INVESTIGATING THE EFFECTS OF STEM CELL THERAPIES IN EXPERIMENTAL MODELS OF RENAL ISCHEMIA-REPERFUSION INJURY

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DECLARATION

I hereby declare that this thesis was composed by myself and that the work presented within it, unless explicitly stated otherwise, is my own. This work has not been submitted for any other degree or professional qualification at Glasgow University, or any other institution.

Henry R. W. Whalen
Glasgow, June 2017

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DEDICATION

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PREFACE

"The next day was a miracle. The young man had been on the brink of death only 24 hours previously. Yet, the morning following surgery he looked at me with bright eyes, and he felt hungry for the first time in months…
The transplanted kidney functioned immediately, with a dramatic improvement in the patient's renal and cardiopulmonary status. This spectacular success was a clear demonstration that organ transplantation could be life-saving."

Joseph Murray, winner of the Nobel Prize in Physiology or Medicine, recalls the first successful kidney transplant, performed at Peter Bent Brigham Hospital, Boston, Massachusetts, 1954.

ABSTRACT

The incidence of end-stage renal disease is increasing in Western Society. Renal transplantation is known to be the optimal treatment for ESRD, being associated with significant reduction in morbidity, mortality for patients and cost for wider society when compared to remaining on dialysis. Unfortunately, the growing number of patients listed for renal transplantation has occurred without a matched supply in the number of suitable organs. This has led to longer average waiting times for increased numbers of patients, who consequently suffer adverse outcomes at considerable cost to the National Health Service as a result of organ shortage.

One strategy employed by clinicians to meet demand for organs has been to transplant 'suboptimal' kidneys' historically rejected as unsuitable for transplantation, which are usually retrieved from older and less fit donors. Sometimes referred to as 'extended criteria' or 'marginal kidneys', such allografts are more prone to damage in the peri-transplantation period, with the major pathological process recognised to be ischemia-reperfusion injury (IRI). Although functioning 'marginal' allografts have been shown to confer benefit to recipients, early transplant failure is associated with negative outcomes. Consequently, there is a real need to develop treatments to mitigate renal IRI, especially since the use of 'marginal' kidneys is likely to increase.

Stem cell therapy has been shown to protect solid organs from IRI in a number of different animal models. Consequently, there is great interest in researching the ability of stem cell-based therapies to ameliorate solid organ damage and perhaps to encourage organ regeneration. However, debate exists regarding the exact mechanism by which stem cells produce their effects. Some researchers suggest that stem cells directly differentiate to replace specialised cell types in damage organs. Other investigators conclude that stem cells produce their effects in a paracrine fashion via the release of extracellular vesicles with the horizontal transfer of genetic material between cells.

Unfortunately, no therapies are currently in widespread use to reduce damage to allografts in the peri-transplant period. In part, this reflects the lack of robust

small animal models for screening potential renal IRI therapies before testing in large animal models. Furthermore, clinical application has been limited by safety concerns, and particularly by the risk of stem cells undergoing malignant transformation and subsequent tumour formation in recipients. However, investigators hypothesise that the use of stem cell-derived, extracellular vesicles may confer similar beneficial therapeutic efficacy, but lack many of the side effects associated with stem cells themselves.

This thesis describes experiments in which stem cell-based therapies are tested in conventional and novel animal models of renal IRI and renal transplantation. In Chapter 3, initial experiments unexpectedly demonstrated the potential of *ex vivo* expanded stem cells to undergo malignant change and induce tumour formation in recipient animals. Therefore, the subsequent research investigated the effects of freshly isolated stem cells or those of novel extracellular vesicle preparations. In Chapter 4, experiments unexpectedly demonstrated the shortcomings of a conventional rat model of renal IRI. Therefore, Chapter 5 describes the development of a novel rat of model of renal IRI, in which stem cell-based therapies may be tested. Using this animal model, Chapters 6 and Chapter 7 describe the investigation of novel stem cell-based therapies and their effects on renal IRI. Some of these treatments were found to protect kidneys from IRI damage with preservation of renal function and structure in the medium to long-term. Chapter 8 describes a rat model of renal transplantation, in which therapies were investigated after being screened for efficacy in the novel rat IRI model. Although no functional difference was demonstrated, renal histology was preserved by treatment, although the mechanisms by which this effect occurred remain unclear.

These findings suggest that stem cells and their extracellular vesicles have the potential to reduce peri-transplantation renal IRI and hence improve long-term outcomes of 'marginal' allografts. However, clinical translation requires the long-term efficacy and safety of these novel therapies to be investigated in large animal models of renal transplantation, before further testing in pilot studies.

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LIST OF ABBREVIATIONS

ADRCs – Adipose-derived regenerative cells

AKI – Acute kidney injury

AS – Albino Swiss

ASCs – Adult stem cells

ATP – Adenosine triphosphate

BD-MSCs – Bone marrow-derived mesenchymal stem cells

B-IRI – Bilateral ischemia-reperfusion injury

BUN – Blood urea nitrogen

c-DNA – complementary DNA

CIC – Constant infusion clearance

DBD – Donation after brain stem death

DCD – Donation after circulatory-confirmed death

DMSO – Dimethyl sulfoxide

d-NTPs – Deoxyribonucleotide triphosphates

DNA – Deoxyribonucleic acid

ECD – Extended criteria donor

ESCs – Embryonic stem cells

ECVs – Extracellular secretory vesicles

ESRD – End stage renal disease

GFR – Glomerular filtration rate

H & E – Haematoxylin and Eosin

HD – Haemodialysis

HLA – Human leucocyte antigen

IHC – Immunohistochemistry

iPSC – Induced pluripotent stem cells

IRI – Ischemia Reperfusion Injury

MHC – Major histocompatibility complex

MSCs – Mesenchymal stem cells

miRNA – Micro ribonucleic acid

mRNA - Messenger ribonucleic acid

MVs - Microvesicles

i-NOS – Inducible nitric oxide synthase

PF – Pathfinder Cells

PF-MVs – Pathfinder cell-derived microvesicles

q-PCR – Quantitative polymerase chain reaction

RNA – Ribonucleic acid

RRT – Renal replacement therapy

TNF-α – Tissue necrosis factor alpha

U-IRI – Unilateral ischemia-reperfusion injury

U-IRI +N - Unilateral ischemia-reperfusion injury with nephrectomy

U-IRI – N - Unilateral ischemia-reperfusion injury without nephrectomy

CHAPTER 1

A REVIEW OF RENAL DISEASE AND KIDNEY TRANSPLANTATION AND THE POTENTIAL OF STEM CELL TREATMENTS TO AMELORIATE ACUTE KIDNEY INJURY

INTRODUCTION

1.1 END-STAGE RENAL DISEASE AND TRENDS IN RENAL TRANSPLANTATION

The rapidly increasing incidence of end-stage renal disease (ESRD) in the developed world is a direct consequence of an ageing population who are more frequently suffering from lifestyle-related conditions such as type II diabetes and obesity. Predictions of global population demographics suggest that the number of people with ESRD will continue to rise, as will the proportion of the population who are elderly.

The kidneys' principle role is the elimination of toxic waste products, and the regulation of both circulating volume and the composition of body fluids. Furthermore, the kidney produces hormones including erythropoietin and renin and plays a crucial role in the metabolism of vitamin D. These functions are lost with the development of renal failure, and those patients with ESRD require renal replacement therapy (RRT) in order to survive. The term RRT encompasses the different modalities of dialysis (both peritoneal and haemodialysis) and kidney transplantation.

In patients undergoing dialysis, waste products are removed from the blood by diffusion across a semi-permeable membrane. Such patients are subjected to dietary and fluid intake restrictions, in addition to regular medication in an attempt to compensate for the loss of normal renal function. Inevitably, patients receiving dialysis have negative outcomes regarding length and quality of life when compared to healthy individuals (Collins et al., 2008, Zelmer, 2007, Wight et al., 1998). Furthermore, survival of dialysis patients has not changed appreciably in the last two decades, with an annual mortality of approximately 20% (Galliford and Game, 2009) and only 35% of dialysis patients alive after five years (Collins et al., 2008). This means that dialysis patients on average have only 20 – 25% the life expectancy of healthy age-matched controls (Collins et al., 2008). Data from the UK renal registry shows that since 2000 there has been an average 5% annual increase in the prevalence of RRT requirement, with 56,940 individuals receiving RRT in the UK at the beginning of 2014 (Pruthi et al., 2013). Of these, 49% of patients were undergoing a form of dialysis. At an annual cost of £35,000 per individual on haemodialysis, and £17,500 on peritoneal dialysis, dialysis provision amounts to 3% of the National

Health Service budget (Pruthi et al., 2013). ESRD patients receiving dialysis therefore create a huge national health and economic burden with sufferers experiencing poorer than average health status and quality of life (van Manen et al., 2002, Wight et al., 1998).

A cursory examination of the long-term outcomes of the current dialysis patient population reveals a minor reduction in morbidity and mortality associated with modern treatment regimes. However, it is clear that more elderly patients are now routinely offered dialysis than was the case two decades ago (Collins et al., 2012, Jager et al., 2003). Comparison of age-matched populations highlights the improved outcomes associated with dialysis in modern practice (Pippias et al., 2015, Marshall et al., 2015).

Nevertheless, kidney transplantation has long been associated with survival and quality of life benefits for recipients when compared with patients receiving other modalities of RRT (Laupacis et al., 1996, Wolfe et al., 1999, Port et al., 1993, Schnuelle et al., 1998, Simmons et al., 1990, Gokal, 1993, Kontodimopoulos and Niakas, 2008, Zelmer, 2007, Whiting et al., 1999, Meier-Kriesche et al., 2005). Life expectancy of patients who receive a deceased donor transplant is ultimately twice that of those remaining on dialysis (Wolfe et al., 1999). The recognised benefits of transplantation, coupled with improved safety in recent years has led to increased numbers of patients on UK transplant waiting lists in the last decade. (See figure 1.1) Older recipients now comprise the highest proportional increase of those patients added to national waiting lists, with almost half of patients awaiting renal transplant being aged > 50 years (Wolfe et al., 2010). High volume studies have shown reduced mortality rates for elderly patients undergoing successful renal transplantation compared with similar patients remaining on waiting lists and receiving dialysis.

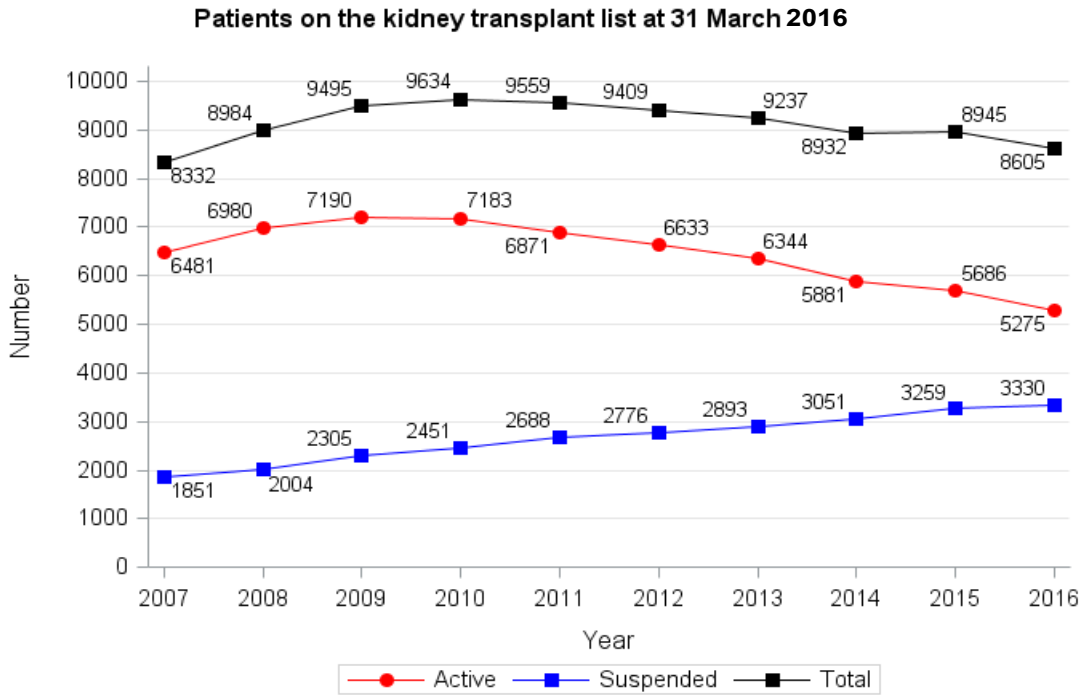


Figure 1.1: shows the trend in the number of patients on the kidney transplant list at 31 March each year between 2007 and 2016. The slight fall in the number of patients actively waiting for a transplant largely reflects the increasing use of 'marginal' kidneys (Transplant, 2016).

Unfortunately, the length of time on dialysis is recognised as an independent predictor of poor transplant survival as ESRD patients are subjected to considerable physiological deterioration due to the systemic nature of their disease (Meier-Kriesche and Kaplan, 2002).

With health economics playing an increasingly important role in modern healthcare provision, it is notable that within 2 years renal transplantation becomes cost effective, saving the NHS an annual of £25,800 per patient transplanted (Pruthi et al., 2013). Renal transplantation is therefore an excellent example of a healthcare technology that provides a reduction in morbidity and mortality, and is at least theoretically, cost saving and cost effective when compared to dialysis. The promotion of renal transplantation to expand the available number of kidneys has additional appeal especially considering evidence that ESRD patients transplanted earlier experience better outcomes (Meier-Kriesche and Kaplan, 2002). Consequently the number of renal transplants performed in the UK has tended to increase annually (see figure 1.2), although there is still a shortfall in the supply of kidneys due to a lack of suitable donors.

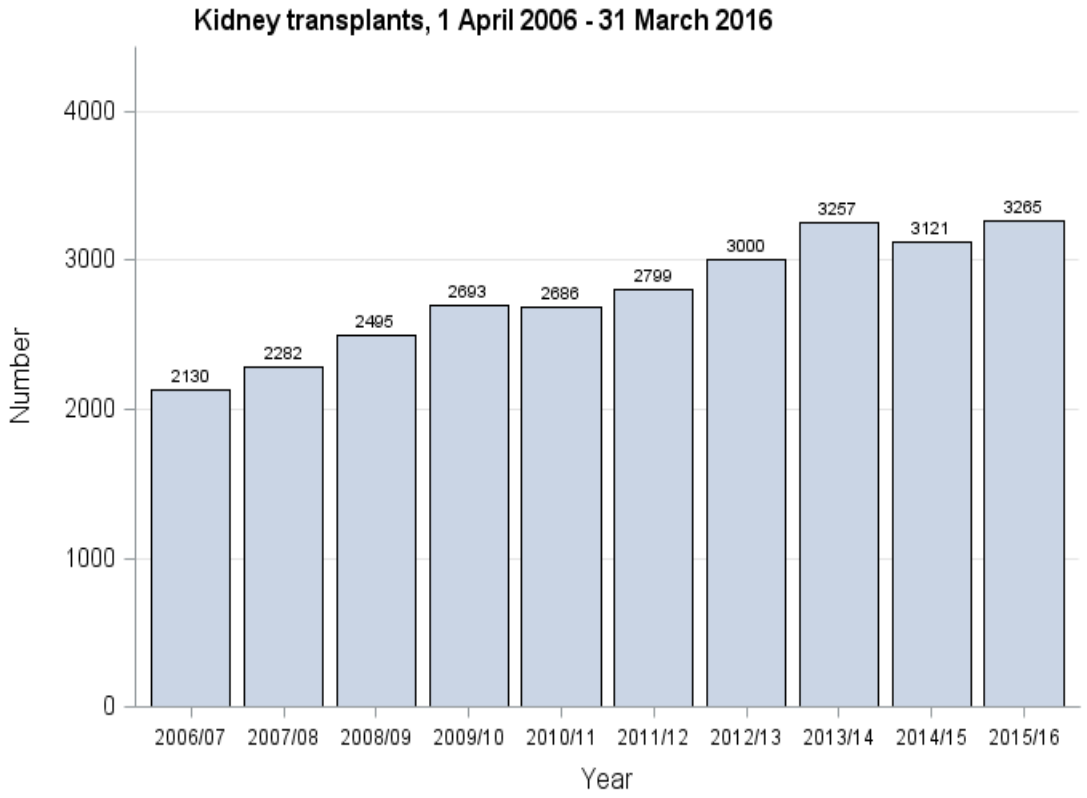


Figure 1.2 shows the total number of kidney transplants performed in the last ten years. The number of transplants steadily increased each year from 2130 in 2006/07 to 3,265 in 2015/16 (Transplant, 2016).

1.11 TRENDS IN KIDNEY DONATION

Unfortunately, the growing demand for transplantable kidneys has occurred without a matched increase in kidney supply, resulting in progressively increasing waiting list times (Matas et al., 2014). This disparity has been a problem since renal transplantation became part of accepted clinical practice. However, with increasing numbers of patients with ESRD listed for transplantation, the shortage of transplantable kidneys has never been so apparent. This shortfall is the result of a number of factors, the most important of which is increased demand due to the success of modern transplantation itself. Coupled with the falling number of kidneys from young, deceased donors, there is a persistent kidney shortage that is the primary factor limiting the wider use of transplantation. This inevitably results in increased morbidity, mortality and cost as ESRD patients remain on dialysis.

1.12 CLASSIFICATION OF RENAL ALLOGRAFTS

To analyse the disturbing current donation trends that lead to thousands of patients languishing on UK waiting lists, one must first understand the types of kidney donor and subsequent classifications of allograft that arise as a result of the donation process.

In essence, kidney donors may be divided into three categories:

Living donors

It is accepted that outcomes from living donors are superior to those from age-matched deceased donors – fully HLA-mismatched live donor kidneys have improved outcomes when compared to fully matched deceased donor grafts (Meier-Kriesche and Kaplan, 2002, Mange et al., 2001). The previous two decades have seen a 3-fold increase in live donation, although in recent years the trend for living donation has plateaued while the total demand for organs has continued to rise. (See figure 1.3)

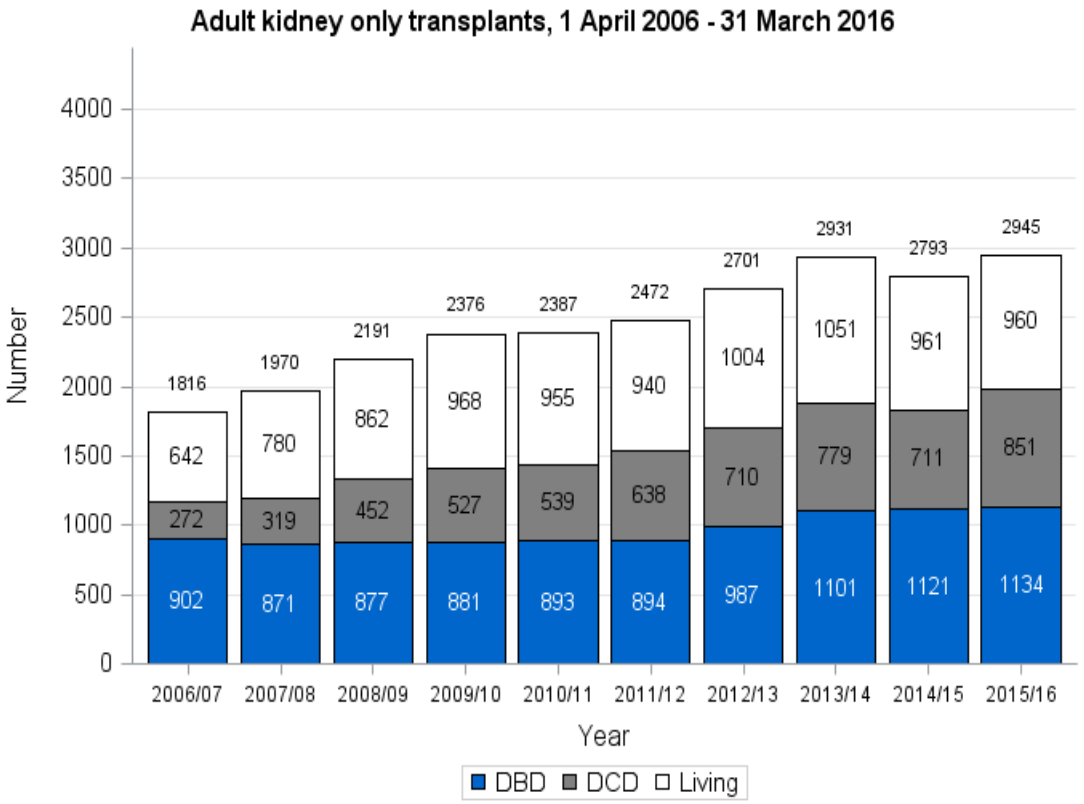


Figure 1.3: shows the total number of adult kidney-only transplants performed in the last ten years, by type of donor. The number of adult transplants from donors after circulatory death (DCD) steadily increased from 272 in 2006/2007 to 851 in 2015/2016. The number of adult transplants from donors after brain death (DBD) has increased in the last four years to 1,134 in 2014/2015 after remaining relatively constant between 2007/2008 and 2011/2012. The recent increase in DBD transplantation is due to the increased used of extended criteria kidneys. The number of adult living kidney transplants performed was steadily increasing over time before plateauing, and subsequently decreasing by 8% to ~ 960 in the two financial years. (Transplant, 2016)

Deceased Donors

Kidneys for transplantation are recovered from deceased donors who:

(1) Donate after brain stem death (DBD)

(2) *Donate after circulatory-confirmed death (DCD)*

Formerly referred to as 'heart-beating' (DBD) and 'non-heart-beating' (DCD) the current terminology more accurately reflects the absence of peripheral pulses and blood pressure over asystole to declare death.

