (2.5 mmol/L stop solution) 50 μL was added to prevent further colour change. The plate was then read at 450 nm with a reference reading at 650 nm in a microplate reader (Dynex Jencons PLS Technologies).

4.2.11 In vivo electroporation (EP)

Mice were treated in according to the UK Home Office Guidance in the Operation of the Animals (Scientific Procedures) Act 1986. Mice were anaesthetized with isoflurane (Concord Pharmaceuticals Ltd, Dunmow, UK) using Boyle's apparatus (BOC Ltd, London, UK). The fur covering the right and left anterior tibialis muscle of the leg was shaved and the exposed skin sprayed with disinfectant. Endotoxin-free plasmid for injection was prepared in a solution of 0.9% NaCl. DNA (25-50 μg) was injected into the anterior tibialis muscle, and Camcare ECG gel (Camcare Gels, Mepal, Cambridgeshire, was then applied to the surface of the skin. Caliper electrodes 384L (BTX Instrument Division, Harvard Apparatus Inc., Ltd, Kent, UK) were applied transversely across the anterior tibialis muscle was electroporated with four pulses at 200 V/cm and 20 ms duration at a frequency of 2 Hz using a BTX Electro Square Porator ECM 830 (Harvard Apparatus Ltd) (Gould *et al*., 2004). The polarity of the electrodes was then reversed and the procedure repeated.

4.2.12 In vitro renal IRI model

As described in chapter 2.2.6.

4.2.13 Cell viability (MTS) assay

As described in chapter 2.2.7.

4.2.14 Caspase-3 assay

As described in chapter 2.2.9.

4.2.15 Materials & statistical analysis

As described in chapter 2.2.13 and 2.2.14 unless specified.

4.3. Results

4.3.1 Naked BMP-7 DNA does not protect against apoptosis caused by in vitro renal IRI model in PTECs

Caspase-3 activity was significantly increased in the lysates derived from HK-2 cells treated with antimycin A (50 μmol/L) and 2-deoxyglucose (10 mmol/L) in HBSS (AA) when compared with lysates derived from control treated cells (HBSS only). Naked BMP-7 DNA (1-50 μg/mL in 2.5 mL DMEM) treatment did not attenuate this increase (Figure 20).

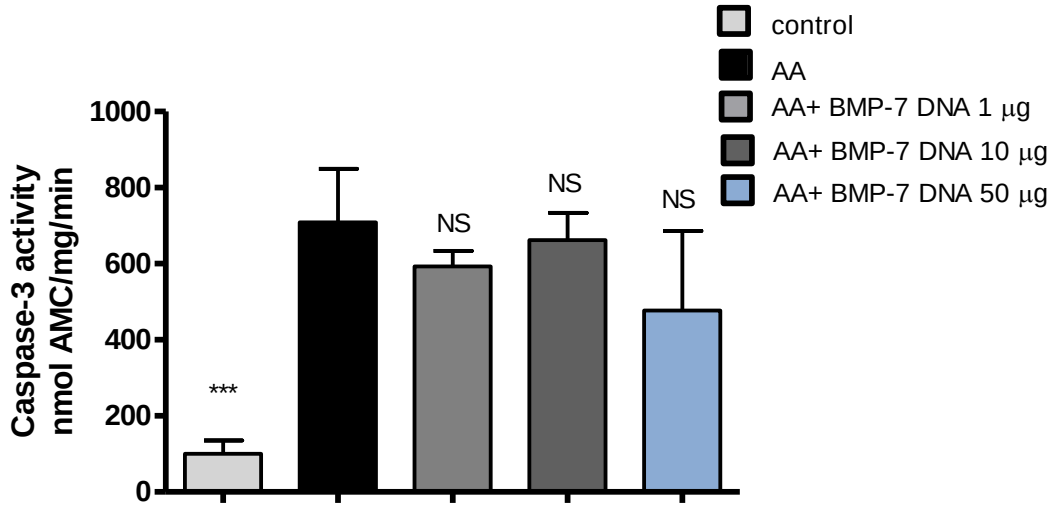


Figure 20. BMP-7 DNA does not protect against apoptosis in HK-2 cells in an *in vitro* renal IRI model.

HK-2 cells were incubated with 50 μmol/L of antimycin A plus 10 mmol/L 2-deoxyglucose with HBSS for 1 h (AA) to induce ischaemic injury *in vitro*. The *in vitro* reperfusion was achieved by incubating cells in DMEM with FCS 10% and antibiotic 1%. Cells were allowed to grow for 24 h in an incubator. HK-2 cell apoptosis was significantly increased in the AA model group compared to control group P <0.001, which was not attenuated by naked BMP-7 DNA 1-50 μg/mL in 2.5 mL DMEM if given at time of ischaemia, ***= P<0.001, n= 3.

4.3.2 Naked BMP-7 DNA does not cause upregulation of BMP-7 protein in PTECs

PTECs (HK-2) were cultured in 6 well plates for 24 h. When around 50-60 % confluent the cell culture media was replaced with or without different concentrations of BMP-7 DNA. After 24 h the cells were scraped from the wells, centrifuged at 3000 *g* for 5 min and the media was collected for BMP-7 quantification. ELISA was performed on supernatants to access the expression of BMP-7 protein. The results demonstrate that there was no upregulation of BMP-7 protein in the BMP-7 group (data not shown).

4.3.3 Expression of human BMP-7 in transfected 293-T and HK-2 cells

Supernatants from transfected 293 T and HK-2 cells with control DNA or human BMP-7 were collected after 24 h and the ELISA was performed to quantify BMP-7 protein. The results showed that the transfection of 293 T cells was much more efficient than for HK-2 cells (Table 3).

Table 3: Transfected 293 T cells show expression of human BMP-7 protein after 24 h.

	Control DNA transfection	**BMP-7 DNA transfection**
293 T cell	Not detectable	598 ± 49 pg/mL
HK-2 cell	Not detectable	Not detectable

Table 3: 293 T cells were found to be more suited to transfection than HK-2 cells. BMP-7 expression was detected by ELISA as mentioned above.

4.3.4: Injected naked BMP-7 DNA causes upregulation of BMP-7 in kidney homogenate

Rats were injected with control PCMV SPORT 6 DNA and human BMP-7 DNA. DNA 5- and 50 μg was diluted with 2.5 mL of saline. Kidneys were harvested after 24 h after of renal IRI and were homogenised with RIPA buffer (see chapter 2.2.9.1). The ELISA was performed to quantify BMP-7 in the kidney homogenate The results of adding 5 μg BMP-7 DNA demonstrated that there was an upregulation of BMP-7 in the BMP-7 group, when compared to controls in a few of rats but was not statistically significant (Figure 21). For the higher dose the ELISA results of adding 50 μg BMP-7 DNA showed that there was significant upregulation of BMP-7 in kidney homogenate compared to the controls (Figure 22).

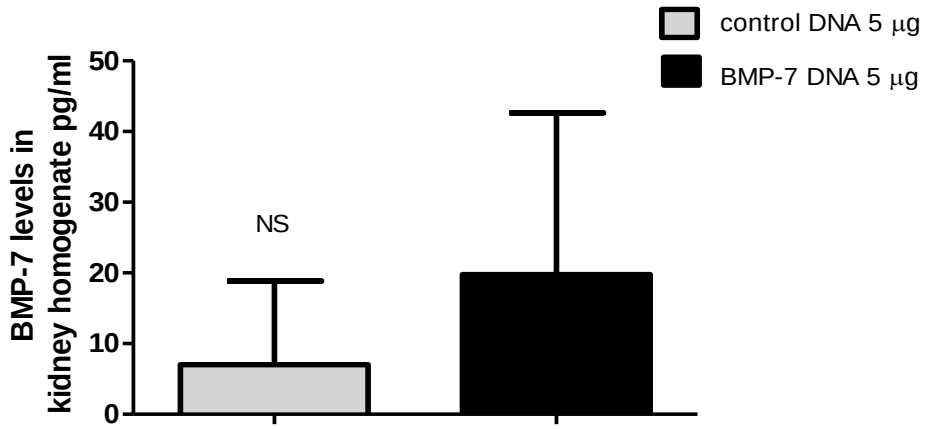


Figure 21. Expression of BMP-7 in the rat kidney homogenate after renal IRI at day 4.

Control and BMP-7 DNA (5 μg diluted in 2.5 mL of saline and injected into the tail vein 3 days prior to renal IRI was performed on rats as recorded in the method. Kidneys were collected on day 4 after renal IRI. An ELISA was performed on kidney homogenate to assess human BMP-7 concentrations. There was an increase in human BMP-7 concentrations on day 4 after renal IRI in rat kidneys of BMP-7 group, when compared to the control group but was not statistically significant. **= P<0.01, n=3, NS = not significant.

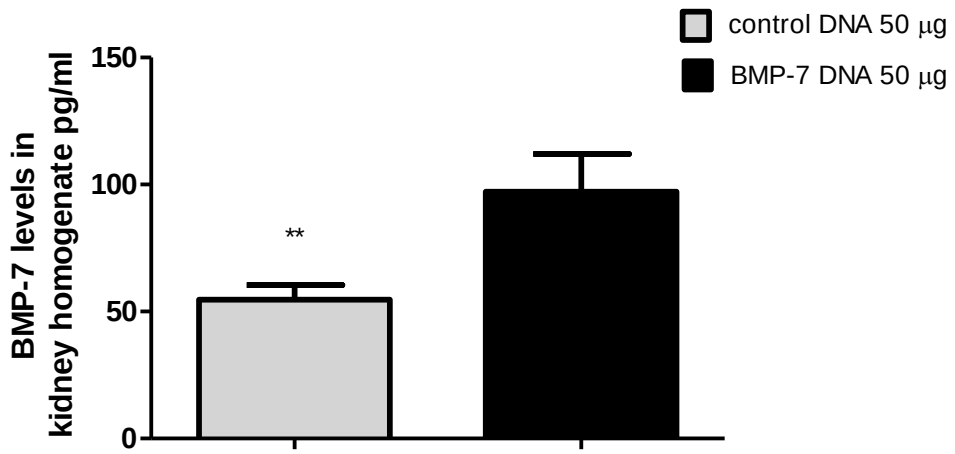


Figure 22. Figure shows that there is significant expression of BMP-7 in the rat kidney homogenate after renal IRI at day 4.

BMP-7 DNA (50 μg) and control DNA (50 μg) was diluted in 2.5 mL of saline and injected into the tail vein 3 days prior to renal IRI was performed on rats as recorded in the method. Kidneys were collected on day 4 after renal IRI. An ELISA was performed on kidney homogenate to assess human BMP-7 concentrations. There was a significant increase in human BMP-7 levels on day 4 after renal IRI in rat kidneys of BMP-7 group, when compared to the control group. **= $P<0.01$, n=3, NS = not significant.