Outcomes from deceased donor kidneys are inferior to those obtained with allografts from living donors. Partly this is probably due to hormonal and cardiorespiratory disturbances that occur in the donor during the retrieval process. Kidneys in patients suffering brain stem death (DBD) are exposed to variations in catecholamine levels and a 'cytokine storm' (Chiari et al., 2000, Gramm et al., 1992, Pratschke et al., 2001). The hypoperfusion that results from this inflammatory milieu may explain the adverse allograft outcomes observed in DBD kidneys when compared to those from living donors. It is unclear if this process is substantially different in the setting of severe, irreversible head injury without herniation that is present in the majority of DCD donors (Brook et al., 2003). However, compared to DBD kidneys, those allografts that result from DCD are subjected to greater periods of hypoxia and hypotension during the progression to circulatory arrest (so-called "agonal phase") and the mandatory 5-minute period of warm pulseless ischemia prior to retrieval (Perera, 2012).

Traditionally, the use of DBD kidneys predominated in most transplant centres. However, due to falling numbers of suitable DBD donors, coupled with increased demand for transplantable kidneys, the use of DCD kidneys has grown in the past decade as a further means of expanding the pool of donor kidneys. (See figure 1.3) DCD kidneys show higher rates of primary non-function and delayed graft function (Neyrinck et al., 2013, Renkens et al., 2005, Singh et al., 2011), but after the immediate postoperative period have been shown to have comparable outcomes when compared to DBD kidneys (Huynh et al., 2015, Weber et al., 2002), provided cold ischemic times are kept below 24 hours (Summers et al., 2010, Summers et al., 2013). Furthermore, DCD transplantation demonstrates a survival benefit to recipients over waiting for a DBD kidney to become available (Snoeijs et al., 2010a, Snoeijs et al., 2010b).

Traditionally, kidneys for transplantation were retrieved from deceased individuals meeting the criteria for 'ideal donors' (Port et al., 2002). These criteria include:

(i) Age between 10 – 39 years.
(ii) No history of hypertension
(iii) Did not die from a cerebrovascular accident
(iv) Pre-donation creatinine < 150μg/dL.

Such 'ideal' kidneys typically came from individuals who had been in excellent health but had suffered an isolated traumatic event. However, during the last 15 years, there has been a steady decline in the numbers of 'ideal' deceased donor kidneys offered for transplantation (Johnson et al., 2010). The cause for the falling numbers of 'ideal donors' is not entirely understood. Reasons cited often include a reduction in high-energy traumatic accidents, reduced intracranial haemorrhage due to improved radiological intervention, and increased reluctance of intensive care doctors to ventilate patients with head injuries whose radiology reveals a poor prognosis (Briggs et al., 1997, Bederson et al., 2009).

1.13 NOVEL STRATEGIES TO EXPAND SUPPLY OF RENAL ALLOGRAFTS

The falling number of kidneys from 'ideal donors', coupled with increased demand for transplantation, has led to persistent kidney shortages resulting in morbidity and mortality for dialysis patients who wait for a transplant to become available. In an attempt to fill this void, clinicians have adopted a number of strategies, involving the use of kidneys that previously would have been deemed un-transplantable.

One such strategy has been the use of kidneys from 'extended criteria donors' (ECD). The criteria for ECD kidneys are based on national registry analysis from the United States for kidneys transplanted between 1995 and 2000. Age and a combination of medical risk factors were found to be associated with a relative risk for graft failure of greater than 1.7 when compared to the ideal donor reference group (Port et al., 2002). Donor elements utilised to define ECDs include:(Port et al., 2002, Metzger et al., 2003)

(i) Donor age greater than 60

(ii) Or donor age between 50 -59 with 2 of the following features
- Cerebrovascular accident as cause of death
- Hypertension
- Preexisting donor renal dysfunction with serum creatinine > 150mg/dL.

Growing demand has necessitated the routine use of DCD and ECD kidneys. Despite controversies regarding allograft survival rates, both DCD and ECD kidney transplants have gained acceptance, although clinicians now acknowledge a higher risk of poor outcome when compared to those allografts that come from 'ideal' donors (Port, 2003, Nathan et al., 2003). However, the risk of a poor outcome needs to be balanced against the benefits provided by a functioning transplant, compared to remaining on dialysis (Merion, 2005). Short-term allograft survival rates have improved continuously in recent years, and most recent studies have reported acceptable medium-term DCD and ECD allograft survival rates. However, less impressive improvements are reported for long-term outcomes (Lamb et al., 2011), and transplanting ECD kidneys with increased donor age is known to result in higher rates of graft failure (Veroux et al., 2012). Inevitably, recipient survival is reduced when a sub-optimal graft fails (Meier-Kriesche et al., 2001).
Nevertheless, many DCD and ECD kidneys perform extremely well, and there appears to be substantial survival, lifestyle and financial benefit in receiving an ECD kidney when compared to remaining on dialysis (Merion, 2005, Meier-Kriesche et al., 2001, Ojo et al., 2001, Martin Navarro et al., 2009, Rao et al., 2007).

As the evidence for the use of kidneys from DCD and ECD sources accumulates, transplantation of such kidneys is progressively increasing in many centres. (See Figure 1.4). Recently > 50% of kidneys transplanted in Europe or North America are from donors aged > 50 years, with many recipients of these often being elderly in an 'old for old' allocation scheme (Wolfe et al., 2010, Moers et al., 2009). Here, older kidneys are considered a solution for more elderly patients, as lower patient life expectancy compensates for the lower expected survival rate of the aged kidney (Foss et al., 2009, Chavalitdhamrong et al., 2008, Giessing et al., 2009).

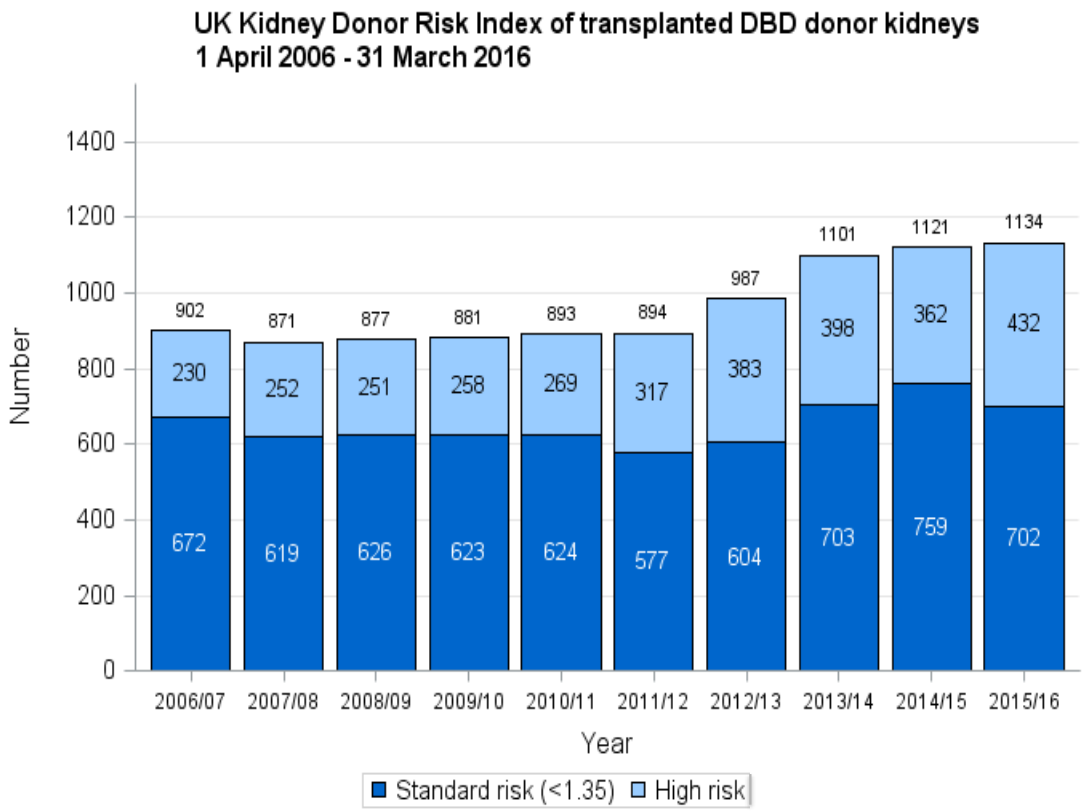


Figure 1.4: shows the use of 'Extended Criteria' DBD kidneys has almost double in the last decade (Transplant, 2016).

The trend of transplanting kidneys that would previously have been deemed unsuitable is likely to continue as clinicians 'push the boundaries' in an attempt to meet demand for transplantation. Transplanting ECD kidneys present unique challenges, and innovative approaches are required to achieve satisfactory results. The future challenges are therefore to predict better which organs are likely to benefit recipients by functioning well, and how best to counter the early, non-specific inflammatory insults to which they have been exposed in the transplantation process.

1.2 MECHANISM OF RENAL REGENERATION IN RESPONSE TO INJURY

From donor to recipient, kidney transplantation entails a series of steps, during all of which allograft damage may result. Long-term function and outcome of kidney transplants are determined by cumulative peri-transplantation injury, arising from such factors as hypoperfusion during organ retrieval, prolonged cold ischemia, warm ischemia, ischemia reperfusion injury, episodes of acute rejection and calcineurin toxicity (Forsythe, 2009). Injury to the allograft in the initial stages is recognised to have a significant influence on long-term allograft function, by predisposing to chronic interstitial fibrosis and tubular atrophy (Pascual et al., 2012, Campistol et al., 2009). Over time this ultimately leads to a reduction in functional nephron mass (Mueller et al., 2011). These changes, often referred to 'chronic allograft nephropathy' have clear implications for transplant survival and hence patient longevity (Nankivell and Kuypers, 2011, Moreso and Hernandez, 2013).

As described, current trends in organ donation have forced clinicians to utilise kidneys from older and less fit donors (ECD kidneys), in addition to kidneys from DCD patients. Such allografts are more prone to peri-transplantation injury (Bagul et al., 2013, Wolfe et al., 1999, Mollura et al., 2003, Meier-Kriesche and Kaplan, 2002, Pomfret et al., 2008, Singh et al., 2011, Renkens et al., 2005), and as a result are more susceptible to acute complications including delayed graft function (defined as the need for dialysis in the first post-transplant week, except for reasons of hyperkalaemia or volume overload (Cantaluppi et al., 2013)) acute rejection or primary non-function (2010, Singh et al., 2011, Renkens et al., 2005, Pomfret et al., 2008). Peri-transplantation injury that results in a long-term reduction in functional capacity of allografts that contained fewer nephrons to begin with is likely to be associated with negative outcomes. Therefore, the minimization of allograft injury in the peri-transplant period and/or strategies to promote the healthy repair of transplant damage seems likely to represent a new field, aiming to optimise transplant function and patient health following graft implantation (Powell et al., 2013, Furuichi et al., 2012). However, the development of new therapeutic strategies necessitates an understanding of both the mechanisms by which renal injury occurs and the process of endogenous kidney regeneration (Wise and Ricardo, 2012).

1.21 RENAL REGENERATION

Different solid organs have a varying capacity for regeneration, and also the rate of cell turnover varies between tissues. Consequently the time it takes different organs to recover after acute injury is variable. For example, the epithelial tissues of the skin and intestine have a high cell turnover and may completely self-renew within days (Blanpain et al., 2007, Blanpain et al., 2004).

In contrast to the skin and intestines, the mammalian kidney is particularly morphologically complicated, containing approximately 26 terminally differentiated cell types that are organised into a highly ordered structure (Miner, 1999). Furthermore, the kidney has a high metabolic rate and is constantly exposed to waste products and toxins. Consequently, the kidney has a relatively low cell turnover rate, and the rate of repair and regeneration after injury is dependent on the specialised cell type involved (Bussolati et al., 2008, Humphreys and Bonventre, 2008). As a result, the mammalian kidney is particularly prone to acute injury. Despite this, the mammalian kidney does have some capacity for self-regeneration and repair, and the ability to recover from severe damage.

The capacity for renal regeneration is variable between different species. For example, adult cartilaginous fish display the capacity to form new nephrons in response to a significant renal insult (Elger et al., 2003, Roufosse and Cook, 2008). In contrast, mammalian kidneys exhibit very limited regenerative ability. Neo-nephrogenesis has been reported in prenatal sheep after unilateral nephrectomy, with the remaining kidney demonstrating up to a 45% increase in nephron number (Douglas-Denton et al., 2002). Initial studies in post natal rabbits illustrated an increase in the total number of glomeruli with the capacity to differentiate following unilateral nephrectomy (Sidorova, 1978), but subsequently these results have not been reproduced. The general consensus therefore is that mammalian kidneys cease the formation of new nephrons after birth, and merely demonstrate compensatory hypertrophy of the remaining nephrons after injury (Sidorova, 1978). However, the mammalian kidney does undergo limited regeneration and remodeling after injury and has the capacity to restore deficits in both structure and function.

1.22 ACUTE KIDNEY INJURY

Native acute kidney injury (AKI) is a syndrome characterised by a rapid decline in glomerular filtration rate, reflected by a rise in serum creatinine and/or a fall in urine output (Khwaja, 2012). The loss of excretory function leads to the rapid accumulation of products of nitrogen metabolism, increased potassium and phosphate and a metabolic acidosis. This is a significant clinical problem, occurring in 5 – 10% of all acute hospital admissions, and accounting for up to 20% of admissions to intensive care units (Erpicum et al., 2014). Overall mortality rates for inpatients suffering AKI is reported to be 35 – 40%, rising to around 75% for patients with multifactorial, sepsis associated renal failure (Nash et al., 2002). A significant proportion of patients with AKI require dialysis, at least on a temporary basis (Lameire et al., 1998). The mortality rate of patients requiring dialysis for AKI (50 - 60%) is nearly twice that of patients without AKI (Ricci et al., 2008, Bellomo et al., 1995).

Aetiology of AKI is often multifactorial. Hypovolemia, sepsis, nephrotoxic injury, autoimmune damage and physical obstruction to urine flow are all well recognised causes of AKI affecting native kidneys.

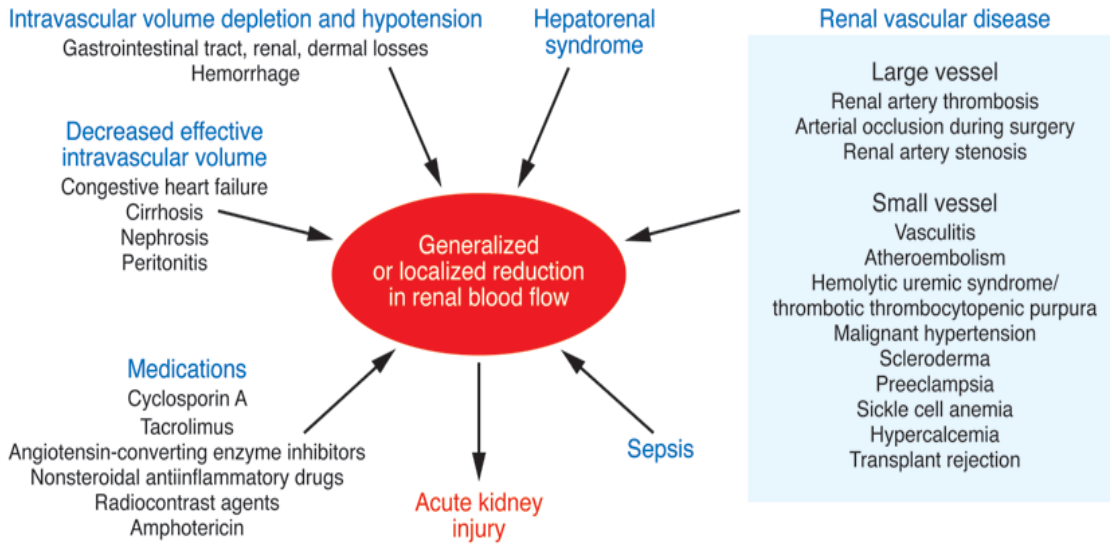


Figure 1.5: Various pathophysiological states and medications can contribute to a reduction of RBF, causing generalised or localised ischemia to the kidney leading to AKI. This figure represents a partial list and points to ischemia as being a common pathway in a variety of clinical states affecting the kidney (Bonventre and Yang, 2011).

Transplanted kidneys are susceptible to all forms of AKI that are seen in native organs, especially in the peri-transplant period when the allograft is ‘bedding in'. However, the ischemia that occurs during allograft retrieval and cold storage, coupled with the warm ischemia and reperfusion injury that occurs during implantation makes IRI an especially important insult affecting renal allografts (Koffman and Gambaro, 2003, Sanchez-Fructuoso et al., 2003, Pascual et al., 2008, Ojo, 2005).

Unfortunately IRI is an unavoidable event in kidney transplantation, as it is technically impossible to move a transplant from donor to recipient without temporary cessation of renal blood flow. IRI is therefore a necessary injury that negatively impacts on short and long-term outcomes by causing direct inflammatory tissue damage and predisposes to enhanced allograft immunogenicity, and hence to episodes of acute cell-mediated rejection (Eltzschig and Eckle, 2011).

1.23 CELLULAR AND MOLECULAR MECHANISMS OF KIDNEY DAMAGE DURING ISCHEMIA REPERFUSION INJURY

The cellular and molecular mechanisms that culminate in ischemic tissue damage after kidney IRI have been extensively studied and well described (Sutton et al., 2002, Bonventre and Yang, 2011, Kosieradzki and Rowinski, 2008). A sustained interruption of renal blood flow is associated with a rapid drop in oxygen partial pressure and nutrient concentration. Anaerobic respiration via glycolysis is necessary to maintain a meager supply of adenosine triphosphate (ATP), but as a by-product of this process, lactate accumulates in the cell cytosol, lowering pH levels such that mitochondrial function is impaired. Interestingly, a recent paper has identified the accumulation of succinate during ischemia as a pivotal step in the generation of mitochondrial ROS during reperfusion (Chouchani et al., 2014)

In this "initiation phase" of IRI, proximal tubular epithelial cells are the primary site of damage. They quickly display a loss of cellular polarity, cytoskeleton disruption, and loss of brush border integrity in response to hypoxia (Ashworth and Molitoris, 1999). Tubular obstruction from sloughed cells and protein aggregation occurs as a result of cellular ATP depletion (Kellerman, 1993, Ashworth and Molitoris, 1999). Proteases and phospholipases become activated and cause extensive cellular injury (Goligorsky, 2005). This eventually leads to functional impairment of tubules, which

are not able to preserve distinct fluid filled compartments with precise electrolyte concentrations (Sheridan and Bonventre, 2000).

Endothelial dysfunction and swelling results in microvascular occlusion increased resistance to blood flow and renal hypoperfusion. This pathological process exacerbates and extends the initial renal injury and is consequently known as the "extension phase"(Sutton et al., 2002). At this stage, sustained hypoxia causes tubular cells and/or glomerular podocytes to undergo necrosis or apoptosis while surrounding cells shift their metabolism from aerobic to anaerobic pathways. The resulting further accumulation of lactate and oxygen free radicals leads to an increase in pro-inflammatory cytokines and the activation of innate immunity (Linfert et al., 2009). Expression of adhesion molecules leads to the infiltration and activation of macrophages and other leukocytes, augmenting an aggressive inflammatory response (Akcay et al., 2009). Subsequently trans-epithelial and trans-endothelial leaks appear at the cortico-medullary junction, an area where high oxygen demand and low oxygen tension at steady state predispose to acute tubular necrosis (Evans et al., 2008).

Paradoxically, the final stage of the ischemic injury occurs during the reperfusion period. This is characterized by re-oxygenation, production of ATP by oxidative phosphorylation and generation of high concentrations of reactive oxygen species that result in hyper-oxidation of proteins, lipids and membranes of both epithelial and endothelial cells. Concurrently, the synthesis of inflammatory cytokines, including tumour necrosis factor alpha (TNF-α), interleukins 6 and 8 and surface adhesion molecules further encourage the non-specific recruitment of inflammatory cells. Leukocyte adherence and lymphocyte activation perpetuate inflammatory cell recruitment to the allograft, leading to tissue damage, apoptosis and inflammation. In addition, up-regulation of major histocompatibility complex (MHC) class II antigen expression increases organ alloreactivity, increasing the probability of acute cell-mediated rejection. Ultimately, the processes of ischemia and reperfusion lead to renal tubular cell dysfunction, the release of donor MHC antigens into the host circulation, increased allograft immunogenicity, tissue injury and apoptotic cell death. These processes contribute to short and long-term allograft dysfunction.

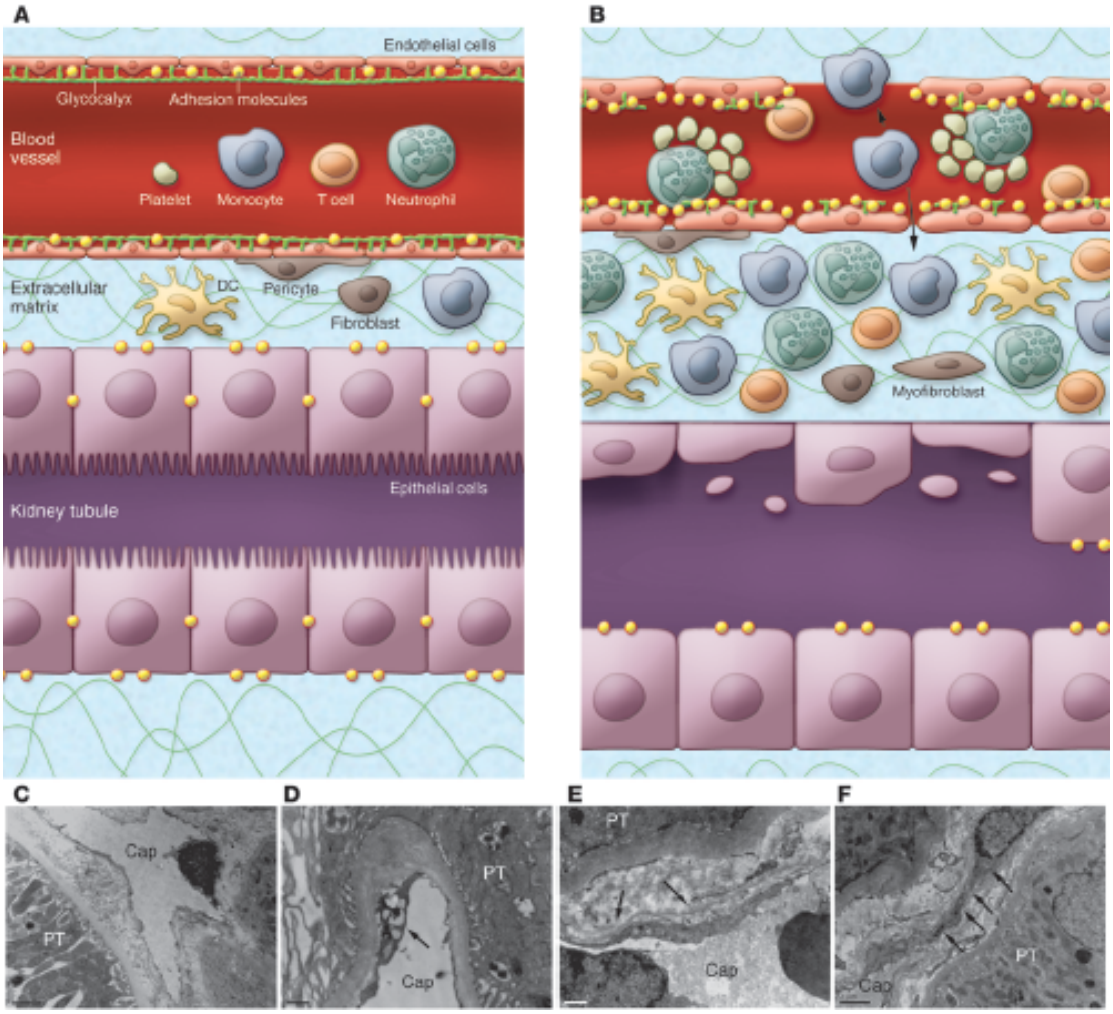


Figure 1.6: Endothelial injury in ischemia/reperfusion AKI. **(A)** Normal epithelium and endothelium separated by a small interstitial compartment. **(B)** Ischemia/reperfusion causes swelling of endothelial cells; disruptions of the endothelial monolayer; and upregulation of adhesion molecules such as ICAMs, VCAMs, and selectins, resulting in enhanced leukocyte-endothelium interactions. There is formation of microthrombi, and some leukocytes migrate through the endothelial cells into the interstitial compartment. The interstitial compartment is expanded with enhanced numbers of inflammatory cells and interstitial oedema forms.
(C) Transmission electron microscopy of normal human peritubular capillary (Cap).
(D–F) Acute tubular necrosis. The peritubular capillaries (PT) show vacuolar degeneration of the endothelial cell (arrow in D), thickening and multilayer basement membrane formation (arrows in E), and attachment and penetration of monocyte-like cells (arrows in F) in the interstitial region. Scale bars: 2 μm (C and F); 1 μm (D and E). From Bonventre and Yang (Bonventre and Yang, 2011).

1.24 RENAL REPAIR

Initial renal repair occurs in the “maintenance phase”, via cellular replacement of the injured tubular epithelium. Under normal conditions, proximal tubular epithelial cells divide at a low rate, which under normal circumstances is enough to balance the routine loss of other epithelial cells (Nadasdy et al., 1994, Prescott, 1966). However, after IRI there is a marked increase in cell turnover, with increased numbers of new epithelial cells needed to replenish those lost by necrosis or apoptosis.

There has been debate regarding the origin of the new renal epithelial cells. Potential sources from which epithelial cells may originate include:

(1) Intra-renal progenitor cells.

(2) Circulating stem cells that localise to the site of injury and differentiate into functional epithelium.

(3) Surviving epithelial cells that divide into new epithelial cells.

Recent evidence suggests mammalian kidneys contain resident stem cells, with progenitor cell populations initially described in embryonic kidneys reportedly identified within the urinary pole of the glomerular parietal epithelium of Bowman's capsule (Ronconi et al., 2009, Appel et al., 2009, Sagrinati et al., 2006). Furthermore, another population of resident stem cell has been localised in papillary "niches"(Oliver et al., 2009), although these have not yet been confirmed to participate in kidney repair (Song et al., 2011). Either of these may represent stem or progenitor cell populations that localise to the urinary pole of the parietal epithelium and could be responsible for podocytes replacement after injury (Ronconi et al., 2009, Appel et al., 2009). Despite these observations, the role of resident stem or progenitor cells within both healthy and injured mammalian kidneys has not been well defined. Given the of presence of stem cells within the embryonic kidney, and the demonstration of neo-nephrogenesis during prenatal life, it is possible that stem cells play a role in the response of adult mammalian kidneys to both minor and severe injury (Reule and Gupta, 2011). However, studies using genetic fate-mapping techniques in transgenic mice have demonstrated surviving tubular cells proliferate to replenish lost tubular cells and are unlikely to come from either resident or circulating renal stem or progenitor cells (Humphreys et al., 2008).

Renal cellular loss leads to the infiltration of bone marrow-derived inflammatory cells that contribute to both tissue destruction or repair depending on the extent of an injury (Ricardo et al., 2008). Circulating stem and progenitor cells, including mesenchymal stem cells and hematopoietic stem cells, are also known to migrate to damaged organs, and may also contribute to renal repair (Patschan et al., 2006, Togel et al., 2004). Early studies suggested that replacement of lost epithelial cells was directly by circulating stem cells of a bone marrow origin (Bonventre, 2003, Witzgall et al., 1994). However, later analysis failed to replicate these findings and demonstrated that bone marrow derived stem cells do not directly replace lost epithelium, but exert paracrine effects that facilitate repair by reducing inflammation (Duffield et al., 2005, Humphreys and Bonventre, 2008).

The formation of new tubular epithelium in response to injury is most likely mediated by surviving epithelial cells near the site of damage (Humphreys et al., 2008, Romagnani, 2009, Duffield et al., 2005). Neighbouring cells are thought to de-differentiate, re-express developmental programs and migrate to areas of denuded basal membrane, where damaged cells have undergone apoptosis, necrosis or detachment. (See figure 1.7) Subsequently proliferation, engraftment and re-differentiation into functional tubular epithelial cells occurs, resulting in tubular cells that exhibit normal polarity and physiological transport mechanisms (Duffield et al., 2005, Bussolati et al., 2008, Bonventre and Yang, 2011, Humphreys et al., 2008, Romagnani, 2009).

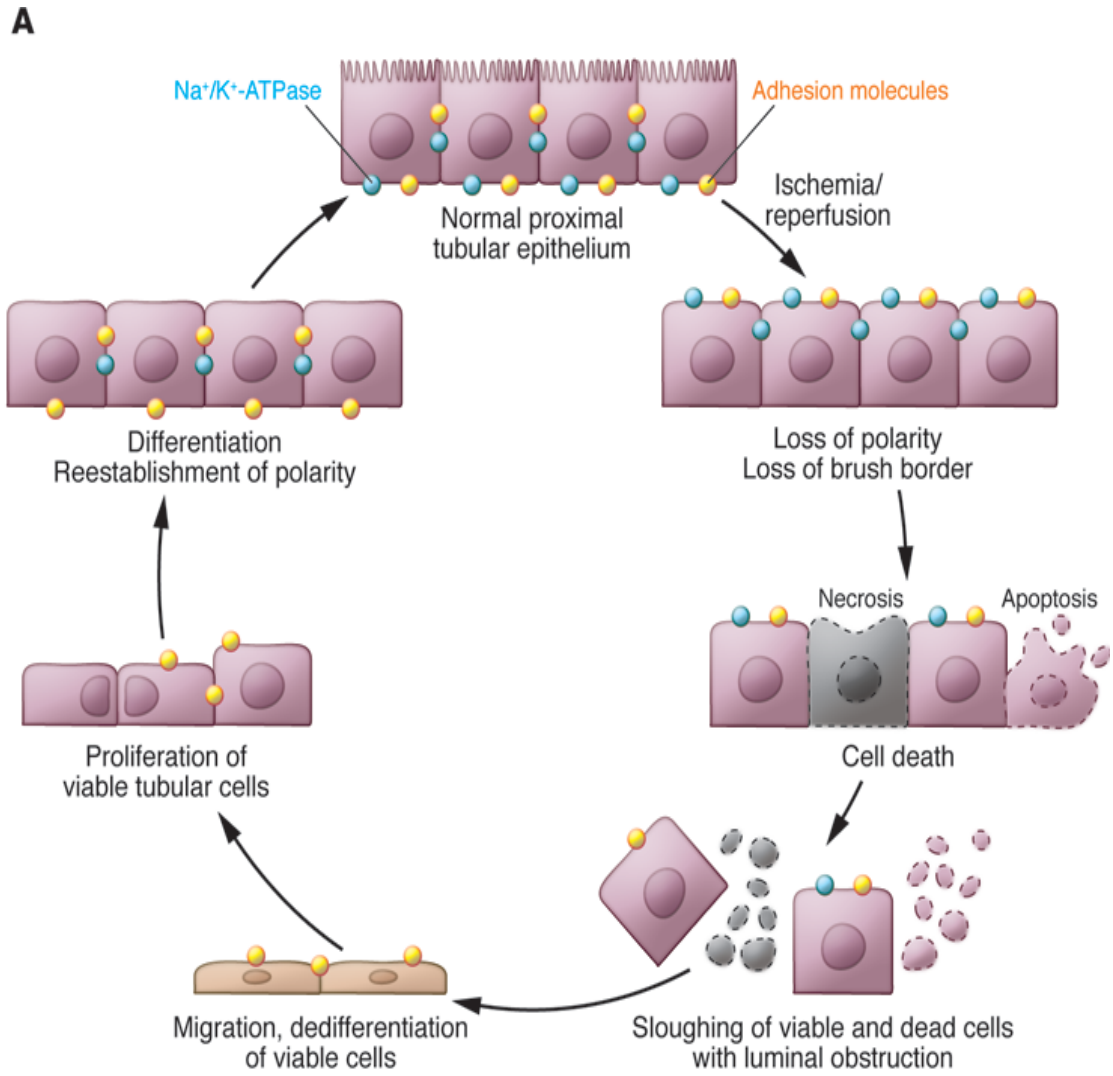


Figure 1.7: Normal tubular cell repair after ischemic AKI. With IRI, the typically highly polar epithelial cell loses its polarity and brush border with proteins mislocated on the cell membrane. With increasing time/severity of ischemia, there is cell death by either necrosis or apoptosis. Some of the necrotic debris is released into the lumen. Viable epithelial cells migrate and cover denuded areas of the basement membrane. These cells undergo division and replace lost cells. Ultimately, the cells go on to differentiate and reestablish the multivesicular polarity of the epithelium (Bonventre and Yang, 2011)

This is associated with the exit of inflammatory cells, downregulation of inflammatory pathways and re-establishment of normal renal blood flow. Apoptotic pathways initiate the removal of damaged and "surplus cells", with subsequent return of physiologic tubular and vascular integrity and function. Unfortunately, severe glomerular injury is irreversible, and progressive glomerular loss leads to expansion of the renal interstitium and the formation of fibrosis (Liu and Brakeman, 2008, Abbate and Remuzzi, 1996).

1.3 RATIONALE FOR THE USE OF STEM CELL-BASED THERAPIES IN THE TREATMENT OF RENAL ISCHEMIA-REPERFUSION INJURY

Regenerative medicine is broadly defined as the study of the repair, regeneration and restoration of diseased or damaged cells, tissues and organs (Mironov et al., 2004). As a field it still very much in its infancy with clinical applications being limited. There are no therapies that reduce injury to native or transplanted kidneys, or treatments that promote the renewal of specific kidney cell types in widespread clinical use. Modern management strategies focus instead on supporting failing organs until recovery, during which time exposure to further systemic or iatrogenic insults may injure a kidney further (Mongardon et al., 2009). ECD and DCD organs are particularly vulnerable to peri-transplantation injury (Koffman and Gambaro, 2003, Metcalfe et al., 2001a, Metcalfe et al., 2001b), and the trend towards increasing use of marginal allografts has highlighted the lack of progress in the regenerative area. Innovative approaches to developing protective and restorative treatments are needed, and reports of stem cell-based therapies mitigating renal IRI in animal models has led investigators to actively pursue novel therapies for AKI that are based on cellular mechanisms of repair and regeneration.

Stem cells are prime candidates for regenerative therapies because they can theoretically replace damaged cells when administered either centrally or peripherally. In addition, stem cells secrete a broad range of trophic growth factors, cytokines and chemokines and release extracellular vesicles that facilitate the lateral transfer of organ-protective messages into target cells. Furthermore, stem cells possess powerful immunomodulatory and anti-inflammatory functions and may enhance the proliferation of endogenous stem or progenitor cells in the repair of damaged organs and tissues (Togel and Westenfelder, 2012). The rationale for stem cell use in clinical renal transplantation is therefore to reduce the severity of IRI, prevention of acute cell-mediated rejection, with the long-term aim of lessening immunosuppression requirement (Franquesa et al., 2012a), encouraging cellular regeneration and reducing chronic allograft nephropathy (Bank et al., 2015).

The potentially beneficial effects of using stem cells in tissue regeneration were first reported by Till and MuCulloch more than 50 years ago (Becker et al., 1963, Till and Mc, 1961). The subsequent enthusiasm for the potential of this novel therapy led to a

variety of different stem cell types being investigated for use in regeneration medicine, with varying degrees of success. The first successful uses of a cell-based therapy for tissue regeneration described the use of bone marrow-derived stem cells to repair damaged muscle fibres in immunodeficient mice (Ferrari et al., 1998). Subsequently, numerous studies have reported beneficial effects of various stem cell-based therapies for mitigation of acute injury and/or restoration of healthy tissue (Monsel et al., 2014).

1.31 DEFINITIONS AND CLASSIFICATION OF STEM CELLS

Stem cells are found in all multicellular organisms and have two characteristic properties by which they are defined (Potten and Loeffler, 1990):
(1) ***Self-renewal*** – the ability to undergo cycles of cell division and remain in an undifferentiated state:
(2) **P*otency*** – the ability to differentiate into both non-renewing progenitor cells or terminally differentiated, functioning specialised cell types.

Stem cells may be classified according to their developmental potential:
• ***Totipotent Stem Cells:*** Produced by the fusion of an egg and sperm cell, these cells are able to give rise to all embryonic and extra-embryonic cell types and therefore can create a complete and viable organism. In mammals only the zygote and the first cleavage blastomeres are totipotent (Donovan and Gearhart, 2001).

• ***Pluripotent Stem Cells*** are able to differentiate into any of the three germ cell layers:

- *Endoderm:* gives rise to tissues of respiratory and digestive tracts.
- *Mesoderm* gives rise to muscle, bone, cartilage, blood and fat.
- *Ectoderm* gives rise to epidermal cells and cells of nervous system (Donovan and Gearhart, 2001, Lovell-Badge, 2001, Spradling et al., 2001, Surani, 2001).

• ***Multipotent Stem Cells****:* Able to give to a subset of cell lineages, and therefore are limited in their ability to differentiate.(Eckfeldt et al., 2005) For example haemopoietic stem cells are able to give rise to different subtypes of terminally differentiated blood cells, but would unable to give rise to cells of neural origin.

• ***Unipotent Stem Cells****:* Able to differentiate into only one mature cell type.

The term ***progenitor cell*** is also often used in the stem cell literature. Unlike stem cells, progenitor cells lack the capacity for repeated self-renewal, and their progeny only gives rise to specific mature cell types (Reule and Gupta, 2011). Progenitor cells' ability to give rise to a few cell types sometimes leads them to be classed as *oligopotent.*

Stem cells from 2 sources are commonly used in biological research:

• ***Embryonic stem cells*** (ESCs) are pluripotent stem cells harvested from the inner cell mass of the blastocyst, around five days after fertilization (Donovan and Gearhart, 2001). This process results in the destruction of the embryo. Research involving ESCs has therefore been subject to considerable ethical debate (Green, 2007).

• ***Adult stem cells*** (ASCs) are undifferentiated cells found throughout the body amongst the differentiated cells of tissues and organs (Spradling et al., 2001). They are best considered multipotent. ASCs appear able to differentiate and replenish damaged cells, thereby repairing the tissue or organ in which they are found. For example, in adult bone marrow there are hematopoietic stem cells (HSC) and mesenchymal stem cells (MSCs). HSC can give rise to all types of blood cell, while MSCs have the ability to differentiate into cells of chondrocyte, osteocyte and adipose lineage.

Unlike ESC based research, isolating ASC lines does not involve the destruction of a fertilized human embryo and is therefore considered less controversial (Green, 2007).

1.32 INDUCED PLURIPOTENT STEM CELLS

The ideal solution to current organ shortages may lie in the development of techniques that allow the growth of organs *ex vivo*, thereby eliminating the need for organ donors per se. This panacea might involve the directed differentiation of pluripotent stem cells into a viable organ, ideally of an identical immunological profile to the intended recipient. This would eliminate the requirement for life-long immunosuppression and the side effects that are associated with this. However, it is unclear how best to stimulate stem cells to differentiate into mature and fully functioning organs, partly because experiments investigating the utility of embryonic stem cells or stem cells derived from human fetal tissue have been the subject of vigorous ethical debate. Furthermore, as yet human fetal stem cells are unproven with regard to their ability to generate the diverse population of differentiated cell types required to produce a viable organ.

However, in 2006 work by Takahashi et al. described techniques that have the potential to circumnavigate both of these barriers (Takahashi and Yamanaka, 2006). The overexpression of four critical transcription factors (Oct 3/4, Sox 2, Klf 14 and c-myc) was demonstrated to potentially return any somatic cell to a pluripotent state. Stem cells that result from this method are referred to as induced pluripotent stem cells (iPSCs) and are thought to have the same potency as ESC lines. Theoretically, iPSCs may be induced from an individual's somatic cells and stimulated to grow into a transplantable organ that has the same immunological profile as the somatic cell donor. Furthermore, this approach is not subject to the same ethical debate as experiments utilising ESCs or cells from human fetal tissue.

After Takahashi's seminal work, studies using iPSCs have described experimental protocols that have allowed the *ex vivo* development of precursor kidneys in a 3D matrix (Takasato et al., 2016a, Takasato et al., 2016b).

Termed kidney "organoids", these precursors are essentially a miniature, self-organizing multicellular structure, which due to tissue structure and function may be regarded as a simplified kidney (Schutgens et al., 2016). However, while the ethical issues surrounding the use of ESCs may be removed by the use of iPSCs, the use of iPSCs for the generation of kidney organoids have several potential disadvantages. Firstly, iPSCs are genetically unstable, as reprogramming factors introduced by lentiviral vectors may incorporate into the cell genome. This poses the risk of later tumour formation (Briggs et al., 2013, Rao and Malik, 2012). Secondly, organs derived from iPSCs may contain cell types that are not fully differentiated, and it is a concern that these may give rise to teratoma formation after transplantation (Gutierrez-Aranda et al., 2010). Finally, organoids derived from iPSCs have been reported to be less efficient that organoids that are derived from ESCs (Freedman et al., 2015).

Nevertheless, while many technical steps require clarification before the successful growth of transplantable kidneys, it is apparent that organoids themselves may have some useful clinical applications (Little, 2016). Firstly, organoids could be utilised to screen drugs for toxicity, and secondly, organoids may prove useful models in which to study genetic aspects of kidney disease. Furthermore, organoids may prove useful sources from which to gather stem cells for use in regenerative therapies (Little and Kairath, 2016).

Several studies have reported the considerable efficacy of cells derived from iPSCs for the treatment of an acute renal injury. However, there are conflicting reports regarding the possible mechanism by which this effect is achieved. Imberti et al. reported that iPSC kidney cell types reduced renal damage by direct kidney integration and differentiation into specialised cell types (Imberti et al., 2015), while another study concluded that this effect was mediated via paracrine factors (Toyohara et al., 2015).