4.3.5 Expression of BMP-7 levels in serum after naked BMP-7 DNA injection:

Rats were injected with control PCMV SPORT 6 DNA and human BMP-7 DNA. DNA 5-50 μg was diluted with 2.5 mL saline. Blood was collected at day 4 after renal IRI and the serum was separated by centrifuging at 9000 *g* for 3 min. The ELISA was performed to quantify the BMP-7 concentration in serum. The results of both the 5 and 50 μg BMP-7 DNA treatments demonstrated there was no upregulation of BMP-7 in the BMP-7 group when compared to controls (data not shown).

4.3.6 Injected naked BMP-7 DNA therapy failed to attenuate the increase in serum creatinine after 24 h of renal IRI:

In comparison with sham rats, IRI+ Control DNA 5-50 μg injected into rats 3 days prior to renal IRI (IRI + control DNA) had significant renal dysfunction at 24 h as suggested by a significant increase in serum creatinine concentration (Figure 23). Rats injected with BMP-7 5-50 μg 3 days before ischaemia in IRI+BMP-7 group did not show any significantly attenuation of this increase in serum creatinine (Figure 23).

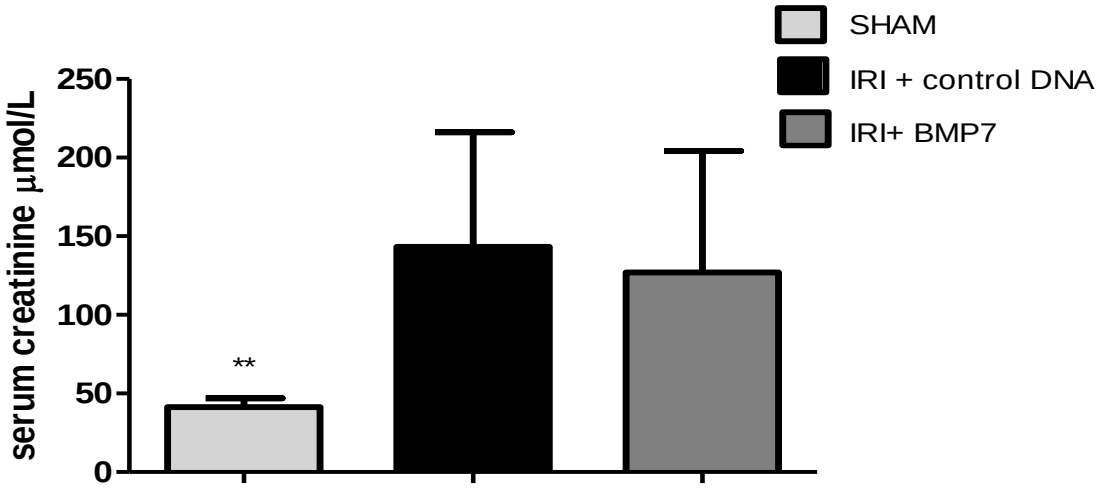


Figure 23. BMP-7 DNA injected by tail vein does not reduce injury and dysfunction caused by renal IRI in rats.

All rats received normal water and standard diet for 7 days. IRI was performed on all rats except sham in which renal pedicle was not clamped. Blood was collected at 24 h after ischaemia. The results provide evidence that pre-treatment with injecting BMP-7 DNA did not attenuate the increase in serum creatinine concentration when compared to IRI+ control group, **= *P* <0.01, n=9, NS = not significant.

4.3.7 BMP-7 ameliorates apoptosis in HK-2 cells caused by an in vitro IRI model:

Caspase-3 activity was significantly increased in the lysates derived from HK-2 cells treated with antimycin A (50 μmol/L) with 2-deoxyglucose (10 mmol/L) in HBSS (AA) when compared with controls (HBSS only). rhBMP7 (10-100 ng/mL) attenuated this increase (Figure 24). This anti-apoptotic effect of rhBMP-7 was translated into preserved cell viability as assessed by MTS assay (Figure 25).

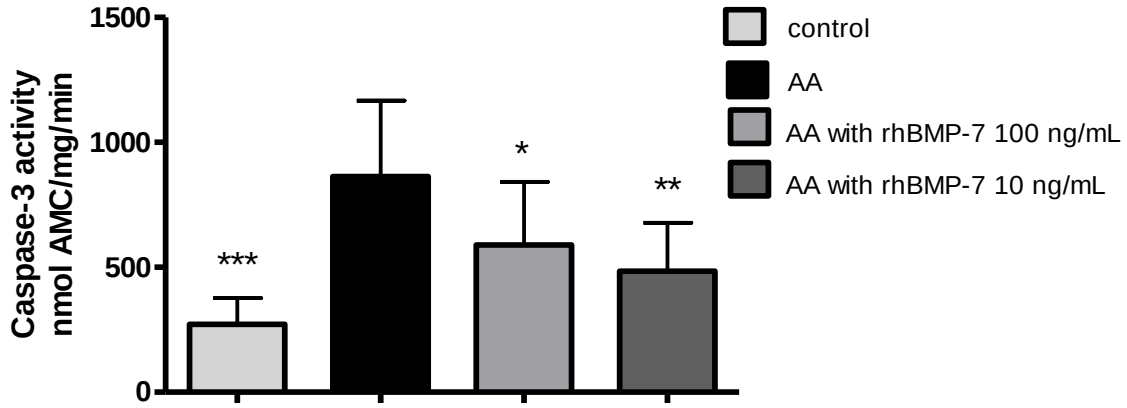


Figure 24. BMP-7 protects HK-2 cells against cellular IRI induced apoptosis.

HK-2 cells were incubated with 50 μmol/L antimycin A plus 10 mmol/L 2-deoxyglucose with HBSS for 1 h (AA) to induce ischaemic injury *in vitro*. The *in vitro* reperfusion was achieved by incubating cells in DMEM with 10% FCS and 1% antibiotic solution. Cells were allowed to incubate for a further 24 h. Caspase-3 activity was significantly increased in AA model group compared to the control group, P <0.01, which was attenuated by rhBMP-7 10-100 ng/mL if given with *in vitro* ischaemia, *= P<0.05, **= P<0.01, ***= P<0.001, n= 4.

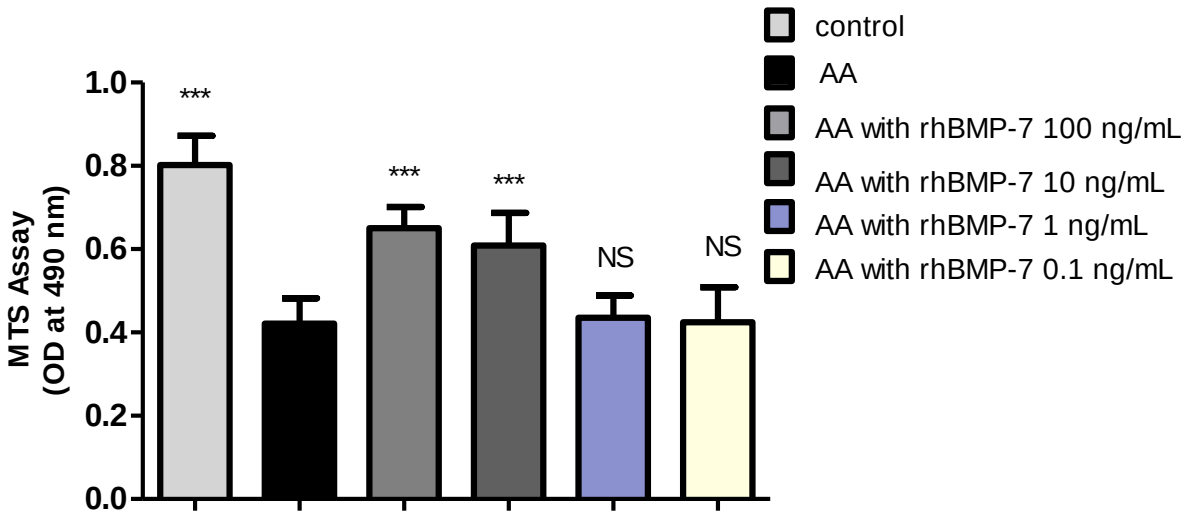


Figure 25. BMP-7 preserves viability of HK-2 cells in the *in vitro* renal IRI model.

HK-2 cells were incubated with 50 μmol/L of antimycin A plus 10 mmol/L 2-deoxyglucose with HBSS for 1 h (AA) to induce ischaemic injury *in vitro*. The *in vitro* reperfusion was achieved by incubating cells in DMEM with 10% FCS and 1% antibiotic solution. Cells were allowed to incubate for a further 24 h. Cell viability was significantly decreased in the AA model group compared to control group P <0.001, which was preserved by rhBMP-7 10-100 ng/mL if given with *in vitro* ischaemia, ***= P<0.001, n= 8.

4.3.8 BMP-7 expression in mice serum after EP on anterior tibialis muscle:

Mice were electroporated (EP) with control PCMV SPORT 6 DNA and human BMP-7 DNA. Blood was collected at day 2 and 4. The ELISA was performed to quantify BMP-7 in the serum. There was significant upregulation of BMP-7 in the BMP-7 group on day 2 when compared to controls. BMP-7 expression remained high on day 4 but this increase was found not to be statistically significant (Figure 26).

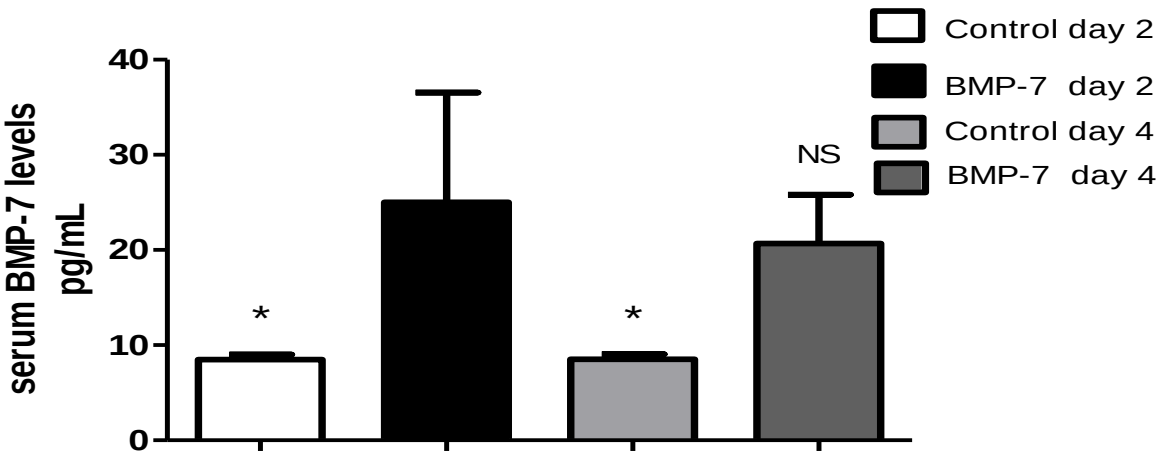


Figure 26. BMP-7 DNA with EP significant increased expression of human BMP-7 on day 2.