1.33 MESENCHYMAL STEM CELLS

Mesenchymal stem cells (MSCs) were originally identified in bone marrow stroma by Friedenstein et al (Friedenstein et al., 1974, Friedenstein et al., 1968). They are a heterogeneous adult population of multipotent stromal cells that can differentiate into cells of the mesodermal lineage. The presence of MSCs has been detected in the tissues of several organs, including peripheral blood, connective tissue, the umbilical cord, amniotic fluid, adipose fat and the kidney (Bruno et al., 2009a, Crisan et al., 2008, Edwards and Hollands, 2007, Flynn et al., 2007). Similarly, the isolation and *in vitro* expansion of MSCs has been described from bone marrow, adipose tissue, the umbilical cord, muscle, fetal liver and lung. Most often isolated from bone marrow, bone marrow derived mesenchymal stem cells (BD-MSCs) constitute only 0.01 – 0.001% of the total bone marrow cell population (Uccelli et al., 2006).

The Mesenchymal and Tissue Stem Cell Committee of the International Society of Cellular Therapy have outlined a combination of phenotypical, morphological and functional characteristics that are required to define MSCs (Dominici et al., 2006). These are primarily based on three criteria:

(1) MSCs must adhere to plastic under standard tissue culture conditions, exhibit fibroblast-like morphology, while displaying the ability to proliferate (Le Blanc, 2006, Tse et al., 2003).

(2) MSCs must express certain cell surface markers such as CD73, CD90 and CD 105, but must not express CD45, CD34, CD14 or CD11b, CD79 CD19 and major histocompatibility complex II (Dominici et al., 2006).

(3) Must have the capacity to differentiate into mesenchymal lineages including osteoblast, adipocytes and chondroblasts when exposed to the appropriate inductive media *in vitro*.

Numerous reports have described the beneficial regenerative effects of MSCs. The majority of these published studies describe the use of MSCs to mitigate injury to solid organs - a reflection of the potential efficacy of MSCs in regenerative medicine. In addition, MSCs possess several advantageous properties that facilitate their use in clinical research (Wise and Ricardo, 2012).

(1) MSC are easily identifiable by their cell surface markers.

(2) They adhere to plastic and are capable of substantial proliferation and expansion in culture *ex vivo*.

(3) They are capable of differentiation into multiple cell lineages.

(4) MSC can be cryopreserved with no loss of phenotype or differentiation potential.

(5) They can be sourced from adult tissue, hence they are free from the ethical issues surrounding ESCs.

1.34 MALIGNANT POTENTIAL OF MESENCHYMAL STEM CELLS

Extensive expansion of MSCs in culture may lead to changes in cell phenotype and function, and it remains unclear if *in vitro* cultured MSCs differ in their properties from populations that have been obtained *in vivo* (Mohseny and Hogendoorn, 2011). The production of sufficient MSCs for clinical use may require consistent *in vitro* expansion, which could lead to spontaneous malignant transformation (Rubio et al., 2008, Casiraghi et al., 2013b). This remains the primary concern regarding stem cell therapy in clinical application. Initially MSCs were reported not to form teratomas following transplantation in rodents (Kuroda et al., 2010), unlike ESCs and induced pluripotent stem cells, which are known to have malignant potential (Blum and Benvenisty, 2008, Baker et al., 2007, Barrilleaux et al., 2006, Kuroda et al., 2013). However, studies have reported the ability of BD-MSCs to stimulate the growth of existing cancers (Studeny et al., 2002) and promote metastases in mice (Karnoub et al., 2007). *In vitro* cultures of mesenchymal stem cells derived from adipose tissue have shown spontaneous malignant transformation after being passaged multiple times over 20 weeks (Froelich et al., 2013, Rubio et al., 2005). This phenomenon was not observed when BD-MSCs were passaged 25 times over 44 weeks (Bernardo et al., 2007), suggesting that the potential for malignant transformation could vary according to source and/or the culture conditions (Mannello and Tonti, 2007). However, whilst some studies have reported malignant transformation of BD-MSCs in culture (Rosland et al., 2009, Rubio et al., 2005), these have subsequently been retracted as the cultures investigated were contaminated with tumor cell lines (Torsvik et al., 2010).

Nevertheless, MSCs therapy applied to clinical practice ought to use cells produced over a short period of time with low passage numbers, and such use would require cautious application and rigorous surveillance (Bartmann et al., 2007).

1.35 BENEFICIAL PROPERTIES OF MESENCHYMAL STEM CELLS

MSC Homing Capacity

In non-injured states, MSCs given intravenously tend to migrate to bone marrow (Gao et al., 2001). However, *in vivo* studies have demonstrated that after systemic administration, MSCs not trapped in the lung have a tendency to localise in sites of inflammation in damaged tissues (Horwitz et al., 2002, Mahmood et al., 2003). Specific and preferential migration to kidneys after renal injury is also reported (Ittrich et al., 2007, Herrera et al., 2004, Morigi et al., 2004).

As MSCs express a variety of adhesion molecules and chemokine receptors(Fox et al., 2007), it is likely that chemokines are regulators of this preferential migration to sites of inflammation and damage. Up-regulated in ischemic or hypoxic conditions, chemokine ligand 12 receptor 4 (CXC-R4)(Togel et al., 2005b, Wynn et al., 2004, Herrera et al., 2007) and platelet–derived growth factor (PDGF)(Burton et al., 1999) have been implicated in MSC migration to acutely injured kidneys. The expression of CD44 also appears to be involved in the localisation of MSCs to injured renal tissue (Herrera et al., 2007). This is based on the observation that MSCs, engineered to lack CD44 expression, are unable to preferentially migrate to an acutely injured kidney, and subsequently, do not aid renal repair (Herrera et al., 2007).

Additionally, matrix metalloproteinase 2 and vascular-cell-adhesion-protein-1 have been shown to be essential in MSC rolling and adherence to endothelial cells and hence MSC engraftment both *in vitro* and *in vivo* (Ruster et al., 2006). Furthermore, expression of these homing-related molecules is up-regulated by inflammatory cytokines, including TNF-α and IL-1 (Ren et al., 2010, Shi et al., 2007). Therefore different tissue inflammatory states, as expressed by different levels of inflammatory cytokines may be pertinent to MSC engraftment and therapeutic efficacy of MSC preparations (Wei et al., 2013).

Immunomodulatory Properties

Administration of MSCs has been found to exert protective effects in response to IRI in a wide range of solid organ animal models. Such models include acute lung injury (Ortiz et al., 2003), AKI (Franquesa et al., 2012b), acute brain injury (Mahmood et

al., 2003) and acute myocardial infarction (Gnecchi et al., 2006). There is general consensus amongst researchers that the benefits of MSC therapy in a wide range of diseases indicate that MSCs produce their effects in different organs by the modulation of common pathways. MSCs are known to dampen inflammation in response to injury, thereby limiting initial tissue damage and subsequently promoting tissue repair (Uccelli et al., 2008, Han et al., 2012).

The immunomodulatory potential of MSCs has been extensively studied (Aggarwal and Pittenger, 2005, Krampera et al., 2003). MSCs poorly present antigen to other cells, lacking the expression of MHC class II proteins or co-stimulatory molecules (Dominici et al., 2006). Consequently, MSCs do not stimulate T-cell proliferation in mixed lymphocyte culture but are known to down-regulate alloreactive T-cell responses *in vitro* via several mechanisms (Di Nicola et al., 2002). Furthermore via a paracrine effect, infiltrating MSCs have been shown to alter both proliferation and cytokine secretion profiles from T helper cells (Krampera et al., 2003, Glennie et al., 2005, Nauta et al., 2006a). Additionally, *in vitro* experiments, demonstrate MSC paracrine action dampens IL-2 and IL-15 driven Natural Killer cell proliferation (Aggarwal and Pittenger, 2005, Spaggiari et al., 2006), and furthermore production of dendritic cells from peripheral blood monocytes (Nauta et al., 2006a, Jiang et al., 2005, Zhang et al., 2004). Data on the role of MSCs on B cell function is less clear (Tabera et al., 2008, Franquesa et al., 2012b). It is postulated that MSCs may reduce B cell proliferation both indirectly via T cell immunomodulation, and directly by up-regulating IL–10 production (Franquesa et al., 2015).

MSC administration has been reported to reduce cell-mediated rejection in rodent transplant models (De Martino et al., 2010). The increasingly used ECD and DCD renal allografts are known to suffer from more severe IRI injury (Bagul et al., 2013) and are consequently more prone to short-term complications including DGF and acute cell-mediated rejection (Pomfret et al., 2008, Renkens et al., 2005, Singh et al., 2011). The immunomodulatory ability of MSCs to reduce inflammatory response to local injury and increase tolerance (Casiraghi et al., 2013a) makes such therapies an attractive option for the treatment of renal IRI (Vanikar et al., 2014). (See figure 1.8)

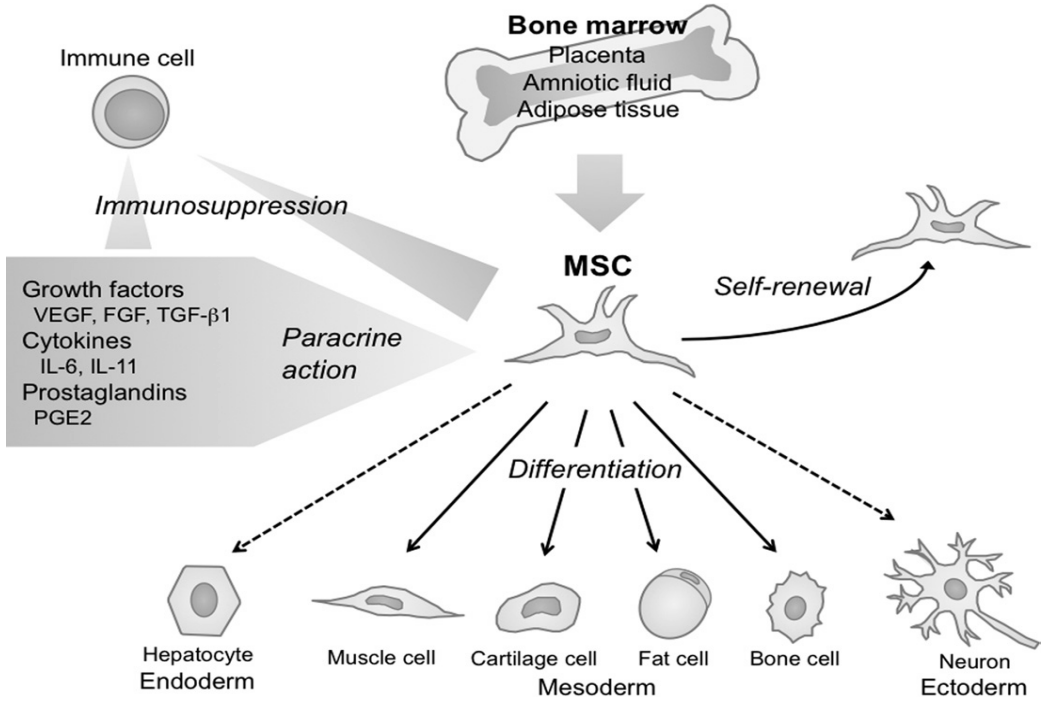


Figure 1.8: MSC origin and function, paracrine function and immunomodulatory properties (Asanuma et al., 2010). Since this publication, it has become apparent that MSCs progeny are limited to bone, cartilage and adipose cell types. MSCs are not able to give rise to neurons, hepatocytes or muscle cells as originally thought.

1.36 MESENCHYMAL STEM CELLS AND CLINICAL TRIALS

Given this potential, a small number of clinical studies been conducted, administering MSCs to patients undergoing kidney transplantation. The timing of MSC preparation administration has tended to be during induction, and most studies have focused on safety and feasibility endpoints (Perico et al., 2011, Mudrabettu et al., 2015). Initial reports suggest a reduction in acute rejection associated with MSC induction therapy in comparison to a more conventional induction regime utilising antibodies directed against IL-2 receptors (Tan et al., 2012). Correspondingly, renal function at one year was reported to be better in the MSC-treated group (Tan et al., 2012). A further randomised study concluded that donor ADRC therapy, when co-infused with hematopoietic stem cells peri-transplant, was safe and associated with fewer episodes of acute rejection (Vanikar et al., 2014). However, another study reported that timing of MSC therapy is critical, with administration prior to transplantation yielding better results (Pileggi et al., 2013).

1.37 PATHFINDER CELLS

There is interest in testing the efficacy of novel stem cells types, that are distinct from conventional MSCs. For example, pancreatic ductal epithelial cells are reported to have the ability to de-differentiate into progenitor cells capable of forming new pancreatic islets and acini (Bonner-Weir et al., 2004). As ductal cells replicate, they transiently express PDX-1, an embryonic transcription factor necessary for pancreatic development and the maturation of β-cells. Cells expressing PDX-1 are reputed to display multi-potency (Bonner-Weir et al., 2004).

The isolation and characterization of a novel population of pancreatic ductal progenitor cells has subsequently been described (Stevenson et al., 2009). These cells, termed 'pathfinder cells' were originally obtained from adult rat pancreatic ducts, and have also been demonstrated to express PDX-1. Two populations of pathfinders, either positive or negative for the MSC surface marker CD90 have been characterized (Stevenson et al., 2009). Pathfinders positive for CD90 have been shown to form islet like structures when appropriately stimulated *in vitro*, in addition to the transcriptional expression of insulin. CD90 negative cells do not form islets but are able to display insulin gene transcription (Stevenson et al., 2009).

Pathfinder cells have been reported to aid organ regeneration in response to injury in a number of animal models. In streptozotocin-induced diabetic mice, administration of intravenous human pathfinders was associated with normalised blood glucose and insulin that was murine in origin (Stevenson et al., 2011). The authors concluded that Pathfinders were able to stimulate native β-cell regeneration, probably via a paracrine mechanism. This conclusion was later supported by additional work by the same group, using extracellular secretory vesicles isolated from pathfinder cells in the same animal model with similar results (McGuinness et al., 2016). Another study, administering intravenous infusion of Pathfinder cells to mice, reported mitigation of renal IRI, again thought to be via a paracrine mechanism (McGlynn et al., 2013).

1.38 EVIDENCE FOR THE MECHANISM OF ACTION OF STEM CELLS

Considerable evidence exists demonstrating the regenerative effects of stem cells as a therapy for the repair of damaged solid organs. However, the precise mechanism by

which the beneficial action of stem cells is conferred is not well understood, and no single mechanism has gained universal acceptance.

1.381 DIFFERENTIATION-DEPENDENT HYPOTHESIS

The initial theory, often termed the "Differentiation-Dependent Hypothesis" stated that the administration of exogenous stem cells resulted in stem cell localisation within the tissues of an injured solid organ. According to this theory, after administration stem cells would migrate to the site of injury, become engrafted into the organ and differentiate into healthy, functional specialized cells and their supporting types (Orlic et al., 2001, Terada et al., 2002, Ferrari et al., 1998).

The main evidence for the Differentiation –Dependent mechanism was provided by the following studies:

- In murine glycerol and cisplatin AKI models, intravenous MSC therapy was reported to promote structural and functional repair of injured kidneys, via MSC engraftment and differentiation into functional tubular epithelial cell (Herrera et al., 2004, Morigi et al., 2004). Up to 22% of injected MSCs were found engrafted within damaged kidneys (Herrera et al., 2004). This indicated MSC tropism for the injured kidney and a potential contribution of the stem cells to aid tubular regeneration and subsequently protect long-term kidney function (Herrera et al., 2004, Morigi et al., 2004).
- In both porcine and rodent animal models of acute myocardial infarction, initial experiments demonstrated restoration of cardiac function after intra-cardiac MSC administration. This led some groups to conclude that MSC differentiation into cardiomyocytes and other cell types was the likely mechanism by which MSCs act (Shake et al., 2002, Tomita et al., 2002, Davani et al., 2003).

Subsequently, evidence from multiple studies utilising different organ injury models emerged to challenge the theory of stem cell differentiation as the possible mechanism by which stem cells act to cause organ regeneration in response to injury.

In summary:

• Using a rat model of acute myocardial infarction Mangi et al reported improved heart function only 72 hours after intra-cardiac MSC administration (Mangi et al., 2003). Due to the short timeframe between exposure and response to MSC therapy, the authors, concluded myocardial regeneration could not be attributed to MSC engraftment and differentiation.

• Herrera et al demonstrated limited replacement of damaged renal tissue by differentiated MSCs, with only 2.0 – 2.5% of injected cells showing engraftment (Herrera et al., 2007). Other studies also reported a negligible percentage (<5%) of administered cells became engrafted in the injured kidney (Imberti et al., 2007, Morigi et al., 2008). These studies also reported administered MSCs did not persist in the kidney in the long-term. This was a direct contradiction to previous reports (Herrera et al., 2004, Morigi et al., 2004) although there was consensus that MSC therapy protected against AKI, reducing tubular cell apoptosis and stimulating proliferation.

• The work by Herrera et al (Herrera et al., 2007) was later substantiated in rat renal IRI models, whereby MSCs were only found to become engrafted transiently. Nevertheless improved renal function, likely as a result of reduced tubular damage by production of anti-apoptotic, pro-mitogenic and vasculotropic factors was observed within 48 hours (Lange et al., 2005). Furthermore, MSC therapy was associated with up-regulation of anti-inflammatory cytokines and growth factors (Togel et al., 2005a, Lange et al., 2005, Togel et al., 2007).

• MSC therapy in animal models had typically been delivered intravenously, due to the minimally invasive nature of this route and the easy of access. However, the majority of intravenously administered MSCs remain in the circulation for less than 60 minutes, before accumulating within filtering organs such as the lung, liver and spleen (Barbash et al., 2003, Kraitchman et al., 2005, Sackstein et al., 2008, Bieback and Brinkmann, 2010).

• Although a limited number of transplanted exogenous cells were seen within the injured tissues, endogenous stem/progenitor cells were reported to become the

majority of the stem cell population that contribute to tissue repair after injury (Usas et al., 2011).

1.382 DIFFERENTIATION-INDEPENDENT (PARACRINE ACTION) HYPOTHESIS

As evidence emerged to challenge the differentiation-dependent hypothesis, complementary studies introduced the concept that stem cells, via a beneficial *paracrine* action, may mediate their effects via the release of biologically active factors.

Paracrine signalling may be defined as a form of communication between two different cells, where one cell releases chemical mediators to its immediate environment, which results in a change in the behaviour of a cell in the adjacent environment.

Supported by a wealth of convincing evidence, the idea that stem cells work via a paracrine mechanism gained widespread acceptance.

• It was reported that intra-myocardial injection of cell-free culture medium conditioned by MSCs, protected cardiomyocytes from ischemic injury to the same extent as medium containing MSCs (Gnecchi et al., 2006). MSC conditioned media improved cardiac function within 72 hours, but no improvement was observed with the use of medium that had never been exposed to MSCs (Gnecchi et al., 2006). This experiment was later replicated in a porcine model of myocardial infarction, concluding that MSC conditioned medium protected cardiomyocytes from ischemic injury (Timmers et al., 2007).

• Lee et al. reported that despite the majority of intravenously administered MSCs becoming trapped in the lungs of treated mice, MSC therapy was associated with significant myocardial injury reduction. They concluded that lung engrafted MSCs were acting in a paracrine fashion by secreting the anti-inflammatory protein TSG-6 (Lee et al., 2009).

• In a rat renal IRI model, Togel et al reported MSC therapy to be associated better outcomes than vehicle only treatment. MSCs were found to up regulate anti-inflammatory growth factors such as IGF-1 and IL-10, whilst causing the down-regulation of pro-inflammatory mediators such as TNF-alpha and inducible nitric oxide synthase (i-NOS) (Togel et al., 2005a). The same group would later demonstrate that MSC-conditioned media increased the survival and proliferation of endothelial cells in vitro, postulating that paracrine factors were responsible for their observations (Togel et al., 2007).

These findings provided strong evidence that stem cells produce their beneficial effects in solid organs by the release of biologically active paracrine mediators. Despite this, some research groups argue that the differentiation-dependent hypothesis is the more likely to be correct, and cite a recent study concluded that stem cell conditioned medium did not protect against AKI in a murine cisplatin-induced AKI model (Gheisari et al., 2011).

Nevertheless, in recent years the differentiation-independent mechanism has gained more supporters. Interestingly, recent studies argue the differentiation-dependent and differentiation-independent mechanisms both play a role in limitation of initial injury and long-term kidney regeneration (Zhao et al., 2014). Whilst acknowledging that the differentiation-dependent mechanism may exist in the organ repair process, the limited cell engraftment and differentiation reported by most studies would make it more likely that stem cells exert the majority of their regenerative effects in a paracrine fashion.

1.39 EVIDENCE FOR A PARACRINE MECHANISM IN MITIGTION OF ACUTE RENAL INJURY

Several studies using different models of acute renal injury have demonstrated the protective effects of exogenous stem cells. In these studies, the use of MSCs has predominated. Although MSCs, when administered systemically, were shown to migrate to the site of injury, the extent of tissue repair did not correlate with the degree of exogenous MSC engraftment. This suggested that MSCs produced their effects in an indirect fashion, leading many of these studies to conclude that stem cells produced their beneficial effects via a paracrine mechanism.

Consequently, the ‘differentiation-dependent hypothesis' began to lose favour. Researchers suspected that the transfer of biologically active factors carried in membrane bound extracellular cellular vesicles (ECVs) between stem and recipient cells could be responsible for the observed results. This realisation, together with the knowledge that cell-free therapy may have a number of benefits over cellular therapy led investigators to isolate preparations of ECVs and utilise them in experiments, in an attempt to demonstrate ECV efficacy.