EP was performed on mice as recorded in the method. Blood was collected on day 2 and 4. ELISA was performed to assess human BMP-7 concentrations in the mice serum. There was a significant increase in human BMP-7 levels on day 2, when compared to the control group.at day 2 and 4 *= P<0.05, n=5, NS = not significant.

4.3.9 Effects of EP of BMP7 in renal IRI in mice:

In comparison with the sham mice, IRI+Control EP mice had significant renal dysfunction at 24 h as illustrated by a significant increase in serum creatinine concentrations (Figure 27). However, EP of BMP-7 3 days before ischaemia (IRI+BMP-7 EP) did not significantly attenuate this increase (Figure 27).

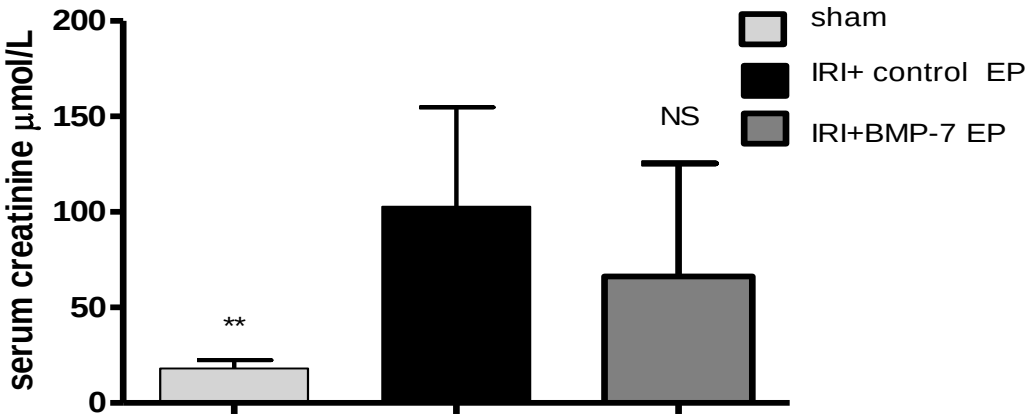


Figure 27. EP of BMP-7 DNA did not attenuate the increase in serum creatinine caused by renal IRI in mice.

There was significant increase in the serum creatinine concentrations in the IRI+Control EP group when compared to sham mice. This increase in serum creatinine at 24 h post-reperfusion was not significantly abrogated by the EP of BMP-7 3 days prior to ischaemia, n=6, ** = P<0.01, NS= not significant.

4.4 Discussion

The main findings of the naked DNA gene therapy study were that (a) naked DNA cannot reduce apoptosis in PTECs, (b) naked DNA without transfection in PTECs cannot up regulate BMP-7 protein levels, (c) naked DNA injection in rats causes the up regulation of BMP-7 protein in the kidney when compared to control DNA but no serum BMP-7 was detected (d) and although,BMP-7 levels were elevated on day 2 after renal IRI, it was not found to attenuate the damage and dysfunction caused by renal IRI.

Previous work conducted on the proximal tubules of rats indicates that gene therapy can be done successfully with retroviral vectors with transfer of the *LacZ* gene into the proximal tubules of rats after injuring the kidney with a nephrotoxic dose of folic acid (Bosch *et al.*, 1993). This injury leads to regeneration of tubular cells that are necessary for infection with the retrovirus; otherwise, the majority of the adult PTECs would be in an inactive state and would not be infected (Bosch *et al*., 1993). Other work shows that the systemic injection of liposome-DNA complexes with mainly transfected endothelial cells (Wissing *et al*., 1997) and that gene expression mostly resides in the tubular epithelial cells following intravenous injection of naked DNA (Wu *et al*., 2005).This study further identifies that specific transient gene expression in the renal tubular epithelial cells can be demonstrated following injection of exogenous, naked DNA via systemic injection. Furthermore, the level of kidney-specific gene expression was concentrated in the corticomedullary region and predominantly in the proximal tubular cells. There was little or no expression of the exogenous gene detected in the distal convoluted tubules and it provides evidence that naked DNA delivery can be a successfully used in

targeting the kidney for gene therapy (Wu *et al*., 2005). This suggests that there can be a potential role of naked DNA gene delivery in the setting of renal IRI and this led us to investigate whether naked DNA injection of BMP-7 can enhance the levels of BMP-7 in serum and kidney and whether it can protect renal IRI.

The results from our study show that there was no upregulation of BMP-7 in the serum after renal IRI but that the renal level of BMP-7 was significantly higher in the group injected with naked BMP-7 DNA 50 μg when compared to the control DNA group. One of the likely explanations for the upregulation of BMP-7 can be that the kidneys have membrane-bound, specific, high-affinity BMP-7 receptors able to mediate BMP-7 actions. The major BMP-7 binding component of kidney may be a long form of BMP type II receptor with a molecular weight of 100 kDa (Bosukonda *et al*., 2000).

Our findings are in accordance with others who have demonstrated that naked DNA can cause upregulation of gene expression in the kidney (Wu *et al*., 2005). Furthermore, previous research shows that the *in vivo* cellular targets for BMP-7 are the convoluted tubule epithelium, the glomeruli in the cortex and the collecting ducts in the medulla region (Bosukonda *et al*., 2000). An alternative reason for the non-detection of BMP-7 protein in serum could be due to the presence of large amount of endonuclease in the circulation and as a result, the plasmid DNA is rapidly degraded following delivery into the blood circulation and therefore no gene expression could be detected (Wang *et al*., 2004). A likely reason for naked plasmid DNA therapy not attenuating renal IRI can be that the gene transduction is very low and expression of transgene is temporary when naked plasmid DNA is injected in animals (Hara *et al*., 1997, Kanemura *et al*., 2008)

which is seen in our study with low levels of BMP-7 expression in the kidney. Research indicates that this problem can be overcome by hydrodynamic gene delivery as it is considered to provide better expression of gene/protein expression after gene therapy (Crystal, 1995, Zhang *et al*., 1997, Liu *et al*., 1999) but this method of gene delivery has its own severe limitations clinically as it can cause heart failure and hepatic injury. Studies have shown that this problem of volume overload caused by hydrodynamic delivery can be overcome by delivering the gene via the inferior vena cava and this study provides hopes for future work (Wu *et al*., 2005).

Although previous research shows that BMP-7 protein can prevent apoptosis (Mitu *et al*., 2007), there have been no other reports showing that using naked DNA BMP-7 gene therapy in the setting of *in vitro* renal IRI model is anti-apoptotic. The results described here show that human naked DNA for BMP-7 cannot protect PTECs from apoptosis caused by an *in vitro* renal IRI model. We further investigated the role of naked BMP-7 DNA on PTECs to assess whether PTECs can take up BMP-7 DNA without transfection and be able to secrete BMP-7 protein. The results demonstrated that PTECs were unable to produce BMP-7 protein.

The main findings of second part of this study suggest that (a) BMP-7 protein can reduce apoptosis and preserve cell viability in HK-2 cells (b) that the transfection of BMP-7 DNA into 293-T cells shows that BMP-7 expression is increased when compared to control transfection. However (c), transfection of HK-2 cells was not able to produce the same effect and (d) the EP of BMP-7 DNA into the anterior tibialis muscle of mice increases its expression in serum which is maximal observed on day 2. Lastly,

EP of BMP-7 cannot prevent the damage and dysfunction caused by renal IRI *in vivo.*

Previous studies have demonstrated that BMP-7 protein can reduce TGF-β induced caspase-3 activity in podocytes through Smad-5 activation and improve cell survival (Mitu *et al*., 2007). Data show that the intravenous administration of recombinant human BMP-7 protein reduced the number of apoptotic tubular cells, suggesting that BMP-7 may play an important role in promoting renal repair and regeneration after an ischaemic insult in renal PTECs (Hruska *et al*., 2000). In a renal IRI model, BMP-7 protein has been shown to ameliorate the injury at a dose of 250 μg/kg (Vukicevic *et al*., 1998). Research indicate that BMP-7 reduces apoptosis in the cortex and medulla, suppresses inflammation by reducing levels of ICAM-1, MPO significantly and reduced the rise in serum creatinine (Hruska *et al*., 2000). In a model of aristochololic acid nephropathy (a progressive fibrosis model), BMP-7 at a dose of 500 ng/mL was found to reduce apoptosis, necrosis and enhances cell viability in PTECs (HK-2) cells (Wang *et al*., 2010). This led us to examine the effects of BMP-7 on HK-2 cells and to see whether it can reduce the apoptosis in the setting of an *in vitro* IRI model. Our results demonstrated that BMP-7 can reduce apoptosis when given at a concentration of 10-100 ng/mL. Furthermore, it also enhanced the cell viability in HK-2 cells in the same model when given at the same treatment dose. Our findings provide evidence that BMP-7 can protect HK-2 cells from *in vitro* renal IRI induced apoptosis thus preserving cell viability. Our results can be considered as being in tandem with others (Mitu *et al*., 2007, Wang *et al*., 2010, Hruska *et al*., 2000).

The transfection of human BMP-7 plasmid DNA into renal epithelial cells (HK-2) with a

liposome method leads to increased expression of BMP-7 protein (Li *et al*., 2006). This led us to investigate whether transfection of HK-2 cells and 293-T cells with OPTIFECT™ transfection reagent and BMP-7 plasmid DNA would work in the same manner. Transfection was performed using the same method following the manufacturer's instructions for both cell lines. Interestingly, our findings are in contrast to the previous study (Li *et al*., 2006). The expression of BMP-7 was not detectable in HK-2 cells but was very high in 293-T cell. One of the explanation for this might be that the 293-T cell are embryonic kidney epithelial cells and are easily transfected as they contain SV40 large T-antigen that allows for episomal replication of transfected plasmids containing the SV40 origin of replication and extended temporal expression of the desired gene products (Harvey *et al*., 1997) and BMP-7 expression is also higher during the embryonic stage.

More recent studies have shown that EP of human BMP-7 DNA into skeletal muscle increases it expression and that EP is considered a useful technique for clinical application (Sardesai and Weiner, 2011). Furthermore, another study has demonstrated that EP on gastrocnemius muscle with BMP-7 also increases BMP-7 expression as detected by PCR (Kawai *et al*., 2006). To examine whether EP of the skeletal muscle can enhance BMP-7 expression in serum, we performed EP on mouse anterior tibialis muscle. The ELISA data showed that there was a significant increase in the expression of BMP-7 protein in serum at day 2 after EP and that BMP-7 levels were higher on day 4 but not significantly different from the control EP group (Figure 26). Renal IRI was performed on mice on day 3 after EP with control and BMP-7 DNA. Blood was collected on day 2 and 4 to determine the concentration of BMP-7 in serum. The results

demonstrate that gene therapy of BMP-7 cannot protect against the damage and dysfunction caused by renal IRI even though serum concentrations of BMP-7 were significantly higher, when compared to control mice at day 2. A possible explanation for this can be that whilst BMP-7 was able to attenuate PTECs apoptosis at nmol/L doses *in vitro*, the amount of BMP-7 seen after *in vivo* EP at day 2 was in the range of pg/L, which is 1000 fold less. Furthermore, previous studies have also demonstrated that a much higher level of BMP-7 protein is required to prevent renal IRI (Vukicevic *et al*., 1998). One the likely reasons for less BMP-7 protein generation can be that EP has been associated with cell death at site of injection which might affect the overall gene therapy procedure and result in less expression of desired gene or protein (Patil P.M, 2012) but further research is needed to confirm whether this was a contributing factor.

In summary, the study presented here is the first one to analyse the possible putative role of gene therapy with BMP-7 in the settings of renal IRI. Two different modes of administration were studied using naked DNA and EP in mice and rats *in vivo*. Initially, *in vitro* experiments were performed and these suggested that BMP-7 protein can prevent apoptosis and preserve cell viability in PTECs. Naked BMP-7 DNA treatment of PTECs was not able to attenuate apoptosis caused by an *in vitro* IRI mode. PTECs were incubated with naked BMP-7 DNA for 24 h to assess whether PTECs can induce protein expression without transfection but ELISA results showed no detectable evidence of BMP-7 protein. Transfections of 293-T cells with BMP-7 DNA showed upregulation of BMP-7 protein at 24 h.

With the *in vivo* study, the positive finding were that naked BMP-7 DNA was able to

upregulate the levels of BMP-7 in kidney in rats at day 4 after injection but was unable to attenuate the degree of injury caused by renal IRI. The results of EP of BMP-7 demonstrate that there was upregulation of BMP-7 in serum at day 2 but again it was not able to prevent the damage resulting from renal IRI. In conclusion, although attempts with gene therapy of BMP-7 were not successful in attenuating renal IRI it does provide evidence that gene therapy could play a crucial role in the future treatment of renal IRI.

CHAPTER 5

GENERAL DISCUSSION

5.1: General discussion

AKI ranges from reversible early mild forms to the most severe requiring RRT (Molitoris *et al*., 2007). It is associated with significant patient morbidity and mortality that is frequently associated following complex surgery, transplantation, sepsis and cardiac surgery (Chertow *et al*., 1998, Uchino *et al*., 2005). Current therapy is limited to supportive measures and preventive strategies, but none have been definitively shown to alter mortality (Bonventre and Weinberg, 2003). One of the main causes of AKI is renal IRI which is largely considered a reversible phenomenon, an observation supported by an excess of pre-clinical data (Bonventre and Weinberg, 2003). However, the translation of these findings into the clinical setting has remained elusive with the failure of several novel therapies tested in clinical trials (Lameire *et al*., 2008). Therefore, treatments to improve the outcome post renal IRI currently represent an unmet therapeutic need, and additional research investigating novel therapies and improving understanding of the mechanisms of disease progression are required. Despite advances in the understanding of pathophysiology of AKI, the available option for treatment has not improved and indeed the mortality associated with AKI has not fallen (Hoste *et al.*, 2006). IRI contributes to acute and delayed graft failure after renal transplantation (Powell *et al*., 2013). Thus, in the thesis presented here an attempt to investigate new strategies to prevent AKI has been undertaken.

Chapter 2 explored the role of NO_2^- during apoptosis in PTECs and its role in the setting of renal IRI. Several studies led us to investigate the role of oral NO_2^- in renal IRI. Firstly, topical NO_2^- application on kidneys has been previously shown to be protective

in renal IRI setting (Tripatara *et al*., 2007). These beneficial effects are dependent on the generation on NO by XOR, acidic disproportion and haem proteins (Tripatara *et al*., 2007). Another study has demonstrated that i.v or i.p administration of NO_2^- is detrimental in AKI or renal IRI (Basireddy *et al*., 2006) whilst other work shows that oral NO_2^- given at a dose of 50 mg/kg in drinking water to rats can significantly protect against myocardial infarction (Bryan *et al*., 2007). It therefore seems likely that NO_2^- can be protective or detrimental depending on its mode of delivery as mentioned previously.

Until now, no research that we are aware has assessed the role of oral NO_2^- in the setting of renal IRI. As MI is associated with ischaemia and oral NO_2^- has been shown to be protective against it (Bryan *et al*., 2007), we decided to investigate the effects of oral NO_2^- in a model of renal IRI. However, surprisingly, the results of our study were in contrast to the finding shown earlier in our laboratory (Tripatara *et al*., 2007). One of the likely explanations for this difference can be that the mode of administration of NO_2^- was topical upon the kidneys once, just before reperfusion in Tripatara study whereas in my study the mode of administration was oral for 7 days prior to ischaemia. Secondly, the dosage used by Tripatara was lower (60 μmol/L) whilst in my study it was high around 50 mg/L which is equal to 724 μmol/L (although it was dependent on how much the animals were drinking). Finally the ischaemia duration was different, 60 min in Tripatara study and 45 min in my study (Tripatara *et al.*, 2007).

I initially examined the role of NO_2^- on PTECs *in vitro*. As there were no well-differentiated PTECs derived from adult human kidney commercially available at the time of the study so we used immortalized HK-2 cells. HK-2 cells are epidermal growth

factor dependent and the cells retain a phenotype indicative of being well-differentiated on the basis of its histochemical, immune cytochemical, and functional characteristics, and it can reproduce experimental results obtained with freshly isolated PTECs (Ryan *et al*., 1994).

As mentioned earlier apoptosis is the leading cause of cell death during renal IRI. I initially chose the serum starvation/deprivation (SS) model for studying apoptosis in PTECs. Research has shown that the elimination of serum from culture medium reduces analytical interference, reduces basal cell activity and provides more reproducible experimental conditions (Sergej Pirkmajer, 2011). Furthermore, SS is used in research to study molecular mechanisms involving protein degradation, cellular stress response, autophagy, apoptosis, and to simulate particular pathological conditions. Researchers have combined SS with hypoxia and/or lowered glucose content in experimental models to mimic clinical conditions such as myocardial infarction and stroke (Sergej Pirkmajer, 2011). We have a well-established SS model which has been used previously to mimic renal ischaemia in PTECs (Kumar et al., 2009, Sergej Pirkmajer, 2011). In the research presented here I firstly explored the role of NO_2^- in PTECs apoptosis at multiple doses of NO_2^- in nmol/L doses with SS. The reason for choosing the nmol/L dose for my research was that previous work with NO_2^- did indicate that NO_2^- given in nmol/L amounts (1.2 nmol/L – 48 nmol/L) was able to reduce infarct size, infarct volume and apoptosis in heart and liver by 50% (Duranski *et al*., 2005, Webb *et al*., 2004). Furthermore a study conducted using a model of stroke in rat shows that NO_2^- supplementation at a dose of 48 nmol/L to 480 nmol/L may reduce cerebral infarcts size by 75% and did attenuate neurological damage (Jung *et al.*, 2006). My

results indicated that NO_2^- is able to prevent caspase 3 activity in HK-2 cells caused by SS at multiple doses if given at time of SS injury. This shows the potential of NO_2^- as a potential treatment therapy in renal ischaemia. As mentioned earlier to our knowledge this is the first study to explore the role of NO_2^- in HK-2 cells apoptosis. These positive finding were strengthened by the results of cell viability assay (MTS) assay which showed that the reduction caused by SS in HK-2 cell viability was preserved by NO_2^- supplementation *in vitro*. My findings are in tandem with previous research that shows that the supplementation of L-arginine increases NO production in epithelial cells and is protective against damage in post-ischemic injury (Jerkic *et al*., 1999). The next step was to evaluate that whether the protection of apoptosis seen in PTECs by NO_2^- is intrinsic or extrinsic. In order to answer this caspase activity assays for caspase 8 & 9 were performed on the lysis from samples derived from HK-2 cells that had injury induced by 24 h of SS. There was found to be an increase of caspase-9 activity (but no caspase-8 activity) after 24 h of SS in HK-2 cells and this increase was abolished by NO_2^- supplementation. The result showed that the apoptosis in PTECs (HK-2 cell) is through the intrinsic pathway indicating mitochondrial involvement.

Our results are in accordance with previous work that shows that NO_2^- is cytoprotective after IRI of the mammalian heart and liver at the mitochondrial level through the transient inhibition of complex I and subsequent limitation of oxidative damage (Shiva *et al*., 2007). NO is a well-characterized regulator of mitochondrial function, with nmol/L concentrations reversibly inhibiting cytochrome c oxidase , regulating ROS formation, initiating biogenesis, and limiting apoptotic cytochrome c release (Shiva *et al*., 2007). This may be one of the possible mechanism by which NO_2^- is attenuating apoptosis in

PTECs. Previous work shows that NO_2^- beneficial effects are because of its conversion to NO via XOR (Tripatara *et al*., 2007), therefore it will be worthwhile for future studies to explore the involvement of anti-apoptotic proteins such as Bcl and IAP and the role of eNOS, XOR and iNOS. Although the results presented here are promising, further research is needed to explore the possible mechanisms behind this protection.

After examining the role of NO_2^- in PTECs apoptosis caused by SS, I further investigated NO_2^- role in necrosis of PTECs by using *in vitro* reperfusion model to elicit injury. Research shows that this is a very good model to study necrosis *in vitro* (Saito *et al*., 2006). Furthermore, a recently conducted study shows that NO_2^- prevents necrosis in a model of diabetic ischaemic injury (Bir *et al*., 2014). However, my results show that NO_2^- was unable to prevent necrosis in PTECs.