With respect to the kidney, an initial study reported the effects of cell media, conditioned by MSCs, injected into the peritoneal cavity of mice with cisplatin-induced renal failure (Bi et al., 2007). Media administration was found to be associated with reduced tubular cell apoptosis and increase tubular cell survival, replicating findings that had previously been observed with MSC therapy itself. As with previous experiments utilizing conditioned cell media, the authors suspected there were active agents residing in the media, released by MSCs during culture.

The first ever study to demonstrate the therapeutic effect of MSC-derived ECVs focused on kidney injury. Here, after centrifugation of MSC conditioned cell media, Bruno et al demonstrated the protective effects of ECVs in a glycerol-induced murine model of AKI (Bruno et al., 2009b). They concluded the ECVs were able to shuttle a subset of micro-RNA (miRNA) that was characteristic of MSC phenotype, thereby influencing cell proliferation, transcription and immune-regulation (Bruno et al., 2009b, Bruno and Bussolati, 2013).

Subsequently, a number of studies, using ECVs purified from conditioned cell media have demonstrated protective effects of ECVs given by an intravenous route.

Gatti et al. reported that rats subjected to renal IRI, were protected by a single dose of ECVs obtained BD-MSC, given immediately after injury (Gatti et al., 2011). ECV use was associated with improved renal function and histology, with reduction in tubular cell apoptosis. Interestingly, ECVs obtained from fibroblasts conferred no benefit to damaged kidneys. These findings were corroborated in a study by Zou et al (Zou et al., 2014). Here, in rat renal IRI model, the use of intravenously administered ECVs isolated from MSCs that originated in umbilical cord tissue, was associated

with improved renal function and histology. The authors concluded that the likely mechanism of ECV action was via a reduction in infiltrating macrophages, due to reduced expression of the chemo-attractant protein CX3CL1 (Zou et al., 2014).

Cantaluppi et al reported that in a rat renal model of IRI, ECVs derived from endothelial progenitor cells, accumulated in peri-tubular capillaries and tubular cells after intravenous injection (Cantaluppi et al., 2012). The use of ECVs was found to accelerate tubular proliferation while reducing apoptosis and leucocyte infiltration. Significantly, improved renal function was associated with ECV administration. The use of RNAase on ECVs prior to administration negated their effects (Cantaluppi et al., 2012). Furthermore this experiment analysed the effect of ECVs harvested from Dicer knockdown endothelial progenitor cells. Dicer is an intracellular enzyme essential for the production of miRNA. ECVs from Dicer silenced endothelial progenitors were not found to be capable of conferring protection against renal IRI.

Taken together with the effect of RNAase treatment on ECV efficacy, these findings clearly implicate the horizontal transfer of miRNA as a key step in the action of ECVs on other cells (Cantaluppi et al., 2012). These results were supported by subsequent studies (Bruno et al., 2012, Milwid et al., 2012), which reported amelioration of neighbouring AKI in SCID mice by repeated intravenous injections of ECVs obtained from BD-MSCs. Again, RNAase treatment negated any protection conferred by ECV administration (Bruno et al., 2012). In addition to these results, He et al. described the reno-protective effects of BD-MSC ECVs, given intravenously to mice undergoing a 5/6 nephrectomy (He et al., 2012).

However, the ECVs obtained in each the above studies were isolated using uniform centrifuge speeds of 100,000g (Bruno et al., 2009b, Cantaluppi et al., 2012, Gatti et al., 2011, Bruno et al., 2012, He et al., 2012, Milwid et al., 2012, Zou et al., 2014). As discussed below, ECVs released from stem cells are far from uniform, differing in size, membrane surface proteins and biological content. Centrifugation at 100,000g is likely to result in the isolation of at least two active vesicle populations that are present in the ECV fraction. Correct identification of the responsible vesicle type is necessary to allow the characterization the active biological factors through which tissue repair and regeneration might be achieved (Han et al., 2016).

1.4 WHAT IS THE EXACT NATURE OF THE PARACRINE MEDIATORS BY WHICH STEM CELLS ACT?

The exchange of information between cells may involve soluble factors or direct cell-to-cell contact. A weight of evidence suggested that stem cells produce their beneficial effects via paracrine factors, and subsequently, researchers have investigated as to what the identity and nature these factors might be.

Extracellular secretory vesicles (ECVs) have emerged as the leading candidates by which stem cells might achieve paracrine signaling. Correctly applied, ECV is term that encompasses the entire spectrum of membranous vesicle derived from cells (Anthony and Shiels, 2013). ECVs are also broadly referred to in the literature using several terms including micro-secretory vesicles, micro-particles and (incorrectly) microvesicles and exosomes. Indeed, a recognised standard needs to be reached with regard to the nomenclature in this area (Pilzer et al., 2005). Too often different studies use different criteria to define ECVs, and due to inconsistent terminology comparison between studies becomes difficult (Anthony and Shiels, 2013, Gould and Raposo, 2013).

ECVs were initially identified from a large number of cell types, both *in vitro* and *in vivo*. They are membrane bound, spherical, submicron factors that are released from the cell membrane. For a long time, ECVs were considered to be inert cellular debris lacking any specific biological purpose (Siekevitz, 1972). Using electron microscopy, ECVs observed *in vitro* were thought to be the result of cell preparatory methods and dismissed as irrelevant artifacts. Similarly, ECVs obtained from biofluids while *in vivo* were regarded as the result of cell damage or routine cell membrane turnover and dismissed as inconsequential (Siekevitz, 1972).

Subsequently, it was demonstrated that circular plasma membrane fragments released from human cells were the result of a specific export process, with ‘membrane fragments’ carrying enzymes in the same ratio as the cells from which they originated (De Broe et al., 1977). More recent evidence has questioned the validity of initial assumptions, and it is now widely recognized that ECVs influence and participate in numerous biological processes (Cocucci et al., 2009). Recent experiments have identified specific functions of ECVs released from various cell types, with the

recognition that ECVs should now be considered carriers of genetic information that is exchanged in cell-to-cell communication (Raposo and Stoorvogel, 2013). Apoptotic vesicles have been demonstrated as being released from both healthy and diseased cells undergoing apoptosis. They range in size from 1 – 5μm (Raposo and Stoorvogel, 2013) and do not appear to have a major role in cell-to-cell signaling (Akers et al., 2013).

However, two broadly distinct types of ECV been reported as participating in the cell signalling process – microvesicles and exosomes (Gyorgy et al., 2011, Raposo and Stoorvogel, 2013, Thery et al., 2009). Despite continuing confusion regarding the nomenclature used to describe these, there is growing acceptance that microvesicles and exosomes are structurally and morphologically distinct. Furthermore, microvesicles and exosomes are thought to contain different biologically active factors, and this has clear potential implications for their effects on target cells (Raposo and Stoorvogel, 2013, Lai et al., 2012).

Microvesicles are sometimes loosely referred to as "shedding vesicles" (Cocucci et al., 2009). They originate by direct budding of small cytoplasmic protrusions of the cell plasma membrane in a process that is dependent on calcium influx. With regard to size, microvesicles are a heterogeneous population that range between 100nm – 1μm (Cocucci et al., 2009, Dragovic et al., 2011). (See Figure 1.9)

On the other hand, exosomes are derived from the endosomal membrane compartment, and after fusion with the plasma membrane undergo exocytosis from activated cells (Hugel et al., 2005, Johnstone et al., 1987). Exosome content entered the cell of origin by endocytosis, before being biologically altered and stored as intraluminal vesicles within multivesicular bodies of the late endosome. They are released when these multivesicular bodies fuse with the cell membrane in a mechanism dependent on cytoskeleton activation. They are a more homogenous population with a size ranging from 30 – 100nm (Heijnen et al., 1999, Rozmyslowicz et al., 2003, Dragovic et al., 2011, Hugel et al., 2005, Raposo and Stoorvogel, 2013).

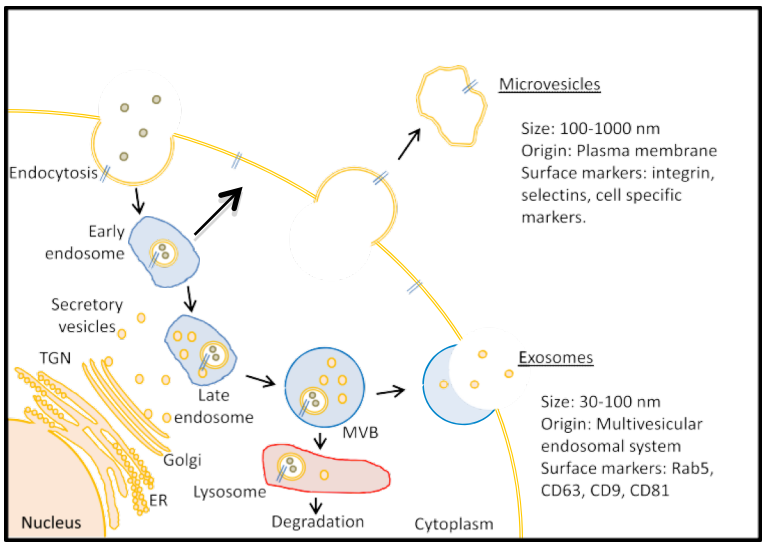


Figure 1.9: Release of microvesicles and exosomes. Microvesicles bud directly from the plasma membrane, whereas exosomes are represented by smaller vesicles that are more regular in size and shape. Exosomes originate from inward budding of the cell membrane and the formation of multivesicular bodies (MVBs). When MVBs fuse with the plasma membrane, exosomes are released. The point of divergence between these types microvesicles and exosomes is drawn at early endosomes, but the existence of distinct early endosomes feeding into these two pathways cannot be excluded. Other intracellular vesicles fuse with lysosomes and are degraded (Anthony and Shiels, 2013).

Interestingly, the release of ECVs from cultured stem cell lines is known to be dependent on conditions in which the cells are maintained. A number of triggers for increased ECV production have been identified, including hypoxia, shear stress, oxidative stress and the presence of inflammatory cytokines, including TNF-alpha and IL-6. Studies report a 15-fold increase in ECV production when cultured stem cells are stimulated with either hypoxia or sheer stress (Bian et al., 2012, Diamant et al., 2004, VanWijk et al., 2003, Ratajczak et al., 2006b).

ECVs have been shown to contain cell surface receptors and cytoplasmic components, including proteins and lipids, which originate from their cell of origin (Ratajczak et al., 2006a, Collino et al., 2010, Deregibus et al., 2007, Yuan et al., 2009). The presence of cell-specific surface receptors suggests that ECVs are an important mechanism by which cells communicate. Furthermore, this is an indication that ECVs target specific cells in the immediate environment and do not communicate with other cells in a random fashion. Analysis has revealed that both microvesicles and exosomes contain biologically active nucleic acids in the form of DNA, messenger RNA (mRNA), and microRNAs. The biologically active content of ECVs

implies that functional modulation and re-programming of recipient cells may occur in response to ECV stimulation. The membrane structure of ECVs suggests that this process is carefully regulated, and consequently is dependent upon the interaction between cell-specific surface protein receptors (Collino et al., 2010, Deregibus et al., 2007, Ratajczak et al., 2006a, Yuan et al., 2009).

Ratajczak et al (Ratajczak et al., 2006a) were the first to show that stem cell derived ECVs are able to reprogram cells resident in neighbouring tissue. In this study, haemopoietic progenitor cells were induced to transcribe proteins in response to ECVs stimulation. Since RNAase treatment ablated this process, the authors concluded that RNA contained in ECVs was critical in this signaling process.

Subsequent reports described the activation of angiogenic pathways in quiescent endothelial cells using ECVs derived from endothelial progenitor cells (Deregibus et al., 2007). Again ECV treatment with RNAase negated this effect. Later, the same group would report that such ECVs contained the pro-angiogenic miRNAs, including miR-126 and miR-296 and hypothesized that delivery of these by ECVs into recipient cells could explain their observations (Cantaluppi et al., 2012).

These studies suggest that stem cells exert at least some of their beneficial effects in damaged solid organs via transfer of genetic information within ECVs (Camussi et al., 2010). The resultant alteration of gene expression may result in the up-regulation of anti-inflammatory cytokines and anti-apoptotic genes, whilst down regulating inflammatory and pro-apoptotic factor expression (Johnstone et al., 1987, Camussi et al., 2010).

Tissue resident cells, rather than circulating stem or progenitors cells, seem to provide the major contribution to regeneration with solid organs. Within the kidney, repair following ischemia is attributed to tubule re-population from resident tubule cells. These are likely to de-differentiate, migrate to the site of injury and differentiate again into mature tubular cells. ECVs released from resident stem cells possibly modulate this chain of events, by re-programming remaining tubular cells to enter the healing process. However, these processes may be overwhelmed in the face of severe injury, especially if the organ is biologically old. Administration of either exogenous stem

cells, or their ECVs, that results in high localized renal ECV concentration may provide a boost to the healing process by both down-regulation of the inflammatory process, and direct stimulation of tissue regeneration. (See Figure 1.11)

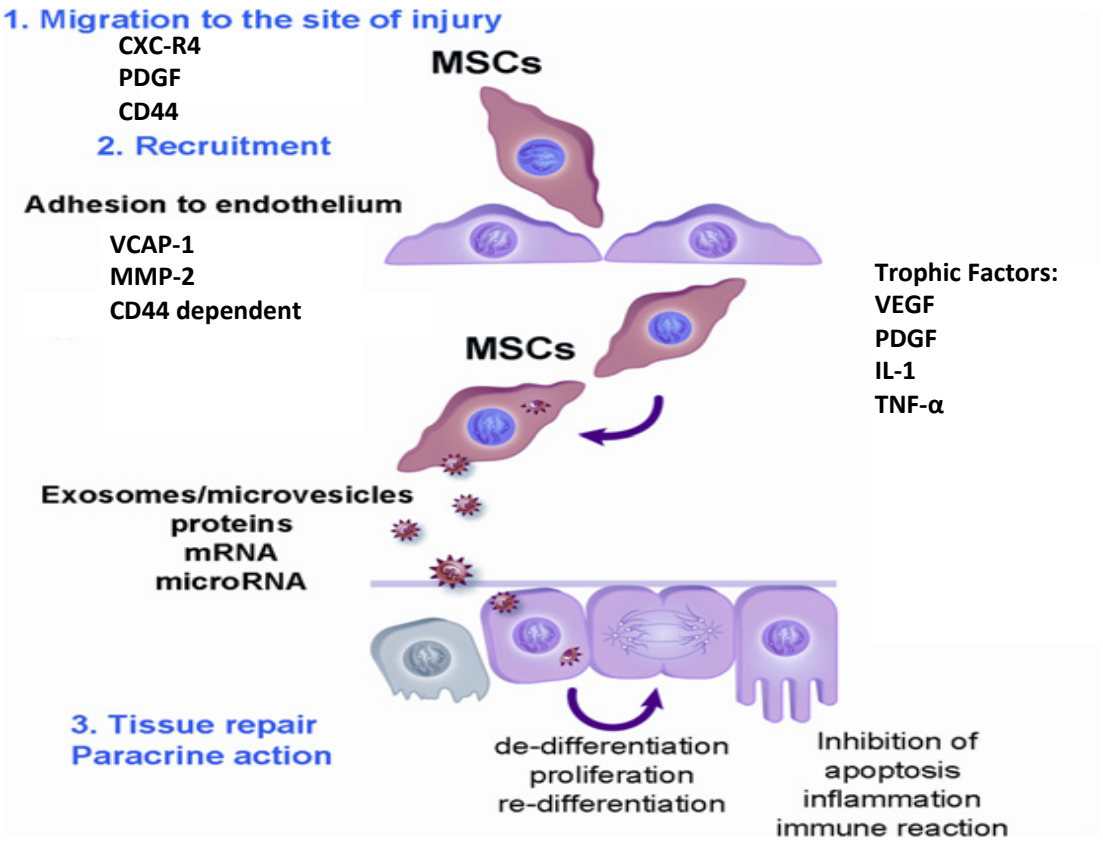


Figure 1.11: Potential mechanism by which stem cells and their ESVs can induce renal regeneration. (Cantaluppi et al., 2013)

1.41 WHICH TYPE OF EXTRACELLUALAR VESICLE IS RESPONSIBLE FOR ORGAN REGENERATION

It has been reported that the microvesicles will sediment at centrifugation speeds lower than 100,000g (Muralidharan-Chari et al., 2010). Due to their smaller size, the exosomal fraction requires speeds of around or higher 100,000g to sediment. Hence it is possible to broadly separate the ECV constituents by the use of differential centrifugation speeds.

Unfortunately, most studies typically report the use of a single centrifugation speed of around 100,000g when preparing their ECV samples. Hence, the many ECV preparations reported to have regenerative effects when applied to injured solid organs, are likely to contain both microvesicles and exosomes. Consequently, any

biological effects associated with the use of such mixed preparations are difficult to attribute to a particular ECV fraction and their specific nucleic acid and protein content (Muralidharan-Chari et al., 2010).

1.5 ADVANTAGES OF CELL-FREE vs. STEM CELL THERAPY

Understanding the identity of the active paracrine factors and how they may influence solid organ repair and regeneration is clearly important in the development of future regenerative therapies. As noted previously, therapeutic administration of stem cells is known to carry the risk of mal-differentiation into malignant cell lines. This is of particular concern in patients undergoing solid organ transplantation as such procedures carry the necessity of life-long immunosuppression to prevent rejection by the adaptive immune system. Immunosuppression is well recognised to increase the risk of malignancy. It is generally accepted that a recent donor or recipient history of a malignant process should be an absolute contraindication to solid organ transplantation. Transplant clinicians recognise the potential risk of using cultured stem cell lines as a therapy to reduce peri-transplantation injury and consequently there is reluctance to expose transplanted, immunosuppressed patients to novel cellular therapies.

ECVs have been shown to be more stable in storage than aliquots containing stem cells. It is also easier to prepare frozen ECVs for delivery than cellular preparations, making ECVs more suitable for everyday use (Vlassov et al., 2012). Moreover, cell-free preparations are thought to carry a much-reduced risk of causing malignancy when compared to preparations containing potent stem cells (Vishnubhatla, 2014) (Han et al., 2016). Identification of the active paracrine factors that mediate protection in the short term and/or tissue regeneration in the long-term may allow these factors to be manufactured synthetically (Vishnubhatla, 2014), reducing the potential for harm to a minimum (Rani et al., 2015). Furthermore, in clinical transplantation, the likely route of administration of any therapy would be into the artery supplying the allograft just prior to transplantation. This would minimise the drug dose needed, and would probably dramatically reduce the systemic distribution when compared to the intravenous route. One potential drawback of intra-arterial therapy is the increased likelihood of microvascular occlusion, a process originally

termed “passive-entrapment” (Walczak et al., 2008). Supra-therapeutic dose of stem cells, administered intra-arterially have since been reported to cause passive entrapment, resulting in renal hypoperfusion and dysfunction (Shih et al., 2013, Lee et al., 2012, Cai et al., 2014). In contrast, a therapeutic dose of smaller, soluble and highly concentrated biologically paracrine factors would be most unlikely to cause such a problem (Rani et al., 2015).

CHAPTER 2

GENERAL MATERIALS AND METHODS

The materials and methods presented in this chapter describe general methodology relevant to all 'results' chapters. Where appropriate, additional materials and methods specific to individual experiments presented within a particular results chapter are addressed within that chapter.

2.1 RODENT HUSBANDRY, ANASTHESIA, SURGERY AND POST SURGICAL CARE

2.11 RODENT DIET AND HOUSING

Albino Swiss rats, their mutant rat substrain (AS-AGU), and Fischer 344 rat strain were used in different experiments reported throughout this thesis. Albino Swiss and AS-AGU rats were bred in the Joint Research Facility, University of Glasgow. Male Fisher 344 rats were bought from Harlan UK Ltd, and housed for at least two weeks before surgery to allow the animals to equilibrate with their new environment.

All animals were housed in the Joint Research Facility under standardised conditions in plastic metal cages, light-dark cycle 12/12 hours, temperature 22^{o}C +/- 2^{o}C, humidity 55 +/- 5%.

Animals were fed a standard diet of rodent chow and had access to tap water *ad libitum* both pre and post procedures.

2.12 RODENT ANASETHSIA

All animals were weighed prior surgery. Anaesthetic induction was achieved by placing the rat in an anaesthetic chamber, and ventilating with 5% isoflurane. Once anaesthetized, the animal was shaved as appropriate.

Unless otherwise stated, anaesthesia was maintained throughout surgery with an inhaled mix of 5% isoflurane and 1 litre/ minute of oxygen given via facemask. The required amount of anaesthestic agent was adjusted by an assistant according to the animal's respiratory rate and pain withdrawal reflexes.

2.13 POSITION AND TEMPERATURE CONTROL DURING RODENT SURGERY

The animal was placed supine under a sterile drape on a corkboard, containing a far-infrared heating mat. A mobile board is useful for microsurgery, as it allows the animals position relative to the surgeon to be easily manipulated during theprocedure.

The animal's core temperature was measured regularly with an infrared thermometer and maintained in the range of 36.9^{o}C - 37.3^{o}C by adjusting the far infrared heating mat.

2.14 SURGICAL STERILITY

The methods used to ensure sterility are recommended by the Home Office and were observed and approved by the Home Office veterinary surgeon. Great care was made to ensure sterility at all times including liberal use of sterile drapes and gloves, autoclaved instruments (30 minutes at 134^{o}C) and sterile surgical technique. After shaving, the animals were cleaned with 2% chlorhexidine solution before being covered with a sterile drape.

2.15 MICROSURGICAL SET-UP AND EQUIPMENT

- Wild Heerbrugg Ltd operating microscope – zoom 10 – 30x with 60mm LED light ring (Microscope Systems Scotland).
- Far infrared heating pad and infrared thermometer. (Kent Scientific: DCT-15)
- Operating Cork Board (30 x 30 cm). (See Figure 2.1) Covered with disposable plastic sheeting, held in place with adhesive tape. Ordinary paper clips, after being autoclaved, were attached to elastic bands and used as tissue retractors, held in place with sterile 16G needles.

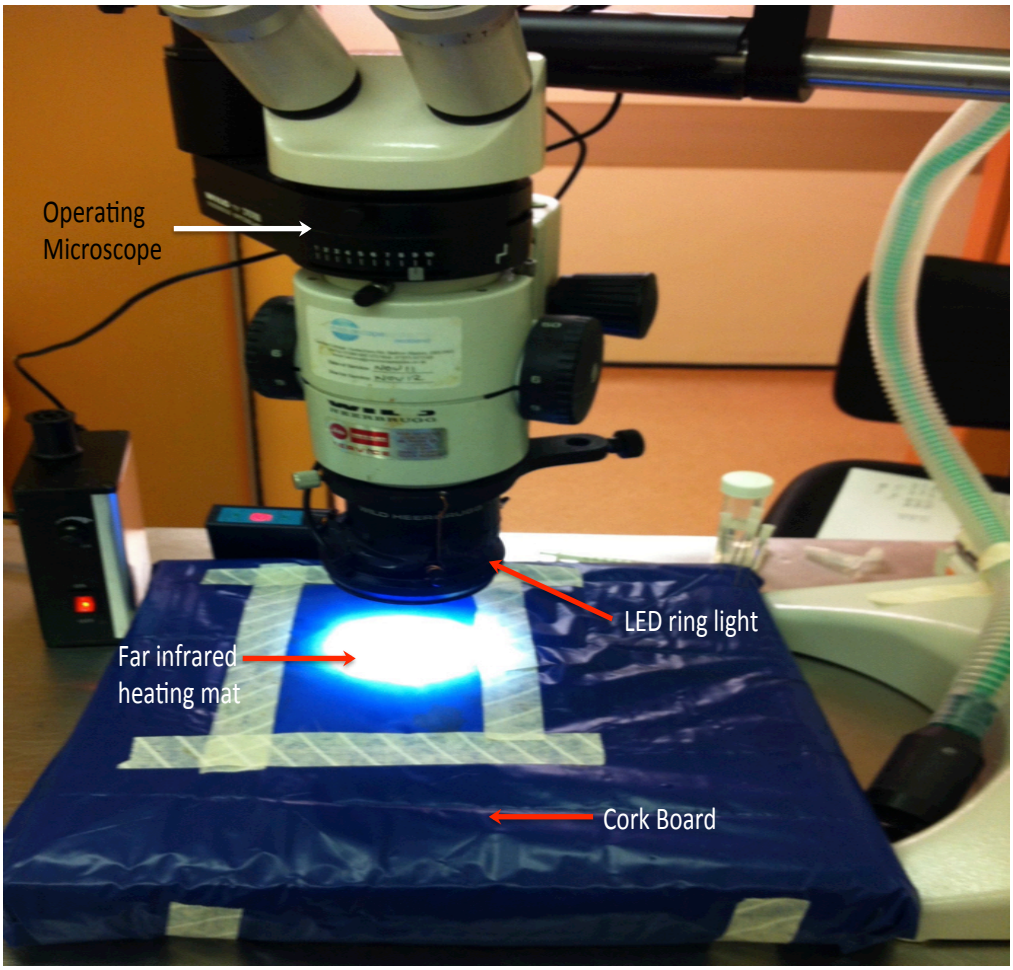


Figure 2.1: Typical microsurgical setup, showing the relationship of operating microscope, cork board and the attached far infrared heating mat.

The following microsurgical instruments were obtained from S&T®

- Microsurgical vessel dilators with 0.1mm tips. (D-5a.1)
- Microsurgical vessel dilators with 0.2mm tips. (D-5a.2)
- Microsurgical vessel dilators with 0.3mm tips. (D-5a.3)
- Angulated forceps with 0.3mm tips. (JFA-5b)
- Dissecting microsurgical scissors with curved tips (SDC-15)
- Adventia microsurgical scissors with sharp tips (SAS-15)
- Microsurgical needle holder without a lock, curved tips 0.4mm. (B-15-8)
- Tubing introducing forceps, tips 0.35mm. (TIF02)
- Clamp applying forceps. (CAF-4)
- Atraumatic vascular clamps. (B1-V and B2-V))
- Aceland Frame Clamp. (ABB-1V)
- Silicon strip visibility background material – essential to place behind vessels during anastomosis, both to protect surrounding structures and to aid visualisation. (VB2 and VB4)
- 0.6mm rubber sloop

Additional equipment used during microsurgery included:

- Electric hair clippers (Oster-A5-00)
- 15G Scalpel. (Ethicon Ltd)
- Sterile Dressing packs (Nu-Care Products)
- Sterile Cautery (John Weiss International Eye Cautery 0111122)
- 30G Rycroft Cannula (0108003)
- Sterile cotton tip applicators (Nu-care Products)
- 10/0 nylon sutures. (Schuco Ltd, ZX-AK-0105, DR4 needle)
- 4/0 polyglactin 910 (vicryl®) sutures (Ethicon Ltd)

2.16 POST-SURGICAL CARE

Post-operatively, all animals were injected with 2.5ml of subcutaneous 0.9% saline (Baxter Healthcare, Thetford UK). Buprenorphine analgesia was injected subcutaneously at 0.0045% per 100g of body weight. This was repeated every 12 hours for a maximum duration of 48 hours post surgery. The animal was placed in a warming box at 38^{o}C for 60 minutes after surgery and then returned to its cage. Animals were allowed free access to rodent chow and water and were weighted daily.

2.17 RODENT VENESECTION

Measurement of serum creatinine is a commonly used to assess kidney function in humans and laboratory animals.

Creatinine is a byproduct of muscle metabolism, formed during the breakdown of phosphocreatine by creatine kinase. For an individual animal, the rate of production of serum creatinine is relatively constant, although this varies between animals according to muscle mass. Creatinine is mainly filtered from the blood in the glomerulus, although it is also secreted by the proximal tubules. When glomerular filtration is impaired, the blood level of creatinine rises.

~ 200μL of blood for creatinine levels was obtained from rat-tail veins by puncture with a 23G needle after anaesthesia was induced as described in Section 2.12.

The blood was spun for 6 minutes at 6000rpm, and the serum stored on ice before being transported to the Diagnostic Veterinary Diagnostic Services Laboratory, University of Glasgow at Garscube. Serum creatinine was measured via a kinetic modification of the Jaffe procedure, using an automated Olympus AU5400 analyzer.

2.2 DETERMINATION OF GLOMERULAR FILTRATION RATE VIA CONTINUOUS INFUSION CLEARANCE WITH FLUORESCENT INULIN

2.21 BACKGROUND

The formation of urine begins in the glomerulus with the generation of plasma ultrafiltrate. The rate of ultrafiltrate formation, known as 'Glomerular Filtration Rate' (GFR) is regarded as the 'gold standard' for determining renal function in both animals and humans (Schock-Kusch et al., 2012, Meneton et al., 2000).

However, the use of the term 'gold standard' implies there are widely employed correct procedures with optimised protocols to measure GFR (Schock-Kusch et al., 2012). In fact, GFR as determined by the administration of an exogenous tracer, may be measured by using two standard approaches:

(1) *Two compartment clearance models* - determination of a tracer's elimination kinetics from plasma after a single bolus injection, requiring timed blood collection (Qi et al., 2004, Sturgeon et al., 1998, Fischer et al., 2000).

(2) *Constant Infusion Clearance (CIC) models* - a tracer is infused at a constant rate until a steady-state tracer concentration is reached in the distribution volume. This allows determination of a tracer's urinary excretion rate via timed urinary and blood collection. To achieve steady state as quickly as possible, a loading dose of tracer is administered in most protocols before constant infusion of tracer begins (Schock-Kusch et al., 2012, Fleck, 1999, Fleck and Braunlich, 1984, Jobin and Bonjour, 1985).

An ideal exogenous tracer for determining GFR should be biologically inert, freely filtered across Bowman's capsule and neither reabsorbed, manufactured nor secreted by the renal tubule (Sturgeon et al., 1998).

Inulin is a biologically inert, uncharged fructan-type polysaccharide molecule. It is unbound by plasma proteins and is not manufactured nor metabolised *in vivo.*

Furthermore, it is freely filtered by glomeruli, and unlike creatinine is neither secreted nor reabsorbed by tubular epithelial cells (Wang et al., 2010). Consequently, inulin is widely regarded as an excellent reporter of GFR, with inulin clearance techniques employed to measure GFR in numerous studies. Recently, the use of fluorescently conjugated inulin has emerged as a reliable technique for GFR characterization, with the use of fluorescent reporter simplifying reporter quantification in the collected biofluids (Fleck, 1999, Fleck and Braunlich, 1984, Qi et al., 2004, Sturgeon et al., 1998).

Throughout this thesis, Glomerular Filtration Rate (GFR) was calculated via a constant infusion clearance (CIC) technique, utilising fluorescently conjugated inulin, in a protocol adapted from Barber et al (Barber and Bourne, 1971).

2.22 PREPARATION OF FITC-INULIN SOLUTION

0.2% Fluorescein Isothiocyanate Inulin (FITC-Inulin, Sigma-Aldrich) solution was made by dissolving 40mg of FITC Inulin in 20mls of 0.9% saline solution. 1% FITC-Inulin was made by dissolving 10mg FITC-Inulin per ml of 0.9% Physiological Saline Solution (Baxter Pharmaceuticals). To facilitate dissolution of inulin into solution, 0.9% saline was warmed in a water bath to 40^{o}C before use.

The solutions were dialysed for 24 hours through a GeBAflex-tube with a 1Kda semipermeable membrane (Gene Bio-Applications) in 0.9% Physiological Saline Solution, to remove unbound fluorescein. The solution was then sterilised by passing it through a 0.22µm syringe filter. At all possible times, inulin solutions were protected from light to prevent dissociation prior to infusion. Furthermore, inulin solutions were universally made and utilised within 24 hours to avoid degradation of inulin solution.

2.23 ANESTHESIA DURING INULIN CLEARNACE STUDIES

Anesthetic induction was with isoflurane as described in Section 2.12. During inulin studies, anaesthesia was then maintained with an initial intraperitoneal injection of thiobutabarbitol sodium (Inactin®) using 12mg per 100g of body weight. Further maintenance doses of 2.4mg per 100g of body weight were given as required. This

dosing protocol has previously been shown to provide stable cardiovascular parameters (Brammer et al., 1993).

2.24 SURGICAL PROCEDURE TO DETERMINE GLOMERULAR FILTRATION RATE

POSITIONING AND TEMPERATURE CONTROL

As described in Section 2.13

FEMORAL VESSEL DISSECTION AND CANNULATION - (Figure 2.2i – 2.2xii)

The left groin was shaved and an incision made inferior and parallel to the inguinal ligament. Dissection was carried out to retract the groin fat pad proximally, thereby exposing the femoral vessels. The femoral artery and vein were fully dissected. (See Figure 2.2) After clamping the femoral vein proximally and ligating distally, microsurgical polyethylene tubing (Smiths Medical), 0.96mm external diameter (ED), was used to cannulate the vein. The tubing was secured using 5/0 ligatures. A 0.5ml bolus of 1% inulin solution was given before starting a constant infusion of 0.2% FITC inulin using a syringe pump (World Precision Instruments, NE300) at a rate of 3ml per hour.

A microsurgical catheter (0.8mm ED) was then placed in the femoral artery. This was used to take blood samples during the experiment. Between sampling, the line was locked with heparin-saline solution.

URETERIC CANNULATION - (Figure 2.2 xii – xvi)

The abdomen was re-opened along the line of the previous midline incision. The bowel was wrapped in damp swabs, the ureters dissected and cannulated using a 7cm length of microsurgical tubing (0.61mm ED) Ligatures were applied to secure the catheters' position.

Via these cannulas, urine was collected into separate tubes during each 30-minute interval. In the middle of each 30-minute collection period, an 80ul blood sample was taken via the arterial line.

Urinary volume for each 30-minute period was measured using a Gilson's pipette after a gentle spin at 2000 rpm for 15 seconds to remove sediment. Once volume was measured, urine was transferred to a fresh Eppendorf tube for subsequently fluorescent measurement. Similarly, immediately after collection, blood was spun down at 6000rpm for 6 minutes, and serum transferred into a fresh Eppendorf tube for later analysis.

The experiment was terminated after six timed urinary/blood samples had been collected. The animal was killed, liver, spleen, heart, lungs and both kidneys were taken and stored in 10% formation, liquid nitrogen and RNA later.

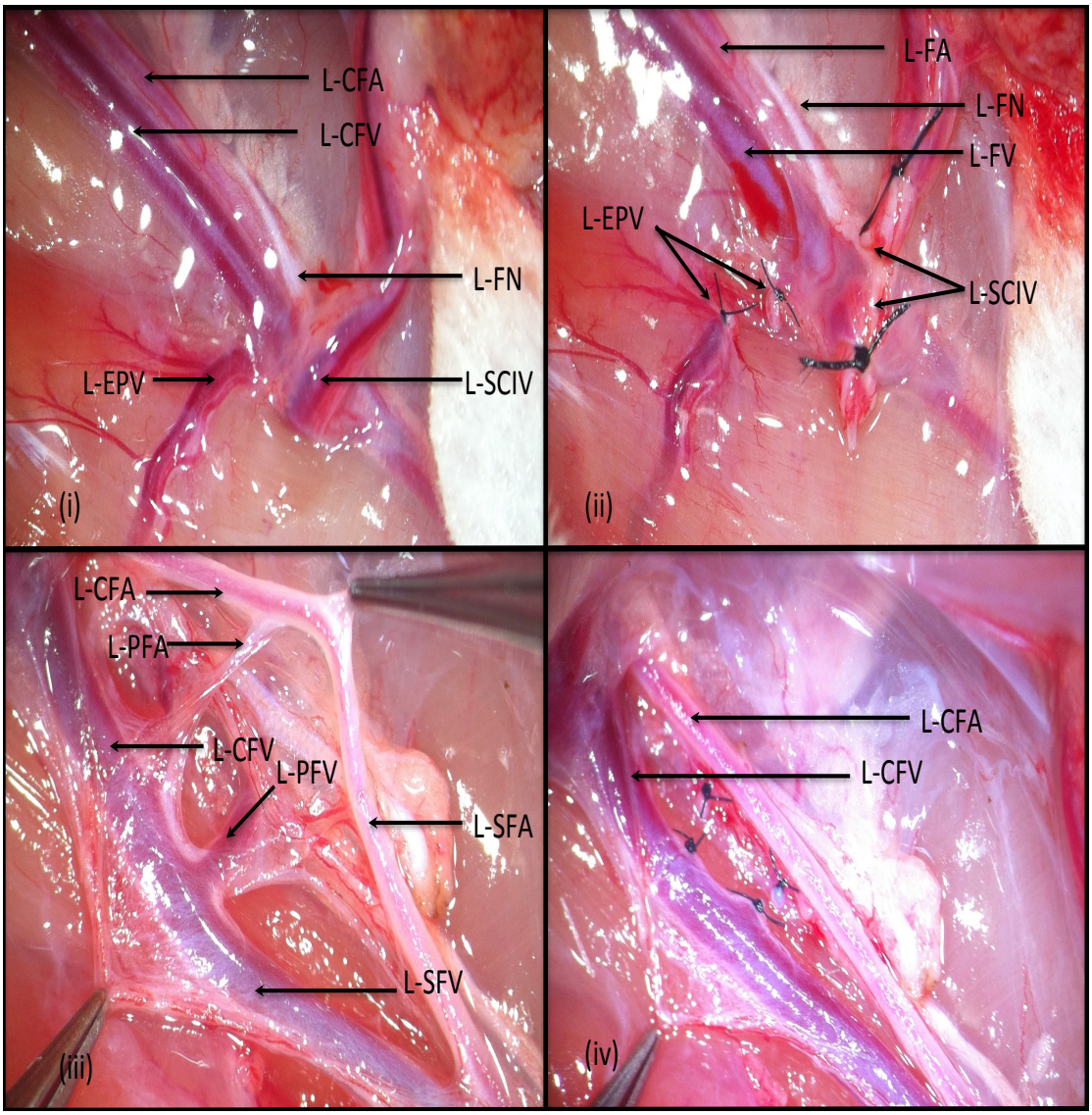


Figure 2.2: Dissection of Femoral Vessels

(i) Dissection of the rodent *left* groin to expose the femoral anatomy. The left common femoral artery (L-CFA), left common femoral vein (L-CFV), left femoral nerve (L-FN), left superficial circumflex iliac vessels (L-SCIV) and left external pudendal vessels (L-EPV) are clearly seen. **(ii)** The L-SCIV and L-EPV are ligated. **(iii)** The femoral sheath is opened, and dissection carried out to separate the femoral artery and vein. The left profunda femoral artery (L-PFA) and vein (L-PFV) are exposed, allowing identification of the left common femoral artery (L-CFA) and vein (L-CFV), and also the left superficial femoral artery (L-SFA) and vein (L-SFV). **(iv)** The L-PFVs are ligated and divided.

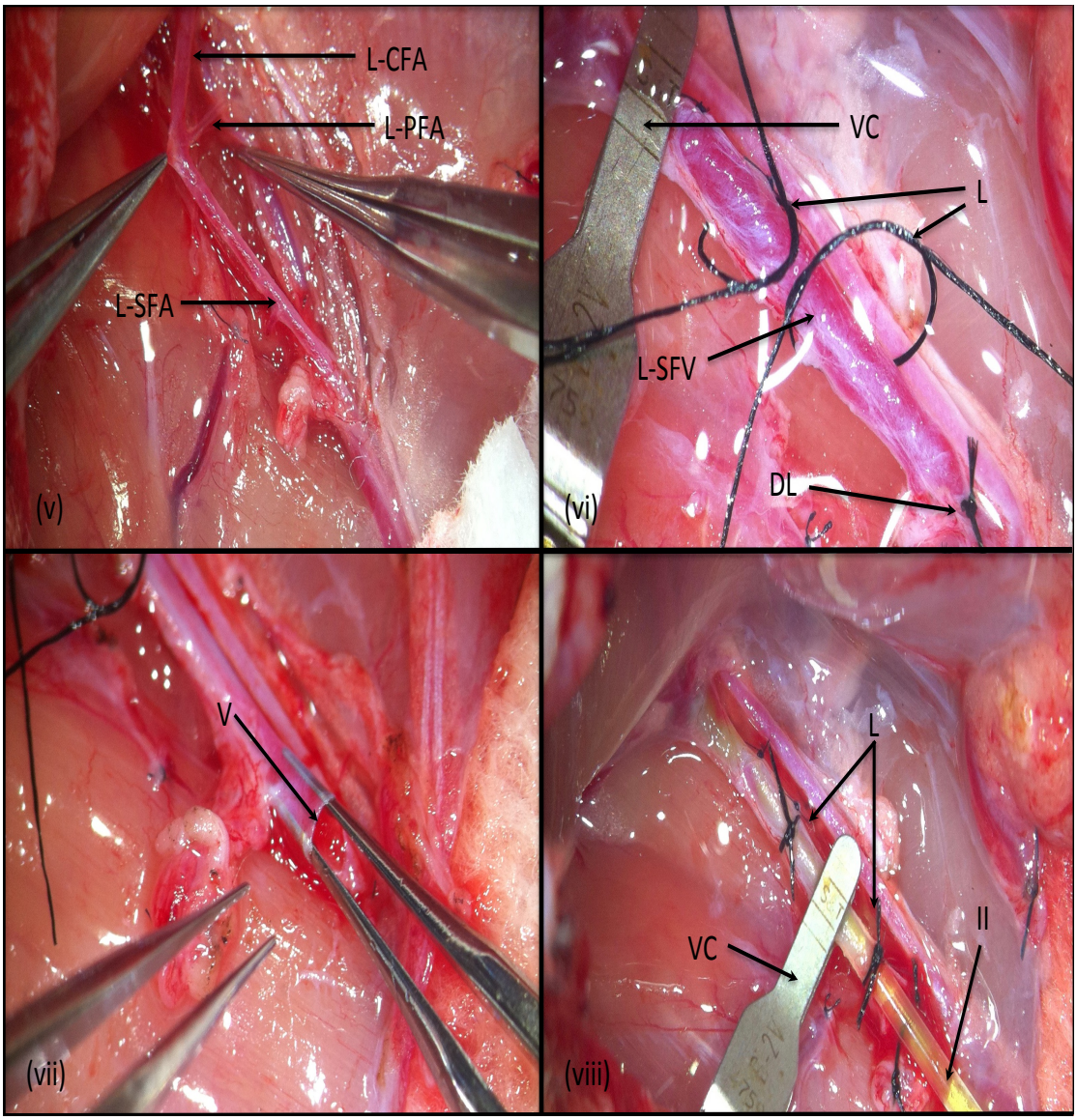


Figure 2.2: Dissection and cannulation of femoral vessels.