Studies have shown that there is a viable *in vitro* model that can mimic renal ischaemia (Lee and Emala, 2002). This model uses antimycin A, which is produced by streptomyces bacteria and is used as an antibiotic commercially. It inhibits ATP production by binding to the cytochrome bc1 complex III of the electron transport chain thus interfering with the oxidation of coenzyme Q and causing irreversible defects in aerobic respiration in the mitochondria (Dagher, 2000, Breggia and Himmelfarb, 2008). Furthermore, inhibition of oxidative phosphorylation caused by antimycin A has been shown *in vivo* to produce large quantities of $O_2^{\bullet -}$, a toxic free radical implicated in kidney injury (Jones, 1986, Breggia and Himmelfarb, 2008) and exposure to high levels of antimycin A for longer period leads to necrosis (Lieberthal *et al*., 1998b). My results provide evidence that NO_2^- at lower doses (nmol/L) can prevent apoptosis in an *in vitro*

IRI model if given with ischaemia or post-ischaemia. Furthermore, NO_2^- was able to preserve cell viability of PTECs in the SS model and attenuated the rise in caspase-9 activity in the NO_2^- treated group when compared to the controls suggesting that the apoptosis in PTECs is mediated via the intrinsic mitochondrial pathway.

Previous research has shown that the benefits of NO_2^- are mediated through the modulation of mitochondrial function by involving the post-translational *S*- nitrosylation of complex I to attenuate reperfusion oxygen radical generation and prevent cytochrome-C release (Shiva and Gladwin, 2009, Shiva, 2013). Other investigations into the effects of pre-treatment with NO_2^- at several doses showed that this pre-treatment was unable to attenuate apoptosis in PTECs in an *in vitro* IRI model. I believe that this is the first evaluation of the role of NO_2^- on PTECs apoptosis but more work is needed before we can understand the underlying mechanism. My findings are in parallel with a study that has shown that NO can inhibit caspase proteases via *S*-nitrosylation, thereby inhibiting apoptosis (Dimmeler *et al*., 1997). This effect may be concentration dependent. Evidence suggests that NO at lower concentrations may inhibit apoptosis whilst higher concentrations may lead to the formation of toxic products such as $ONOO^-$ or other ROS which lead to cell necrosis and apoptosis (Dimmeler *et al*., 1997, Siriussawakul *et al*., 2010).

Studies have shown that NO_2^- at low levels can attenuate apoptosis and has cytoprotective effects (Sugimoto *et al*., 2012, Gonzalez *et al*., 2008). Our findings also indicate that NO_2^- in nmol/L doses during ischaemic or reperfusion phase have anti-apoptotic properties and can be cytoprotective. Furthermore, previous studies show that

NO can also induce apoptosis when there are high levels of NO donors (Messmer and Brune, 1996) such as NO_2^-/$NO_3^-$and increased expression of iNOS (Lysiak *et al*., 1995) in macrophages (Messmer and Brune, 1996, Albina *et al*., 1993), neurons (Dawson *et al*., 1996) and thermocytes (Fehsel *et al*., 1995). The results of my pretreatment of NO_2^- group indicating apoptosis are consistant with above studies.

One of the likely explanations for this NO dual nature can be that NO or its reaction product induces apoptosis by a mechanism independent of caspase-3 in some cell types (Kim *et al*., 1997). Secondly, NO reacts rapidly with $O_2^{\bullet-}$ to form the highly toxic $ONOO^-$, which can induce apoptosis (Lin *et al*., 1995). This interaction of NO with $O_2^{\bullet-}$ leads to the formation of more toxic radicals and can also remove NO from the system. So, NO may protect cells from apoptosis or may induces cell death and this mostly depends on the rate of NO formation and the prominence of caspase-3-like protease activation in the apoptotic process as well as other factors such as the presence of other radicals and antioxidants (Kim *et al*., 1997). I believe that the work presented here is the first study to identify the anti-apoptotic role of NO_2^- in PTECs. Whilst I have shown that NO_2^- supplementation protects PTECs from apoptosis mediated through intrinsic pathway the mechanism behind this protection is still unknown.

The *in vivo* NO_2^- study was conducted using a rat model of renal IRI in which rats subjected to renal IRI demonstrated the characteristic signs of renal dysfunction and injury by the development of increased serum creatinine concentrations. Previous research has shown that NO_2^- can prevent IRI by the conversion to NO during ischaemia by XOR (Tripatara *et al*., 2007). Another study has demonstrated that the

beneficial effects of topical NO_2^- on kidneys in a renal IRI setting but the systemic administration of NO_2^- has been shown to be detrimental (Basireddy *et al*., 2006). One of the likely explanations of this is thought to be that the topical route provides a higher local concentration of NO_2^- to the kidney than systemic administration (Tripatara *et al*., 2007). When NO_2^- is administered by a systemic route, it is probable that substantial amounts of NO_2^- are metabolized to NO by deoxyhaemoglobin in the circulation before it reaches the kidney (Tripatara *et al*., 2007). Others have demonstrated that very high levels of NO derived from iNOS is detrimental in renal IRI (Basireddy *et al*., 2006). In chapter 2, the *in vivo* results demonstrated that systemic application of NO_2^- via oral route was able to increase the levels of plasma NO_2^-/ NO_3^- but it was not able to prevent the damage and dysfunction caused by renal IRI. Similar results were seen with low NO_2^- diet group. One of the likely explanation is that insufficient NO_2^- might have reached the kidney, as it gets blood supply only from renal artery, which, if clamped, may prevent the achievement of sufficient tissue levels of NO_2^-(Basireddy *et al*., 2006). Research suggests that in the liver and the heart the direct exposure of $NO_2^-$to the organ surface was protective and resulted in elevations in NO_2^- in the plasma (Duranski *et al*., 2005). Secondly, excess of NO can lead to the formation of $O_2^{\bullet-}$ and $ONOO^-$ which is very toxic at cellular levels and can worsen injury (Basireddy *et al*., 2006). Another study demonstrate that significant NO production may be induced by the single cytokines IL-1β and TNF-α or their combination in the presence or absence of LPS and that this NO production is associated with damage of PTECs (Sugimoto *et al.*, 2012). Furthermore, NO can be generated by two different pathways: one is NOS dependent NO synthesis is the main pathway of NO generation and it requires physiological pH and oxygen (Giraldez *et al*., 1997). Contrary to this, the second NOS-independent

pathway generates NO (reduction of NO_2^- by XOR) under ischaemic conditions. NOS and XOR are both important enzyme for NO synthesis and they can be either protective or damaging to cells, depending on the conditions. These contradictory functions can be explained by the dual roles of these enzymes; both NOS and XOR can generate either NO or ROS (Lundberg and Weitzberg, 2005, Sugimoto *et al*., 2012). Evidence exists suggesting that NOS generates $ONOO^-$ and $O_2^{\bullet-}$ whilst in shortage of L-arginine or cofactors resulting in cellular injury (Xia and Zweier, 1997). Similarly, XOR reduces oxygen to $O_2^{\bullet-}$ and generates other damaging ROS during the reperfusion phase contributing to oxidative tissue injuries (Berry and Hare, 2004). Although Tripatara's study (Tripatara *et al*., 2007) did demonstrate that NO generation via XOR can protect renal IRI via topical route studies have shown that inhibition of XOR can also protect kidney from the damage associated with it (Rhoden *et al*., 2000). Although, in our studies NO_2^- was given to rats for 7 days prior to ischaemia in drinking water and it led to significant increase in levels of NO_2^-/ NO_3^- compared to the control animals before renal IRI, it still remains unclear what level of NO_2^- there was present in the kidney at time of ischaemia and at the 24 h reperfusion phase. It would be of some importance to investigate whether the pretreatment of NO_2^- leads to the generation of NO or to the generation of ONOO- or ROS before ischaemia. Although one study in a cardiac MI model shows that when NO_2^- is given in the drinking water it is able to prevent MI in rats and that there is NO generation in NO_2^- treated group compared to the control treated animals (Bryan *et al*., 2007), it is still not justified to assume that the same is happening in the kidneys.

A recent study with NO_2^- shows that it can reduce liver cold IRI in rats at a dose of 250

μmol/L (Li *et al*., 2012) which is lower when compared to the dose used in my study. Although previous work indicates that NO_2^- at low levels can protect against IRI, the mode of administration was different for each study (Tripatara *et al*., 2007). For future research I think the key line of investigation would be study the levels of NO before and after ishaemia and to assess whether pretreatment through oral administration prior to ischaemia leads to the generation of ONOO- or ROS.

In summary the results shows that NO_2^- treatment can prevent apoptosis and preserve cell viability in PTECs if given at time of SS or ischaemia but cannot reverse damage caused by necrosis in the same conditions. Furthermore, apoptosis in PTECs is mediated through the intrinsic pathway as shown by caspase-9 activation in these same models. Furthermore, pretreatment with NO_2^- did not attenuate the degree of apoptosis caused by the *in vitro* IRI model at multiple doses when compared to the control treated cultures.

My findings suggest that high and low NO_2^- doses when given through an oral route cannot attenuate renal IRI even though the plasma levels of NO_2^-/NO_3^- were significantly higher in the high NO_2^- group when compared to the controls. Further research is needed to understand the mechanism of NO_2^- and NO when given orally with renal IRI.

In chapter 3 I investigated the role of thiamine and its lipid soluble analog benfotiamine in the renal IRI setting. Previous research shows that thiamine can protect cardiomyocytes from hypoxic stress by causing a sustained expression of Hsp70 and

inhibiting apoptosis (Shin *et al*., 2004). Thiamine has been shown to have protective effects on several disorders of the heart (Larrieu *et al*., 1987, Vinogradov *et al*., 1991) and the brain (Naito *et al*., 1999, Tanaka *et al*., 1997). Hypoxic/ischaemic insult can disturb metabolic homeostasis, leading to protein instability and loss of anti-apoptotic signals, and can cause necrosis and apoptosis (Shin *et al*., 2004). Thiamine deprivation causes focal necrosis and apoptosis, and reduces myocyte contractility ultimately leading to myocardial dysfunction (Cappelli *et al*., 1990). Although research show that thiamine can prevent apoptosis in cardiomyocyte (Shin *et al*., 2004), nothing as yet is known regarding its role in the proximal tubular epithelial cells (PTECs). This led us to investigate the role of thiamine in PTECS that whether it can prevent apoptosis in *in vitro* renal IRI model and necrosis in *in vitro* reperfusion model. Our results demonstrate that thiamine can prevent apoptosis and preserve cell viability in PTECs but it is unable to reverse the progression to necrosis in PTECs. Our findings are in tandem with an earlier study that showed that thiamine treatment significantly increased cardiomyocyte survival after a hypoxic insult by protecting the cells from hypoxia-induced apoptosis associated with caspase-3 activation, PARP cleavage and DNA fragmentation (Shin *et al*., 2004).

Earlier work (Larrieu *et al*., 1987) suggested that thiamine is protective against experimental MI. It has been argued that many organs, including the kidneys are deficient in thiamine at the moment of transplantation (Klooster *et al*., 2007). Another study has shown that cardiac oxidative stress is involved in heart failure induced by thiamine deprivation (Gioda *et al*., 2010). This study further identifies that there is lower oxygen consumption, increased ROS generation and increased apoptosis in thiamine

deficient rats compared to normally fed rats (Gioda *et al*., 2010).