(v) The L-PFA is dissected before being divided with cautery. Both femoral artery and vein have now been dissected, and their side branches ligated. **(vi)** The L-SFV is ligated distally (DL). A vascular clamp (VC) is applied to the L-CFV proximally. Ligatures (L) are loosely placed in preparation for cannulation. **(vii)** A venotomy (V) is made in the distal part of the L- SFV. **(viii)** The vessel is cannulated, and the ligatures tightened before being cut to size. The vascular clamp is re-positioned onto the cannula to provide extra security against slippage. A bolus is inulin is given at this point, and the FITC-inulin infusion (II) is started.

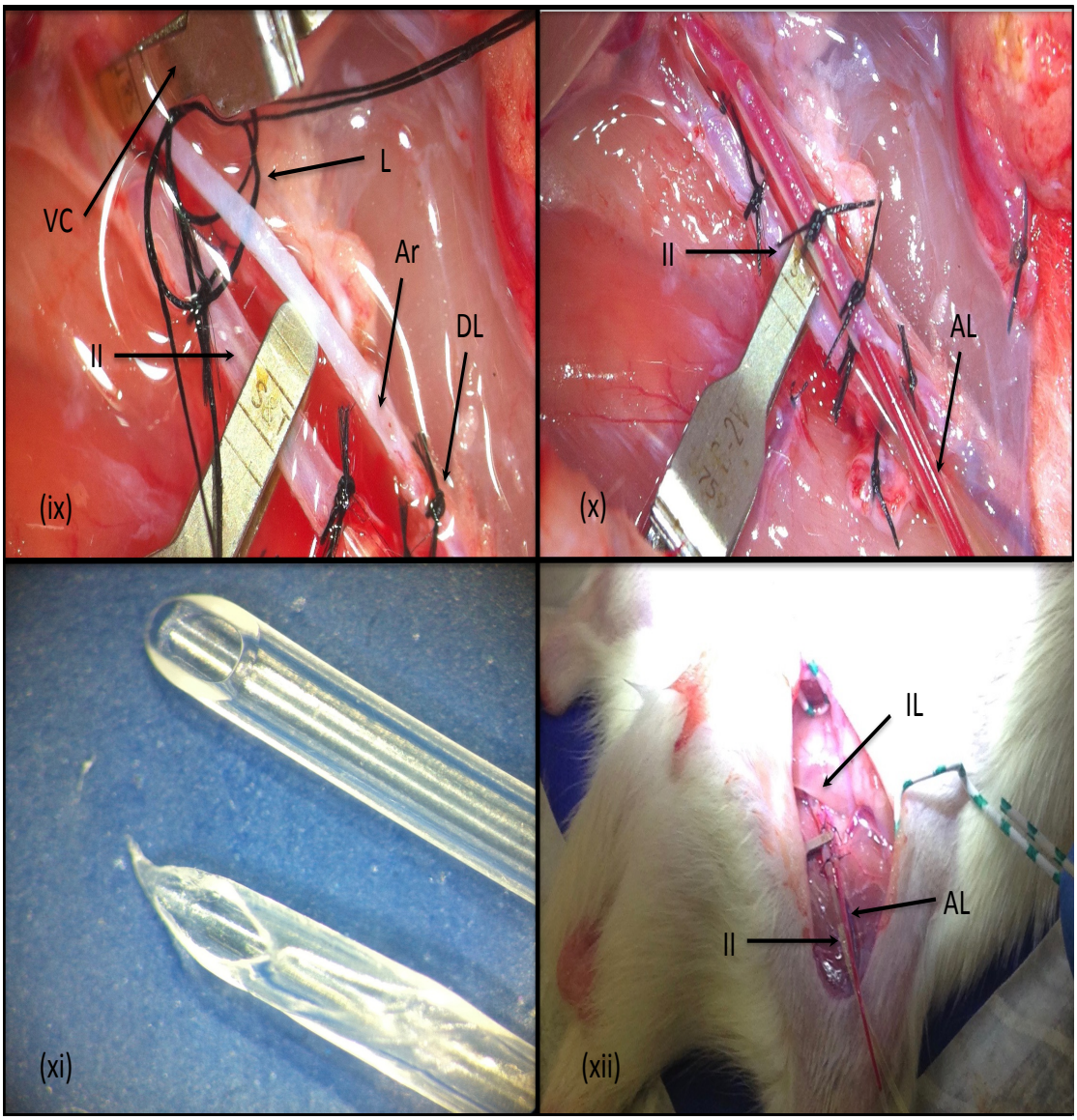


Figure 2.2: Cannulation of Femoral Artery.

(ix) The L-CFA has a vascular clamp (VC) applied proximally and ligature applied distally (DL). Loose ligatures are applied in preparation for cannulation. An arteriotomy (Ar) is then made distally. **(x)** An arterial line prepped with heparinized saline, the vessel cannulated and ligatures tightened to secure the line in place. The vascular clamp is released and blood flows into the arterial line allowing a sample to be taken. The inulin infusion (II) is continuously running. **(xi)** The smooth cannula tip achieved by cutting with a scalpel (top) versus the ragged edge made with sharp scissors (bottom). **(xii)** Overview of rat position with left groin dissection during inulin clearance.

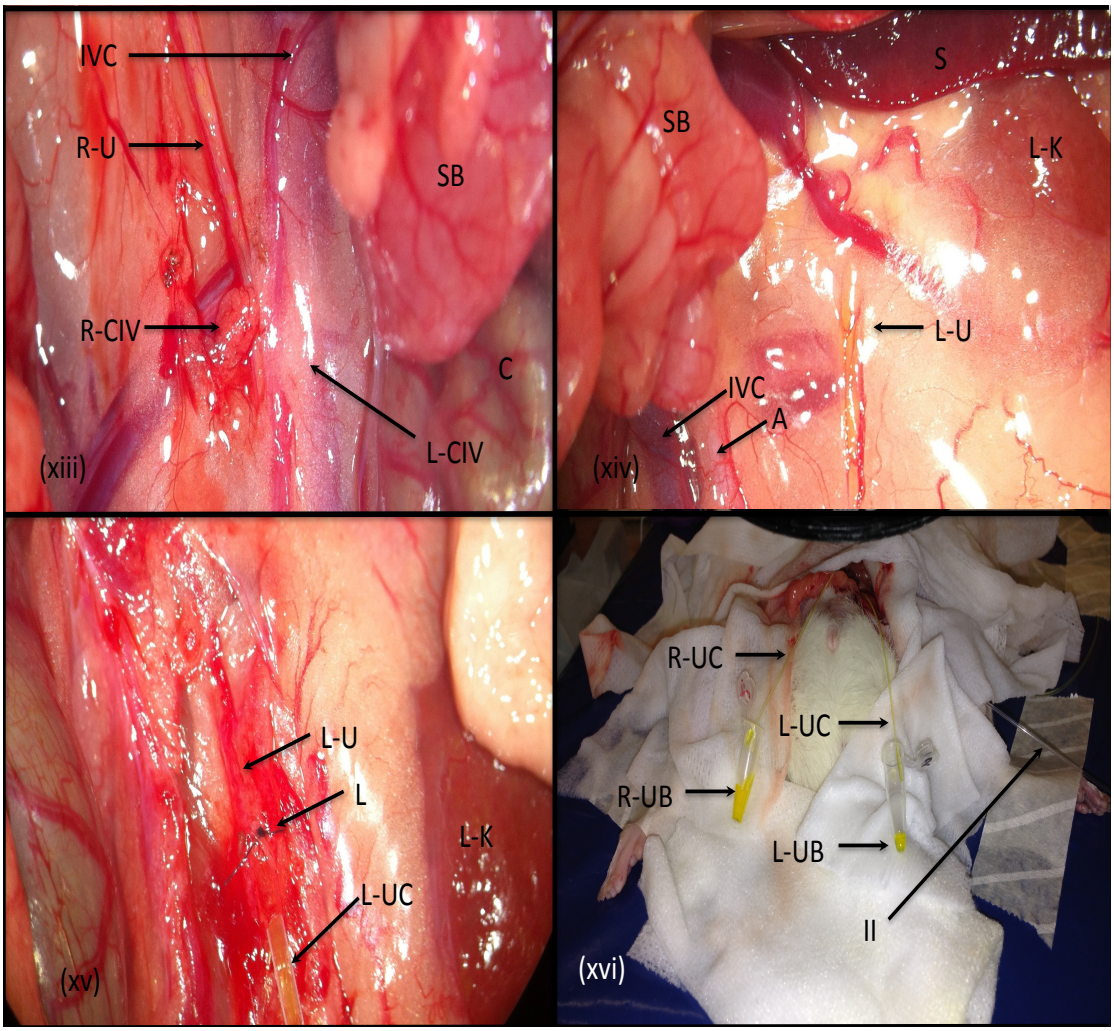


Figure 2.2: Ureteric cannulation.

(xiii) After a midline incision, retroperitoneal dissection is carried out to expose the right ureter (R-U). This involves mobilising the small bowel (SB) by incision of it posterior attachments. The ureter, containing fluorescent urine, is easily identified by its characteristic vermiculation. Cannulation is easiest at the point the ureter runs over the right common iliac vein (R-CIV). The inferior vena cava (IVC) and left common iliac vein (L-CIV) are easily visible. **(xiv)** Dissection is carried out to expose the left ureter (L-U), again easily identified by the fluorescent urine. The IVC and aorta (A) are easily seen. The left kidney (LK) and, spleen (S) are also visible. **(xv)** The left ureter is cannulated and urine is seen to freely flow into the cannula. **(xvi)** The position of the rat after ureteric cannulation. The urine in collected into Eppendorf tubes (UB), placed to catch under ureteric cannulas as it flows. Note the inulin infusion line (II).

2.25 MEASUREMENT OF BIOFLUID FLUORESCENCE

Plasma and urine fluorescence were measured by pipetting a10µL sample of biofluid onto with a Fluorescent Plate (Thermo-Scientific F96 Microwell plate). To this, 40µL of HEPES 500mM buffer solution (dissolved 59.6g of HEPES buffer in 500ml of water, adjusted to pH 7.4 by adding Sodium Hydroxide solution) was added to give a total volume in each well of 50µL. The addition of buffer is necessary, as fluorescence of biofluids has been shown to vary according to pH (Lorenz and Gruenstein, 1999). Each sample was assayed in triplicate to identify pipetting error.

Serum and urinary fluorescence were measured using a fluorescence microplate spectrometer (BIO-TEK flx-800) measuring emission and absorbance wavelengths of 495nm and 430nm respectively.

From triplicate assays, mean fluorescence values were obtained and mean fluorescent values calculated.

Steady state was reached in the plasma when the amount of inulin infused was equal to the amount filtrated by the kidneys. This is defined as a <5% change in inulin plasma concentration over a 30-minute period (Sturgeon et al., 1998). Data indicated that steady state is typically reached 60 - 90 minutes after the inulin infusion had commenced.

2.26 CALCULATION OF GLOMERULAR FILTRATION RATE

At steady state, the amount of inulin removed per minute from the plasma is equal to the urinary inulin concentration (U_i) multiplied by the urinary volume per minute (U_v). As inulin is freely filtered at the glomerulus, measuring the plasma inulin concentration at steady state (P_i) allows the volume of plasma filtered per minute (GFR) to be calculated according to the formula:

$$GFR = U_i \times U_v / P_i$$

Where U_i = Urinary fluorescence: U_v = Urine volume per minute: P_i = Plasma fluorescence.

2.27 KEY FACTORS IN ACHIEVING RELIABLE GFR RESULTS

Avoiding unnecessary blood loss is key to ensuring accurate GFR results. To achieve this, it is advisable to ligate all branches of the femoral vessels and to provide proximal and distal control of vessels before making an incision for cannulation. Bleeding points on the re-opened abdominal wall should be cauterised or over-sewn immediately. Cauterisation of small retroperitoneal vessels encountered during dissection of the ureter is advised, as these may bleed profusely over the time course of the study if left uncontrolled.

2.28 TIPS FOR SUCCESSFUL MICROSURGICAL CANNULATION

Cannulation is easier if a precise hole is made in the front wall of the structure to be cannulated. A horizontal cut with a pair of pointed microsurgical scissors is most effective. This manoeuvre allows the structure to remain under tension, while the created defect gapes open, allowing easy access for the passage of the cannula. (See Figure 2.2 (xii) and (ix)) Fully dividing a structure causes proximal retraction, whereby cannulation becomes extremely problematic.

It is useful to have ligatures to secure a catheter loosely applied prior to cannula insertion. This allows simple tightening of the ligature as soon as the cannula is inserted, reducing the chance of the cannula inadvertently slipping out. (Figures 2.2 (xi) and (ix))

When the microsurgical tubing is cut to size, the use of a scalpel blade allows a smooth, angled but non-pointed end of the tube to be fashioned. (Figure 2.2 (xi)) The use of scissors leads to a ragged pointed tube that is difficult to insert and will tear holes in vessel walls when advanced.

2.3 RODENT RENAL TISSUE ANALYSIS

2.31 PROTOCOL FOR EMBEDDING FORMALIN-FIXED KIDNEYS IN PARAFFIN

Kidneys, fixed in 10% formalin, were halved prior to embedding in paraffin. The remaining half of the kidney was returned to formalin.

In order to dehydrate the tissue:

(1) Kidneys were placed in 80% ethanol for 30 minutes
(2) Kidneys were placed in 95%% ethanol for 30 minutes
(3) Kidneys were placed in 95% ethanol for 45 minutes
(4) Kidneys were placed in 100% ethanol for 40 minutes
(5) Kidneys were placed in 100% ethanol for 40 minutes
(6) Kidneys were placed in 100% ethanol for 40 minutes
(7) Kidneys were placed in xylene for 40 minutes
(8) Kidneys were placed in xylene for 40 minutes
(9) Kidneys were placed in hot paraffin for 60 minutes
(10) Kidneys were then finally placed into hot paraffin for 30 minutes before being allowed to cool on a ‘cold plate', and blocks turned out.

Using a hand-operated microtome, blocks were then cut into 5μm sections, and floated in a warm water bath, before being placed on poly-L-lysine coated glass slides.

To fix paraffin sections to the sides, slides were then placed in a drying oven for 90 minutes at 60^{o}C, before being stored at 4^{o}C in a cold room.

2.32 PROTOCOL FOR STAINING RENAL SECTIONS WITH HAEMATOXYLIN AND EOSIN

Haematoxylin and Eosin (H & E) staining are the most commonly used histological stains. Cell nuclei are stained blue, and cell cytoplasm is stained pink.

METHOD

2% Eosin was made up by dissolving 20g of Eosin in 1000ml of distilled water, using magnetic stirring. A 20ml scoop of Calcium chloride was added to the mixture. This helps eosin fix to the tissue sections.

Paraffin-fixed tissue was cut to 5μm and mounted on poly-L-lysine coated glass slides as previously described in Section 2.31.

In order to remove paraffin from the sections:

(1) Slides were placed in xylene for 5 minutes
(2) Slides were then replaced in a fresh xylene for a further 5 minutes

In order to re-hydrate tissue sections:

(3) Slides were then placed in 100% ethanol for 3 minutes
(4) Slides were placed in 100% ethanol for a further 3 minutes
(5) Slides were placed in 90% ethanol for 2 minutes
(6) Slides were placed in 70% ethanol of 2 minutes
(7) Slides were placed in running water for 2 minutes
(8) Slides were then stained Harris haematoxylin for 8 minutes
(9) Slides were then rinsed in running tap water for 2 minutes
(10) Slides were then placed in 'Scott's' tap water for 45 seconds until blue
(11) Slides were then rinsed in running tap water for 2 minutes
(12) Slides were then placed in 2% Eosin stain for 10 minutes.
(13) Slides were then rinsed in running tap water for 2 minutes
(14) Slides were quickly dipped in 90% ethanol
(15) Slides were quickly dipped in 100% ethanol
(16) Slides were quickly dipped in 100% ethanol
(17) Slides were placed in xylene for 1 minute
(18) Slides were placed in xylene for 1 minute
(19) Slides were mounted with coverslips and fixed using DPX.

2.33 RENAL HISTOLOGY ASSESSMENT

IRI-related changes in renal histology are most severe in the S3 segment of the proximal renal tubule, located at the outer stripe of outer medulla. Such changes typically include denudation of tubular basement membrane, loss of brush border, and sloughed debris in the tubular lumen that results in hyaline cast formation.

To assess renal histology and quantify ischemic related damage, 5μm kidney sections were routinely stained with hematoxylin and eosin as described in Section 2.32. Histology scoring was a method described in a previous study (Melnikov et al., 2002) and subsequently modified in later reports.(Wang et al., 2012, Gatti et al., 2011, Allam et al., 2012) Using a 200x objective [high-power field (HPF)] 10 non-overlapping fields were randomly selected by a single blinded observer. Within each field, the following were counted in a 60 second period:

(i) Number of hyaline casts

(ii) Number of epithelial breaks (denudation of tubular basement membrane)

2.4 IMMUNOHISTOCHEMISTRY PROTOCOL

Immunohistochemistry (IHC) was carried out in a standardised fashion for the proteins of interest listed below. Primary antibody type and dilution are expressed in brackets.

Kidneys sectioned to 5μm and fixed to poly-L-lysine coated glass slides as described in Section 2.31, were removed from storage at 4^{o}C, placed in a plastic slide rake, and baked for 30 minutes at 56^{o}C.

2.41 DE-WAXING AND REHYDRATION OF PARAFFIN FIXED TISSUES

(1) Slides were placed in xylene for 2 minutes.
(2) Slides were placed in xylene for 2 minutes.
(3) Slides were placed in 100% ethanol for 2 minutes.
(4) Slides were placed in 100% ethanol for 2 minutes.
(5) Slides were placed in 90% ethanol for 2 minutes.
(6) Slides were placed in 70% ethanol for 2 minutes.
(7) Slides were placed in running tap water for 2 minutes.

During this process, solution for antigen retrieval was prepared.

2.42 ANTIGEN RETRIEVAL

(1) 1000ml of distilled water was placed in a plastic pressure cooker
(2) 0.37g of EDTA (Sigma E-5134) and 0.55g Tizma Base (Sigma T-1503) were added to the water
(3) Using a pH probe, the pH of the solution was adjusted to 8.0. This usually necessitated the addition of a few drops of hydrochloric acid via a Pasteur pipette.
(4) This solution was then heated for 13.5 minutes in the microwave at high power
(5) Slides were then placed in the hot solution, and the lid locked onto the pressure cooker to provide a tight seal.
(6) The slides were then heated in the microwave at high temperature, and under pressure for 5 minutes.

(7) Slides were left in antigen retrieval solution to cool for 20 minutes at room temperature, before being rinsed in distilled water.

2.43 SLIDE STAINING

A 3% solution of hydrogen peroxide (H_2O_2) solution was made by mixing 360ml of distilled water with 40ml of 30% H_2O_2.

(1) Slides were placed in 3% H_2O_2 solution for 10 minutes, the solution being gently agitated with a magnetic stirrer.
(2) Slides were washed in running tap water for 2 minutes.
(3) Slides were then 'ringed' with a DAKO marker to create a hydrophobic barrier.
(4) 200µl of a 1x casein solution made up at a 1 in 10 dilution in TBS was then applied to each section. This was incubated for 60 minutes at 25^{o}C in a humidified chamber.
(5) Casein solution was then blotted from sections.
(6) Primary antibody for the protein of interest was made then up to the stated dilution in 5% casein.
(7) 200µl of primary antibody solution was applied to each slide, which were then incubated overnight at 4^{o}C.
(8) Slides were washed in TBS for 2 x 5 minutes
(9) Secondary anti-Rabbit Envision antibody was made up to 1:200 dilution in 1x casein in TBS. 200µL was applied to each slide.
(10) Slides were incubated at 25^{o}C for 30 minutes in a humidified chamber.
(11) Slides were washed in TBS for 2 x 5 minutes
(12) DAB substrate (Vector SK4100) was made by mixing two drops of stock buffer with 5ml of distilled water, four drops of DAB substrate and two drops of hydrogen peroxide.
(13) Sections were incubated at room temperature until colour developed. Typically this took around 7 minutes.
(14) Slides were then washed in water for 10 minutes.

2.44 COUNTER-STAINING OF SLIDES

(15) Slides were stained for 45 seconds in Harris Haematoxylin.

(16) Slides were then rinsed in running water.

(17) Slides were de-stained in acid alcohol for 30 seconds.

(18) Slides were then rinsed in running water.

(19) Slides were then stained in Scot's tap water substitute for 1 minute.

(20) Slides were then rinsed in running water.

2.45 SLIDE DEHYDRATION AND MOUNTING

(21) 1 minute in 70% ethanol

(22) 1 minute in 90% ethanol

(23) 1 minute in 100% ethanol

(24) 1 minute in 100% ethanol

(25) 1 minute in xylene

(26) 1 minute in xylene

(27) DPX and coverslips were added.

2.46 P-16 IMMUNOHISTOCHEMISTRY PROTOCOL WITH VECTOR LAB IMPRESS PEROXIDASE POLYMER KIT MOUSE-ANTI-MOUSE (MP2400)

The protocol used for p16 IHC differed slightly from the standardised IHC technique in that a specific, pre-prepared reagent kit (MP2400) was utilised for the primary and secondary antibody applications steps. De-waxing, antigen retrieval, counterstaining and mounting of slides were as described in Sections 2.41, 2.42, 2.43 and 2.44.