Initially a pilot study was performed by gavaging with 125 mg/kg of thiamine and this showed a possible trend that thiamine can play a crucial role in renal IRI settings in rats. Further experiments were performed at a higher dose of 500 mg/kg of thiamine by gavaging once daily for 3 days prior to renal IRI. The results provide evidence that thiamine can protect renal IRI at a dose of 500 mg/kg. This is the first study to identify that thiamine can prevent renal IRI. As our *in vitro* results provided proof that thiamine can prevent apoptosis in PTECs in renal IRI model and preserve cell viability we further investigated the role of thiamine in the setting of renal IRI in rats. Our results suggests that thiamine can protect against damage and dysfunction caused by renal IRI and apoptosis post renal IRI. Thiamine also attenuated the histological kidney damage caused by renal IRI.

These results led us to investigate further the mechanism behind this protection caused by thiamine. Previous research indicates that thiamine deficiency in the brain leads to the upregulation of inflammatory COX-2 which leads to synthesis of prostaglandins from arachidonic acid (Gu *et al*., 2008). COX-2 can play an important role in multiple conditions such as inflammation, fever, cancer, vascularization, anaphylactic shock, childbirth, bone metabolism, and apoptosis (Matsuyama *et al*., 2005). Furthermore, under normal conditions COX-2 is rarely expressed, but it is upregulated in macrophages, fibroblasts, synovial cells, endothelial cells and neurons in response to cytokine (e.g., IL-1) stimuli as well as carcinogenesis promoters, endotoxins, and hormones (Matsuyama *et al*., 2005, Yamada *et al*., 2008) . Studies show that there is

upregulation of COX-2 after renal IRI that is maximal at around 3 to 5 h and remains significantly higher at 24 h compared to controls and that this increase plays an important role in causing the tissue damage after renal IRI (Matsuyama *et al*., 2005). Other work shows that in contrast to this study that COX-2 levels after 24 h of renal IRI were not significantly higher when compared to the controls but they did not evaluate COX-2 at the earlier time points (Villanueva *et al*., 2007). As there is upregulation of COX-2 due to thiamine deprivation in the brain (Gu *et al*., 2008) our hypothesis was that thiamine supplementation might prevent COX-2 upregulation caused by renal IRI. Our western blotting data provided evidence that there is upregulation of COX-2 expression in the kidney after renal IRI and that thiamine supplementation significantly reduces the expression compared to renal IRI group. These results are in accordance with an earlier study that showed that COX-1 and COX-2 blockade ameliorates the renal tissue damage triggered by IRI (Feitoza *et al*., 2005).

Inflammation plays a pivotal role in renal IRI and research shows that cytokines such as TNF-α and IL-1 upregulate the adhesion receptors such as ICAM-1 on endothelia and that these in turn localise white blood cells to the site of inflammation/injury (Kelly, 2003). ICAM-1 on endothelial cells interacts with its counter receptors on leucocytes and immobilizes leucocytes on the endothelium. Furthermore, during heart ischaemia there is increase of TNF-α , IL-1 and an increase of mRNA for ICAM-1.These increases were accompanied by increases of MPO activity, increased leucocyte infiltration and activation in the heart (Kelly, 2003). There is a possibility that anti-ICAM-1 antibody might prevent leucocyte infiltration in heart ischaemia. Furthermore, anti-ICAM-1 has been shown to prevent damage and dysfunction caused by renal IRI (Kelly *et al*., 1994).

MPO is a 140 kDa protein which is stored in the lysozymes of monocytes and in the azurpophilic granules of PMN and it is released abundantly upon neutrophil activation. MPO has the capacity to catalyse the formation of hypocholrous acid HOCL from H_2O_2 and chloride ion and makes it a powerful tool in the bactericidal armament of these cells (Klebanoff, 2005, Matthijsen *et al*., 2007). However, clinical research shows harmful effects of MPO in the immune mediated inflammatory syndromes, such as multiple sclerosis, acute coronary syndrome, and renal disease (Matthijsen *et al*., 2007). Furthermore, studies have shown that MPO and MPO derived oxidants play an important part in causing atherosclerosis and organ damage after MI. A study with $MPO^{-/-}$ mice shows that these mice are protected from renal IRI (Matthijsen *et al*., 2007). This protection is mediated by reducing PMNs infiltration in MPO deficient mice. As there is an increase in ICAM-1 and MPO during renal ischaemia and that thiamine deficiency further contributes to their increase (Calingasan *et al*., 2000), in this work we therefore attempted to assess whether supplementation of thiamine can reduce the levels of ICAM-1 and MPO activity during renal IRI.

The results show that there is significant upregulation of ICAM-1 and MPO activity in renal IRI rats when compared to controls and this increase was significantly attenuated in the thiamine treated rats. Furthermore, we can presume that the reduction in MPO levels could have reduced the levels of PMN activation and adhesion, thereby reducing the infiltrating neutrophils at the site of injury and have prevented damage. NIMP-R14 is a neutrophil infiltration marker which is not influenced by MPO reduction (Matthijsen *et al*., 2007) and by studying it might be possible to demonstrate the lack of neutrophil infiltration in the renal IRI+ thiamine group. Another possible mechanism can be that

MPO generated products such as HOCL and chloramines can inactivite α ketoglurate dehydrogenase complex and cause ATP depletion (Jeitner *et al*., 2005). Although all these mechanisms could be involved further work is needed to establish a correlation. Due to limited time I was unable investigate these mechanisms but it is an area of great interest for future study.

To summarise, our findings provide evidence that thiamine has anti-inflammatory properties as demonstrated by the significant reductions of COX-2, ICAM-1 and MPO in renal IRI group compared to vehicle group and it is one of the mechanisms by which it attenuates the damage and dysfunction caused by renal IRI. During IRI there is significant amount of ROS generation beyond the scavenging capacity of organs (Ye *et al*., 2010). Furthermore, thiamine deficiency can cause impairment of chronic oxidative metabolism and it is associated with generation of lipid peroxidation product, 4-HNE (Calingasan *et al*., 1999). Research shows that lipid peroxidation and ROS both plays a major role in renal IRI and antioxidant therapy can protect the damage associated with renal IRI (Zou *et al*., 2013).

Another study shows that treatment with an antioxidant can prevent against oxidative stress caused by ischaemia (Yuan *et al*., 2011). Studies with anti-oxidant treatment have been shown to prevent AKI in clinical and experimental models (Lloberas *et al*., 2002). Furthermore, lipid peroxidation plays one of the most pivotal roles during oxidative stress, and research provides evidence indicating its implication in renal IRI (McCord, 1985, Eschwege *et al*., 1999). It can cause oxidative destruction of cellular membranes associated with the production of toxic, reactive aldehydic metabolites and

cell death (Niki *et al*., 2005). Malondialdehyde (MDA) and 4-hydroxynonenal (HNE) are the most important aldehydic metabolites. Evidence exists suggesting that excessive production of these highly cytotoxic metabolites may cause their diffusion from the site of origin to attack distant targets, through which they form covalent links with various molecules (adducts) to mediate the inactivation of enzymes and inhibition of DNA, RNA and protein synthesis (Yuan *et al*., 2011). As thiamine has anti-oxidant properties we further investigated whether thiamine supplementation can reduce oxidative stress and attenuate ROS generation as shown by others (Yuan *et al*., 2011).

Our results provided evidence that thiamine can attenuate the ROS generation and oxidative stress (HNE-4).and this is one of the mechanism by which it reduces damage and dysfunction caused by renal IRI. Although researchers have used different models and experiments to assess oxidative stress such as levels of MDA (Yuan *et al*., 2011). We chose to assess lipid peroxidation by measuring the levels of 4-HNE due to its correlation with thiamine deficiency (de Andrade *et al*., 2014). Our results are in tandem with previous work showing that oxidative stress reduction can protect damage associated with renal IRI (Zou *et al*., 2013, Yuan *et al*., 2011). Furthermore the findings are also in parallel with recently published data which shows that thiamine pyrophosphate can reduce oxidative stress caused by renal IRI (Altuner *et al*., 2013).This can be one of the possible mechanisms by which thiamine affords protection in renal IRI. Another important factor concerns the thiamine-HCl dose of 500 mg/kg chosen for our study. Research conducted on mice indicates that this dose is lower than the maximum tolerated dose of thiamine used previously with no toxic effects seen (Leuschner, 1992). In comparison to this study we have additionally used a low dose of

thiamine. Clinical data suggests that thiamine toxicity is very low but in some cases side effects are seen at doses above 7000 mg/day. Research conducted on Alzeimer's patients indicates that thiamine is well tolerated at a dose of 6000-8000 mg/day for 6 months with no adverse effects reported (Meador *et al*., 1993). Collectively these studies justify our approach and there appears to be an urgent need to conduct a clinical trial in humans with thiamine in AKI patients to see if our findings in rodents are translatable to the clinical environment. For future work it will be good to assess the concentrations of thiamine or its derivatives in blood if possible and search for a technique or method that is easy to perform, inexpensive and accurate.

In summary this is the first study to show that thiamine can attenuate apoptosis and preserve cell viability in the PTECs in SS and *in vitro* IRI model. Furthermore our *in vivo* data shows that thiamine prevents damage and dysfunction caused by renal IRI by anti-apoptotic, anti-inflammatory and anti-oxidant mechanisms.

As we had already investigated the role of thiamine in renal IRI we thought next to evaluate the role of benfotiamine, which is considered as a lipid soluble analog of thiamine and has been shown to have better bioavailability than water soluble thiamine (Bitsch *et al*., 1991). Previous research shows that benfotiamine supplementation can improve the post-ischaemic healing of diabetic mice by attenuating apoptosis and stimulating reparative angiogenesis and both these effects are mediated via activation of PKT/Akt (Gadau *et al*., 2006). However, most of the research conducted with benfotiamine does not mention the carrier for it (Hammes *et al*., 2003). Researchers have either used a special benfotiamine diet or they have solubilized it and force fed it.

A paper by Volvert (Volvert *et al*., 2008) shows that benfotiamine is sparingly soluble in water and they tried many different approaches to dissolve it. They found that benfotiamine at 100 mg/kg can be dissolved in HP-β-CD to a concentration of 200 mmol/L and its derivatives ThDP reaches a peak plasma level after 2 h after administration and decreases slowly afterwards. As this study identified a new method to dissolve benfotiamine we herein tried to mimic what the Volvert group has shown (Volvert *et al*., 2008). We dissolved benfotiamine in HP-β-CD and rats were gavage with 100 mg/kg to 250 mg/kg of benfotiamine for 3 days prior to renal IRI. The renal ischaemia was performed exactly 2 h after garaging so to achieve maximal thiamine concentrations (Volvert *et al*., 2008). The target of this was to explore whether benfotiamine can protect renal IRI at a lower dose than thiamine. The results showed that benfotiamine at a dose of 250 mg/kg cannot prevent injury and dysfunction caused by renal IRI. One of the likely explanation for this inability to prevent renal IRI at a lower dose than thiamine can be that benfotiamine according to some workers should not be considered as a lipid soluble thiamine (Volvert *et al*., 2008). This study indicates that benfotiamine cannot diffuse through cell members unassisted (Volvert *et al*., 2008) despite previous research having shown that benfotiamine can attenuate the injury caused by glucose toxicity (Hammes *et al*., 2003) but still there is no proof that it can cross the cell membrane on its own (Volvert *et al*., 2008). Further research is needed to understand the mechanism behind this dual nature of benfotiamine. I believe future work should investigate other lipid soluble thiamine derivatives such as sulbutiamine, allithiamine and frusultiamine as research has shown that they can have better bioavailability than benfotiamine and might prevent renal IRI at much lower dose than

thiamine (Volvert *et al*., 2008).

In my final results chapter I investigated the role of gene therapy of BMP-7 in the setting of renal IRI and the investigated the direct effects of DNA or BMP-7 protein on apoptosis and cell viability in PTECs. I firstly investigated the effect of naked BMP-7 DNA on apoptosis in PTECs. Research shows that proximal tubular cells can take up and express foreign naked DNA (Bosch *et al*., 1993). Another study shows that BMP-7 reduces tubular apoptosis and necrosis (Wang *et al*., 2010). Research to assess the gene therapy in the kidney has met with success (Bosch *et al*., 1993) but the role of direct naked BMP-7 DNA on apoptosis in PTECs in unknown. So, I investigated the effects of BMP-7 naked DNA on PTECs. The results shows that direct naked BMP-7 cannot protect PTECs against the apoptosis caused by *in vitro* renal IRI model. To assess whether PTECs can directly or by transfection take up BMP-7 DNA and upregulate BMP-7 protein *in vitro* I performed an ELISA which showed that PTECs cannot upregulate BMP-7 protein *in vitro* with or without transfection. One of the likely explanations for this is that foreign DNA has been shown to be detrimental and causes DNA dependent cell death. Other research shows that introduction of single stranded DNA into the cell induces DNA damage and upregulation of apoptotic factors. The uptake of DNA at cellular level is restricted by cell defence enzymes such as DNA endonucleases (Wu *et al*., 2005). Although transfection using lipofectamine has identified that PTECs can be transfected (Li *et al*., 2006) I was unable to detect any BMP-7 protein formation in my study. To test further whether this was due to any technical issues I transfected PTECs simultaneously with the 293 T cells, which are kidney embryonic cells and are considered as the ideal cells for transfection under

similar conditions. The results of that experiment showed the upregulation of BMP-7 in 293 T cells but no upregulation of BMP-7 protein in PTECs. My findings are in tandem with previously published data that also shows that there is no expression of BMP-7/Lac z transgene in PTECs and it is higher in the medullary region than in the cortical region of the kidney (Gould *et al*., 2002). Although there was no expression of BMP-7 seen this study identifies that PTECs do possess a BMP-7 receptor which can respond to exogenously delivered BMP-7 (Gould *et al*., 2002). Others have also demonstrated that there is less or no BMP-7 expression in PTECs (HK-2) (Kitten *et al*., 1999, Bramlage *et al*., 2010). Although in contrary there is some published data which suggest that there is high BMP-7 mRNA expression in PTECs (Rudnicki *et al*., 2007).

One of the likely explanation of BMP-7 expression not seen in PTECs can be that DNase I and Endo G are endonucleases found in PTECs and research indicate that plasmid DNA in host cells can be subject to endonucleolytic attacks both outside and inside the cell leading to the destruction of plasmid DNA by the endonuclease (Buzder *et al*., 2009). Although we think these might be the possible reasons further research is needed to understand the exact cause of PTECs not showing protein expression of BMP-7 after transfection. Previous research indicate that exogenous administration of naked DNA leads to gene expression at cellular levels (Bosch *et al.*, 1993). Although, I could have investigated further the gene expression of BMP-7 by PCR methods on transfected PTECs but due to time constraints it was not feasible. For future work it will be important to investigate the gene expression by all of the possible delivery methods. Although, the results of direct naked DNA are not as yet promising it is still of significance as it is the first study to investigate naked BMP-7 DNA transfection and its

role in PTECs apoptosis.

Lastly I investigated the role of naked BMP-7 DNA in the setting of renal IRI *in vivo*. The rats were injected with naked BMP-7 DNA 3 days before renal IRI and blood was collected after renal IRI. The results confirm the *in vitro* data in that naked BMP-7 DNA cannot reduce damage and dysfunction caused by renal IRI. To assess whether there was any upregulation of BMP-7 protein in the kidney or in serum an ELISA was performed on kidney homogenates and serum. The result show that there was a significant upregulation of BMP-7 protein in the kidney but no upregulation was seen in the serum. Although there was upregulation of BMP-7 protein in the kidney, it was still not able to prevent the damage and dysfunction associated with renal IRI. One of the likely explanation for this upregulation of BMP-7 in the kidneys can be that the kidneys have a membrane bound, specific, high affinity BMP-7 receptors which may help in mediating its action (Bosukonda *et al*., 2000). Secondly, previous work does indicate that there is expression of BMP-7 in the kidney and PTECs do possess BMP-7 receptor which may respond to exogenously relieved BMP-7(Gould *et al*., 2002). Further work is needed to investigate the possible mechanism behind this finding. However, previous research shows that BMP-7 protein in the setting renal IRI can protect against damage and dysfunction caused by renal IRI (Vukicevic *et al*., 1998). My findings are in contrast to it. One possible reason might be that I used naked BMP-7 DNA whilst previously it was given i.v directly in protein form (Vukicevic *et al*., 1998) and renal IRI was performed straight immediately whereas in my study renal IRI was performed after 3 days of injecting BMP-7 DNA. Although unsuccessful, it still shows some promise as there was direct uptake of BMP-7 DNA by the kidney. Secondly the amount of BMP-7

protein injected was 250 μg/kg in the Vukicevic study (Vukicevic *et al*., 1998), and they were able to detect 1.4 μg/ml of BMP-7 in the circulation which is very high compared to the 100 pg/mL which I achieved in the kidney homogenates after BMP-7 DNA injection. Thirdly the time point is also very critical, I have injected BMP-7 DNA 3 days prior to ischaemia whilst Vukicevic injected BMP-7 protein 10 min before ischaemia (Vukicevic *et al.*, 1998). Overall, both studies are very different from each other. Although for future work it will be of great interest to assess the role of BMP-7 DNA delivery and the amount of protein present in kidney homogenate at earlier time points. If it shows a significant amount of BMP-7 upregulation at earlier time points than renal IRI should be performed at that time point.

In summary this is the first study to my knowledge to investigate the role of BMP-7 DNA on apoptosis in PTECs and renal IRI. Although, there was no protection against apoptosis and renal IRI with BMP-7 DNA still there was upregulation of BMP-7 protein seen in the kidney homogenate. For future work it will be of great importance to investigate BMP-7 DNA and its effect of BMP-7 protein formation at earlier time points and then to target it for renal IRI. Furthermore, with new techniques of gene delivery available such as electroporation, hydrodynamic gene delivery and gun needle it would have been helpful to investigate BMP-7 gene delivery with these alternative methods.

As I had already investigated the effects of direct BMP-7 DNA on apoptosis and renal IRI I next investigated the role BMP-7 protein on PTECs and cell viability. As mentioned before earlier studies have shown that BMP-7 can attenuate the apoptosis and necrosis in the PTECs (Hruska *et al*., 2000). Our results demonstrate that BMP-7 protein can

reduce apoptosis in PTECs at a dose of 100 ng/mL and this finding is in tandem with the studies mentioned previously (Wang *et al*., 2010). Furthermore, BMP-7 protein was also able to preserve the PTECs cell viability at 24 h after ischaemia.

Previous research on the effect of BMP-7 on human renal artery SMC primary cultures showed that BMP-7 maintains the expression of markers specific for SMC phenotype and protects against cytotoxicity induced by the ATP-depleting agent, antimycin A (Dorai *et al*., 2000). This led us to investigate the role of BMP-7 in this antimycin A model and the findings show that the degree of apoptosis was attenuated by 10-100 ng/ml of BMP-7 protein which is in accordance with above mentioned study (Dorai *et al*., 2000). Furthermore, I also demonstrated that there was PTECs viability was lost with this antimycin A model and that the BMP-7 supplementation during that ischaemic phase preserved the cell viability. Past studies have shown that BMP-7 counteracts TNF-α-induced apoptosis in human PTECs (Hruska *et al*., 2000). My *in vitro* results are in the same vein as those reported by Vukicevic and co-workers, who demonstrated decreases in apoptotic cells in BMP-7 rodents with acute renal failure (Vukicevic *et al*., 1998). However, high concentrations of BMP-7 were necessary to achieve significant changes. Furthermore, previous studies have shown protection at higher dose of BMP-7 protein 500 ng/ml (Wang *et al*., 2010) but I have found that BMP-7 can attenuate apoptosis in PTECs at a lower dose of 10-100 ng/ml.

In summary BMP-7 attenuates apoptosis in PTECs and preserve cell viability. Although, I have shown protection of apoptosis by BMP-7 protein, further research is needed to investigate the mechanism behind this protection. After proving that BMP-7 can reduce

apoptosis in PTECs and can preserve cell viability, I next investigated whether gene therapy of BMP-7 with EP technique can upregulate BMP-7 at serum level and protect against the damage and dysfunction caused by renal IRI. Previous research shows that non-viral gene delivery systems are potentially useful in gene therapies for tissue regeneration or repair (Nishikawa and Huang, 2001). In particular, EP is attractive, because it is an easy and inexpensive method that requires only a plasmid and a device for performing electroporation (Brown *et al*., 2009, Ferber, 2001). In addition, this method does not require viral vectors, expensive proteins, or carrier matrices (Kawai *et al*., 2006). Recent study shows that simultaneous gene transfer of BMP-7 with BMP-2 in the gastrocnemius muscle by EP is successful and induces bone formation (Kawai *et al*., 2006). So, I investigated the role of BMP-7 by EP in mice. Initially the mice were EP with control or BMP-7 DNA and blood was collected at different time points to assess whether EP was successful or not. ELISA was performed in order to detect any BMP-7 upregulation.

The results showed that there was significant upregulation of BMP-7 on day 2 compared to the controls. The renal IRI was performed on day 3. Blood and organs were collected on day 4 as end-points and serum was used to assess the degree of renal IRI injury. The results demonstrated that there was significant rise in serum creatinine in renal IRI group when compared to sham mice but this upregulation was not attenuated by the EP of BMP-7 DNA. Although, ELISA on serum showed that there was upregulation of BMP-7 protein in serum at pg/mL doses but it was unable to protect against the damage and dysfunction causes by the renal IRI. There are many factors that could account for the difference in the results obtained such as the amount of BMP-

7 protein detected in the Vukicevic study (Vukicevic *et al*., 1998) was very high in μg/ml range than that achieved in our study after EP. Secondly, the gene delivery with EP is an entirely different protocol that is still developing as more research is undertaken. Thirdly, the lower amount of BMP-7 protein expressed can be due to the difference in the choice of muscle that I used in my study. I performed the gene therapy on the anterior tibia muscle which is a small muscle when compared to the gastrocnemius muscle. Fourthly, it could be due to lack of BMP-7 at the time of renal IRI as I measured it on day 2 and renal IRI procedure ended on day 4 and the ELISA results shows that the BMP-7 levels were not significantly higher when compared to the controls EP mice on day 4.

This is the first time in my knowledge someone has investigated the role of gene therapy with BMP-7 in the setting of renal IRI. Although EP of BMP-7 in renal IRI did not show significant protection from injury it was still successful at increasing BMP-7 expression. For future work I would recommend that gene therapy of BMP-7 should be done with nanoparticles as recently it has emerged to be successful in inhibiting corneal fibrosis in rabbits (Tandon *et al*., 2013). Nanoparticles are defined as particulate dispersions or solid particles with a size in the range of 10-1000nm. The drug is dissolved, entrapped, encapsulated or attached to a nanoparticle matrix. Nanoparticles are prepared mostly by three methods: (1) dispersion of preformed polymers; (2) polymerization of monomers; and (3) ionic gelation or coacervation of hydrophilic polymers. Furthermore research indicates that nanoparticles can be loaded with plasmid DNA and they can serve as an efficient gene delivery system due to their ability to escape from the degradative endo-lysosomal compartment to the cytoplasmic

compartment. Furtermore, after their intracellular uptake and escape from endolysosomal degradation, nanoparticles could release DNA at a sustained rate resulting in sustained gene expression (Mohanraj, 2006). Nanoparticles have the potential be used as gene delivery vectors because to their ability to carry therapeutic molecules with high efficiency and low toxicity into targeted cells/tissues. A variety of nanoparticles have recently been tested for their potential as a gene therapy vectors for various cell types and BMP-7 gene delivery via nanoparticles may provide a potential way for successful future research (Tandon *et al*., 2013).

In conclusion the main focus of my research was on apoptosis and necrosis in PTECs and renal IRI induced AKI using novel therapeutics strategies. All the treatments were able to attenuate apoptosis and preserve cell viability in PTECs but were unable to protect against necrosis *in vitro*. Thiamine was able to attenuate the damage and dysfunction caused by renal IRI *in vivo* but other therapeutic drugs such as NO_2^-, benfotimine and BMP-7 were unable to protect renal IRI. It would be necessary to mention here that one of the main draw back of this study can be the fluctuations seen in serum creatinine levels in IRI group of rats, which were higher in NO_2^- study and were getting lower as my technique was getting better with time. However, every experiment was conducted in parallel with their untreated controls to circumvent this issue.

5.2: Further studies

To progress my work in chapter 2 regarding the oral administration of NO_2^- in renal IRI I would make use of stored tissue samples from this study to analyse the degree of nitrotyrosine staining (as a marker of $ONOO^-$) histologically. Furthermore, I would also like to investigate the mechanism involved by performing western blot to look at eNOS, iNOS and XOR levels in the kidney homogenate after renal IRI. In the thiamine study I would like to assess the circulating concentrations of thiamine in the blood pre and post renal IRI via an HPLC method. From the study presented in chapter 4 I would like to perform PCR on the anterior tibialis muscle in order to confirm BMP-7 expression after EP. Furthermore, histological analysis and TUNEL staining would be performed on stored mice kidneys with western blot analysis performed to assess for the expression of SMAD-5 and SMAD-1 in the kidney.

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Poster Communications:

Shoab Memon, Thiemermann C, Muhammad Yaqoob.Sodium nitrite inhibits apoptosis in PTECs and protects against renal Ischaemia/ reperfusion (I/R) injury in the rats. The Renal Association meeting; 2009 Liverpool, UK

Shoab Memon, J E kieswich, Thiemermann C, Muhammad yaqoob. Thiamine protects the kidney against the injury and dysfunction caused by ischaemia reperfusion by multiple mechanisms. ASN Oct 2009 San Diego, USA

Shoab Memon, J E kieswich, Thiemermann C, Muhammad yaqoob. Thiamine protects the kidney against the injury and dysfunction caused by ischaemia reperfusion by multiple mechanisms. The Renal Association meeting; 2010 Manchester, UK

Oral administration of sodium nitrite protects against Ishaemia/Reperfusion (I/R) injury in the rat kidney.

SA Memon, JE Kieswich, DA Allen, SM Harwood, NSA Patel, I Khan, MJ Raftery, C Thiemermann and MM Yaqoob. Centre for Translational Medicine and Therapeutics, William Harvey Research Institute Charterhouse Square London.

Our previous study showed that topical application of sodium nitrite directly to the kidney prevents ischaemia/reperfusion injury in the rat by conversion to nitric oxide by xanthine oxidoreductase. Intravenous injection of sodium nitrite also ameliorates cardiac injury following myocardial infarction. In the present study we examined the role of sodium nitrite on apoptosis in renal proximal tubular epithelial cells (HK-2) as well as I/R injury in the rat kidney after dietary administration sodium nitrite. PTECs were cultured for 24 h in growth medium with or without foetal calf serum (serum deprived) in the presence or absence of sodium nitrite (50 – 500 nM). The cells were scraped and whole cell lysates prepared using modified RIPA buffer. Caspase-3 activity was measured on lysates using a fluorescent peptide substrate (DEVD-AMC). Results are expressed as nmol AMC/mg/min and are mean±SD. Caspase-3 activity was significantly increased in cells that were serum deprived when compared with controls (serum deprived = 208.5±65.3; control = 30.2±20.3, n=3, $P<0.05$) whereas sodium nitrite (100 nM) attenuated this increase (serum deprived plus nitrite = 95.1±33.4, n=3, $P<0.05$). Although 100 nM sodium nitrite was optimal for this effect there was significant reduction in caspase activity at 50 nM. To further test this hypothesis in vivo, we carried out a pilot study in which male Wistar rats were divided into three groups; sham operated rats, rats subjected to ischaemia/reperfusion of the kidney (I/R group) and I/R plus pre-treatment with sodium nitrite (50 mg/L in drinking water for 7 days). There was a significant reduction in renal injury in rats given sodium nitrite prior to I/R when compared with those subjected to I/R alone (serum creatinine □mol/L, mean±SD; I/R = 226±70 compared with I/R plus nitrite = 88±29; n=4, P<0.05). Taken together, our data show that sodium nitrite attenuates the increase in apoptosis in serum deprived PTECs

and can prevent renal I/R injury in the rat. These interesting findings may have important clinical implications.

Thiamine attenuates apoptosis and injury caused by I/R of Kidney

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Renal ischemia-reperfusion (I/R.) injury is a major cause of acute kidney injury (AKI) and is a consequence of acute tubular injury initiated by a combination of reactive oxygen species (ROS) generation and ATP depletion. Thiamine has been shown to play an essential role in the scavenging of ROS and regeneration of ATP. It has been hypothesized that thiamine depletion during I/R injury is a cause of AKI and can be prevented by thiamine supplementation. To test this we cultured a human proximal tubular cell line (HK2) +/- antimycin (to induce ischemia), and +/- thiamine (10uM). Caspase assays showed a significant ($P<0.05$) reduction in caspase 3 activity in thiamine treated cells (n=3). An in vivo study using male Wistar rats administered thiamine orally for 3 days prior to induction of ischemia (45 minute clamping of both renal pedicles) showed a significant ($P<0.05$) reduction in serum creatinine and urea levels 24hrs post ischemia in a dose dependent manner (125-500mg/kg) compared to animals receiving vehicle (n=6/group). Ex vivo analysis of the kidneys showed a significant improvement in terms of histological scoring of the tubular injury score ($P<0.05$), TUNEL positive cells ($P<0.01$) and reduction of COX-2 and ICAM-1 proteins ($P<0.05$) in the thiamine treated group compared to control group. Assays for MPO activity, HNE and ROS demonstrated significant reductions in the thiamine treated group. We conclude that thiamine protects the kidney against the injury and dysfunction caused by I.R. by anti-apoptotic and anti-oxidant mechanisms. This study identifies a role of thiamine or its analogue in the prevention of AKI in multiple clinical settings and calls for large scale randomized control trials.

Thiamine attenuates apoptosis and injury caused by I/R of Kidney by multiple mechanisms.

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ABSTRACT:

Renal ischaemia-reperfusion (I/R.) injury is a major cause of acute kidney injury (AKI) and is a consequence of acute tubular injury initiated by a combination of reactive oxygen species (ROS) generation and ATP depletion. Thiamine has been shown to play an essential role in the scavenging of Reactive Oxygen Species (ROS) and regeneration of ATP. Thiamine deficiency causes up-regulation of COX- 2 which is associated with inflammation and causes cell death. It has been hypothesized that thiamine depletion during I/R injury may be the cause of AKI and can be prevented by thiamine supplementation. To test this we cultured a human proximal tubular cell line (HK2) +/- antimycin (to similate ischaemia), and +/- thiamine (10uM). Caspase assays showed a significant ($P<0.05$) reduction in caspase 3 activity in thiamine treated cells (n=3.Fig:1). An in vivo study using male Wistar rats administered thiamine orally(500mg/kg) for 3 days prior to induction of ischemia (45 minute clamping of both renal pedicles) showed a significant ($P<0.05$) reduction in serum creatinine and urea levels 24hrs post ischaemia compared to animals receiving vehicle (n=6.Fig:2a&2b). Ex vivo analysis of the kidneys showed a significant improvement in terms of histological scoring of the tubular injury score ($P<0.05$), TUNEL positive cells ($P<0.01$) and reduction of COX-2 and ICAM-1 proteins ($P<0.05$) in the thiamine treated group compared to control group. Assays for MPO activity (Marker of inflammation), HNE (marker of lipid peroxidation.Fig:3) and ROS (Fig:4) demonstrated significant reductions in the thiamine treated group. We conclude that thiamine protects the kidney against the injury and dysfunction caused by I/R. by anti-apoptotic, anti- inflammatory and anti-oxidant mechanisms. This study identifies a role of thiamine or its analogue in the prevention of AKI in multiple clinical settings and calls for large scale randomized control trials.