2.461 SLIDE ANTIBODY STAINING

A 3% solution of hydrogen peroxide (H_2O_2) solution was made by mixing 360ml of distilled water with 40ml of 30% H_2O_2.

(1) Slides were placed in 3% H_2O_2 solution for 10 minutes, the solution being gently agitated with a magnetic stirrer.

(2) Slides were washed in running tap water for 2 minutes.

(3) Slides were then 'ringed' with a DAKO marker to create a hydrophobic barrier.

(4) Three drops of Protein blocking solution (Reagent 1) was added to each slide to completely cover the tissue section. This was incubated for 30 minutes at 25^{o}C in a humidified chamber.

(5) Protein blocking solution was then blotted from sections.

(6) MaxHomo TM blocking reagent (Reagent 2) was then added to completely cover tissue sections. This was incubated for 60 minutes at 25^{o}C in a humidified chamber.

(7) Slides were washed in TBS for 5 minutes

(8) Slides were washed again in TBS for 5 minutes

(9) Primary antibody (Mouse anti-mouse p16 (F12) Santa Cruz sc-1661) was made up to a 1:150 dilution in DAKO diluent. 200µl of primary antibody solution was applied to each slide to cover tissue sections.

(10) Sections then incubated overnight at 4^{o}C in a humidified tray.

(11) In the morning, slides were washed in TBS for 5 minutes.

(12) Slides were washed again in TBS for 5 minutes.

(13) Mouse Antibody Amplifier (Reagent 3) was added to cover tissue sections. Slides were then incubated for 30 minutes at 25^{o}C in a humidified chamber.

(14) Slides were washed in TBS for 5 minutes

(15) Slides were washed again in TBS for 5 minutes

(16) Ready to use Polymer HRP Secondary antibody (Reagent 4) was added to slides to cover tissue completely. Slides were then incubated for 30 minutes at 25^{o}C in a humidified chamber.

(17) Slides were washed in TBS for 5 minutes

(18) Slides were washed again in TBS for 5 minutes

(19) DAB substrate (Vector SK4100) was made by mixing two drops of stock buffer with 5ml of distilled water, four drops of DAB substrate and two drops of hydrogen peroxide.

(20) Sections were incubated at room temperature until colour developed. Typically this took around 7 minutes.

(21) Slides were then washed in water for 10 minutes, counterstained and mounted as described in Sections 2.44 and 2.45.

2.47 CALCULATING HISTOSCORES

Histoscores from each immunohistochemistry slide were calculated in standard fashion as previously described (Hirsch et al., 2003). Using a semi-quantitative approach, immunohistochemistry slides were assigned a histoscore by two blinded observers. This was achieved by evaluating both nuclear and cytoplasmic staining intensity. This is graded as follows:

Grade 0 = non staining; Grade 1 = weak staining; Grade 2 = medium staining; Grade 3 = strong staining.

The percentage of each staining intensity level is estimated and a histoscore assigned using the following formula:

[1 x (%cells @ Grade 3) + 2 x (%cells @ Grade 2) + 3 x (% cells @ Grade 3)]

The final score for each tissue section may range from 0 to 300.

2.5 ISOLATION, PURIFICATION AND QUANTIFICATION OF NUCLEIC ACIDS

2.51 RNA EXTRACTION FROM TISSUE USING TRIZOL®

Unless otherwise stated, reagents used during RNA extraction, manufacture of cDNA and q-PCR were obtained from Invitrogen Ltd (Paisley, UK)

Workspace and tools were cleaned with RNAase away spray prior to starting RNA extraction.

(1) 50 – 100mg of tissue, previously stored at -20oC in RNAse later, was added to a sterile tube containing 0.5ml of Trizol.
(2) This was homogenised with a sterile fashion, using an electric homogenizer.
(3) A further 0.5ml of Trizol was added and the solution vortexed for 20 seconds.
(4) Samples were incubated at room temperature for 10 minutes.
(5) 0.2ml of ice-cold chloroform was added, and the tube vortexed for 20 seconds, and incubated for 5 minutes at room temperature.
(6) Tubes then underwent centrifugation for 10 minutes (14,000rpm at 6^{o}C).
(7) The colourless upper phase was then transferred to a new tube, and 1x volume of phenol/chloroform/ isoamyl alcohol at pH 6.6 was added.
(8) This was vortexed for 20 seconds, incubated for 5 minutes at room temperature, before further centrifugation for 10 minutes (14,000rpm at 6^{o}C).
(9) The colourless upper phase was then transferred to a new tube, and 1x volume of ice-cold isopropanol added. Tubes were inverted a few times to mix the solutions.
(10) RNA was precipitated at – 80^{o}C for 30 minutes.
(11) Tubes were centrifuged for 30 minutes (14,000 rpm at 6^{o}C).
(12) RNA pellets were obtained, and supernatant removed without disturbing the pellet.
(13) The pellet was washed with 1ml of 95% ice-cold ethanol.
(14) The sample underwent centrifugation for 10 minutes. (14,000 rpm at 6^{o}C).
(15) The supernatant was removed.
(16) The pellet was washed with 75% ice-cold ethanol.

(17) The sample underwent centrifugation for 10 minutes. (14,000 rpm at 6^{o}C).

(18) The supernatant was removed.

(19) Samples were left to air-dry for 5 minutes.

(20) Pellets were dissolved in 40ul of 0.1% diethylpyrocarbonate-treated (DEPC) water (ThermoFisher Scientific).

Nanodrop® spectrophotometry analysis was then carried out, to measure RNA concentration and purity.

(1) The machine is 'zeroed' using a 1.5ul sample of DEPC water

(2) A 1.5ul RNA sample is placed on the pedestal. The machine automatically adjusts to create a column of fluid, forming the optimum path length, through which the absorbance of the sample is measured.

(3) Nucleotides, including RNA, single-stranded DNA and double-stranded DNA absorb at 260nm and contribute to the total absorbance of the sample. The ratio of absorbance at 260nm and 280nm is used to assess the purity of RNA. A ratio of ~2.0 is accepted as pure for RNA. If the ratio is lower, it may indicate the presence of protein or phenol, which strongly absorb at 280nm.

(4) Samples were stored in – 80^{o}C freezer.

2.52 DNAase TREATMENT

(Promega RQ1-RNase Free DNase®)

Before the conversion of mRNA to cDNA, it is necessary to perform DNAase treatment to remove all genomic DNA. This was achieved in the following standard fashion.

(1) Samples were retrieved from the – 80^{o}C freezer and thawed at room temperature.

(2) The volume of RNA solution required to give 2μg was calculated for each sample, and this was added to fresh 200μl Eppendorf tube.

(3) The total volume in each tube was then made up to 15μl by adding the requisite volume of DEPC water.

(4) DNAase 'master-mix' was made, containing 40% 10x DNAase buffer, 40% RNAase Out and 20% RQ DNase by volume.
(5) 5µl DNAase 'master-mix' was added to each Eppendorf tube, so that each tube held exactly 20µl containing:
    - 2µg of RNA
    - 2µl 10x DNAase buffer
    - 2µl RNase OUT
    - 1µl RQ DNase
(6) Samples were then incubated at 37oC for 30 minutes
(7) 1µl of STOP solution was added
(8) Samples were incubated at 65oC for 10 minutes to inactivate DNAase.
(9) Samples were kept on ice, and Nanodrop measurement repeated to assess RNA concentration without genomic DNA contamination.

2.53 cDNA SYNTHESIS

(Roche Transcriptor Reverse Transcription).

After purification of RNA, conversion to cDNA is necessary to proceed to q-PCR. It is crucial to have equal concentrations of cDNA for q-PCR, and to do this the RNA concentration during reverse transcription must be kept constant. This was achieved via the following standardised protocol:

(1) After establishing RNA concentration with Nanodrop, exactly 1µg of RNA from each sample was added to a separate 200µl Eppendorf tube.
(2) DEPC water was added to make the total volume of RNA and water to 20µl.
(3) 2µl of random hexamer solution was added.
(4) 2µl of dNTP solution was added
(5) The solution was then incubated for at 65^{o}C for 10 minutes before being placed on ice.
(6) RT 'master-mix' containing, by volume 50% 5x Buffer, 25% 0.1M DDT, 12.5% RNAase, and 12.5% reverse transcriptase was made.
(7) 16µl of RT 'master-mix' was then added to each sample so that each Eppendorf contained 40µl solution made up of:
    - 1µg RNA
    - 2µl random hexamer solution

- 2µl dNTP solution
- 8µl 5x RT buffer
- 4µl 0.1M DTT solution
- 2µl RNase Out
- 2µl Reverse Transcriptor

(8) Samples were mixed and then incubated for at 25^{o}C for 12 minutes, 42^{o}C for 50 minutes and 70^{o}C for 15 minutes.

(9) Samples were then stored at -20^{o}C until q-PCR.

2.54 TAQMAN REAL-TIME POLYMERASE CHAIN REACTION

Polymerase Chain Reaction (PCR) is a method that allows exponential amplification of short DNA sequences (usually 100 – 600 bases) within a longer double-stranded DNA molecule. PCR entails the use of a primers pairs that are complementary to a defined sequence on each of the two strands of DNA and specific to the gene of interest. During PCR cycling, these primers are extended by a DNA polymerase, so that a copy is made of the designated gene sequence. Repeating the process leads to logarithmic amplification. During each cycle, the strands of DNA are separated by heat denaturation, during which the primers become annealed to their complementary sequence. The use of heat-stable polymerases (Taq-polymerase) eliminates the need to added fresh polymerase after each cycle.

TaqMan probes are oligonucleotides that have fluorescent reporter dye attached to the 5' end and a quencher to the 3' end. When the probe is intact, the reporter dye emission is quenched due to the physical proximity of the reporter and quencher fluorescent dyes. During the extension phase of the PCR cycle, the 5' nuclease activity of the Taq-polymerase cleaves the hybridization probe and releases the reporter dye from the probe. With each cycle of PCR amplification, there is an increase in fluorescence emission that is monitored in real-time (RT –PCR) using the ABI 7700 sequence detector.

TaqMan RT-PCR was done using a Roche Lightcycler. Using a standardised RT-PCR 'master-mix', so that each sample was measured in triplicate, using specific

probe and primers for the genes of interest. Each PCR plate well contained the following:

(1) 1µl cDNA
(2) 5µl 2x Taqman Universal Master Mix
(3) 3.5µl DEPC water
(4) 0.5µl of probe/primer mix

Quantification of RT-PCR was via the comparative CT method (ΔΔCT). A threshold was set at a point where the amplification appeared linear for both the gene of interest and the housekeeping gene HPRT. The resultant cycle number (CT) for both of these genes was recorded for each sample and also for a control tissue sample. The CT values of both the sample of interest and control are then normalised to the housekeeping gene HPRT and quantified according to the following equation:

$$\Delta\Delta CT = \Delta CT_{sample} - \Delta CT_{reference}$$

Where ΔCT_{sample} is the CT value for any sample normalised to the endogenous housekeeping gene ($\Delta CT_{sample} - CT_{housekeeping\ gene}$), and $\Delta CT_{reference}$ is the CT value for the control also normalised to the endogenous housekeeping gene ($\Delta CT_{reference} - CT_{housekeeping\ gene}$)

2.6 ISOLATION OF PANCREATIC DERIVED PATHFINDER CELLS

Laboratory staff, using methods described in a previous report, conducted pathfinder isolation and colony maintenance (Stevenson et al., 2011, Stevenson et al., 2009). The author did not perform this task, but was provided with cell preparations, used for the experiments described in Chapter 3.

Briefly, minced pancreatic tissue was obtained from 12-month-old Albino Swiss rats. Seeding into CMRL medium (Invitrogen, Paisley UK) produced a confluent monolayer containing Pathfinder cells after ~ 5 weeks, which was then harvested and washed in phosphate buffered saline. Cells were maintained in CMRL-1066 medium, supplemented with 10% fetal bovine serum ((FBS), Sigma, Poole UK). Cells were characterised as described in previous reports. Pathfinder cells were comprised of a mixed CD90 cytotype, positive for expression of c-met, CD147, CD44, CD49f and CD71. Cells were negative for CD31, CD34, CD45, CD105, CD73 and c-kit expression.

2.61 PREPARATION OF PATHFINDER DERIVED MICROVESICLES

The microvesicles used throughout this thesis were isolated by Dr Diane Anthony.

The methods employed have been previously described in detail (McGuinness et al., 2016).

Briefly, pathfinder cells, grown as described above in T50 flasks, CMRL-1066 medium and FBS, and were centrifuged for 2.5 hours at 120,000 x *g* and then supernatant was carefully removed. Pathfinders were then cultured without medium change until 80% confluent and the supernatant harvested.

Under aseptic conditions, the supernatant was centrifuged at 1000 x *g* for 10 minutes to remove cell debris. The supernatant removed and centrifuged at 16,000 x *g* for 2.5 hours at 4^{o}C. The cell pellet was removed, re-suspended in sterile PBS and centrifuged for 2.5 hours at 16,000 x *g* at 4^{o}C. The yield was a cell free fraction, made up predominantly of microvesicles. These were kept at -80^{o}C until required for use.

2.7 STATISTICAL ANALYSIS

Statistical analysis was performed using Graphpad Prism 6.0.
The specific parametric and non-parametric statistical tests used to analyse each experiment are detailed within each results chapter. Data are presented as means ± Standard Deviation (SD) with the level of significance set at $p<0.05$.
Error bars on graphs represent SD unless otherwise stated.

2.71 DETERMINATION OF ANIMAL NUMBERS IN EXPERIMENTAL GROUPS

Most previous rodent renal IRI studies have utilized a group size of 6 – 8 animals. However, these experiments have exclusively used serum creatinine / BUN to measure kidney functional status, with these being recognized as less sensitive indicators than GFR. Furthermore, there appears to be a complete absence of reported data describing the effect of stem cells and / or ECVs on GFR in renal IRI experiments.

A recent paper, outlining a similar model to the novel renal IRI model described in Chapter 5, used a power calculation to determine a sample size requirement of 6 animals per group (Le Clef et al., 2016). Furthermore, studies using ADRCs (Chen et al., 2011, Shih et al., 2013, Wang et al., 2013b, Iwai et al., 2014) or ECVs (Gatti et al., 2011, Cantaluppi et al., 2012) have employed 6 or 8 animals per group to demonstrate significant findings.

The experiments presented throughout the course of this thesis were novel. As such, it was not thought feasible to perform formal power calculations to determine group numbers, as effect size of therapeutic agents and standard deviation were unknown due to a lack of previous pilot data. Therefore, based on the findings / methods of the previous studies noted above, it was decided to employ 6 animals per group, but to review this requirement based on experimental findings.

CHAPTER 3

TESTING THE RENAL REGENERATIVE EFFICACY OF PATHFINDER CELLS IN A RODENT MODEL OF CHRONIC RENAL FAILURE AND PREMATURE AGING

3.1 INTRODUCTION

The increase in ESRD seen in the Western world is mainly related to chronic disease processes that are a consequence of lifestyle in individuals with a genetic predisposition to cardiovascular disease and diabetes. While there has been much interest in developing cell-based therapies to mitigate acute events affecting native and transplanted kidneys, there has been less interest in the potential of stem cells to combat disease processes that culminate in patients with chronic renal failure progressing to end-stage disease.

In part, this is due to a lack of suitable animals models that display chronic renal failure in association with increased cellular senescence. However, within Glasgow University exists the mutant rat substrain (AS/AGU), which arose spontaneously in a colony of Albino Swiss rats (Clarke and Payne, 1994). As a result of a mutation in the gene encoding the protein kinase PKC_{Υ}, the AS/AGU rat is predisposed to excessive levels of oxidative stress, manifested by increased levels of cellular senescence and hence premature ageing (Craig et al., 2001). This is most obliviously apparent in a Parkinsonian type movement disorder that is the result of the progressive loss of dopaminergic neurones (Payne et al., 2000). However, the AS/AGU strain has been shown to develop chronic renal failure, with associated renal senescence in an age-dependent fashion (Gingell-Littlejohn, 2014). It is therefore feasible to hypothesise that the AS/AGU rat provides a good model in which to study the ability of multiple doses of stem cell-based therapy to halt the progression of chronic renal disease in the long-term.

A previous study reported that intravenous therapy with Pathfinder cells reduces senescence in acutely injured kidneys (McGlynn et al., 2013). Furthermore, in mice subjected to streptozotocin-induced diabetes, intravenous Pathfinder therapy is reported to initiate native islet regeneration, with subsequent long-term normoglycemia observed in treated animals (Stevenson et al., 2011).

3.2 HYPOTHESIS

(1) AS/AGU rats treated with multiple doses of intravenous Pathfinder cells may show improved renal function and reduced molecular marker of renal ageing 12 months after treatment is initiated.

3.3 METHODS

3.31 ANIMAL HOUSING AND HUSBANDRY

Animals were housed and fed as described in Section 2.1. Animals were weighed weekly and assessed on a daily basis for signs of distress or illness.

3.32 GROUP SELECTION

16 AS/AGU rats (eight male and eight female) aged from 4 -12 months, were randomly assigned to treatment and control groups so that each group contained four male and four females rats of similar ages.

All animals were weighed at baseline and bled for baseline serum creatinine levels one week before the first injection.

3.33 STEM CELL TREATMENT

Animals randomised to treatment received 3 x 10^6 rat pathfinder cells in 0.9ml phosphate buffered saline (PBS) via tail vein injection. Animals randomised to the control group received 0.9ml PBS vehicle via tail vein injection.
Injections were repeated every two weeks.

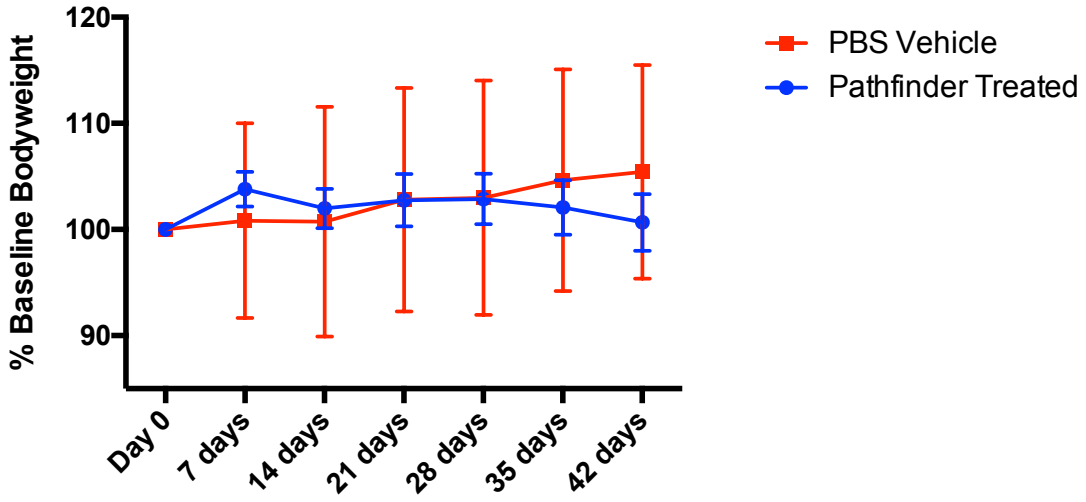
3.34 RENAL FUNCTION ASSESSMENT

Rats underwent tail vein bleeding (Section 2.17) every two weeks. Bleeding and tail vein injection weeks were alternated, so that no rat was both injected and bled in the same week.
Animals were culled in terminal GFR studies (Section 2.2)

3.4 RESULTS

3.41 ANIMAL WEIGHTS

No significant weight difference was noted between Pathfinder treated and control animals at any point. (See Figure 3.1) However, a trend of weight loss in the Pathfinder treated group was observed prior to the experiment being halted.



3.42 ANIMAL HEALTH

35 days after the experiment began, a Pathfinder treated male rat was found dead in its cage. For the previous five days, this animal had displayed weight loss, and signs of illness (marked piloerection, increased respiratory rate, reduced movement and food intake). A post-mortem examination was carried out, with the finding of multiple pulmonary nodules, highly suspicious of disseminated malignancy. (See Figure 3.2 (i))

By this time, other animals in the Pathfinder treatment group were displaying similar signs of weight loss, illness and distress. Accordingly, both the University Veterinary Officer and the Home Office inspector were asked for an opinion. It was deemed that all treated animals were distressed and consequently an order was made to immediately cull the animals. It was permitted to do this in terminal GFR experiments. A post-mortem examination was performed on each Pathfinder-treated animal, with the universal finding of multiple pulmonary lesions. In addition five animals displayed macroscopic evidence of cardiac, renal or splenic involvement. (See Figure 3.2). Formal pathology reports, produced by the Veterinary Diagnostic Services, University of Glasgow identified the macroscopic lesions as highly

infiltrating neoplastic tumours, and these were present in the lungs of all examined animals, in addition to the heart, spleen and kidneys of specific individuals. These tumours were highly anaplastic, displaying signs of bone formation although the cells of origin could not be determined. **(See report attached: Appendix 1)**
These features are most frequently observed in teratoma or osteosarcomas.

No sign of illness or distress was observed in the control group animals, which were also culled in terminal GFR experiments. Post mortem examination revealed no evidence of pulmonary or abdominal malignancy in any animal treated with PBS vehicle.

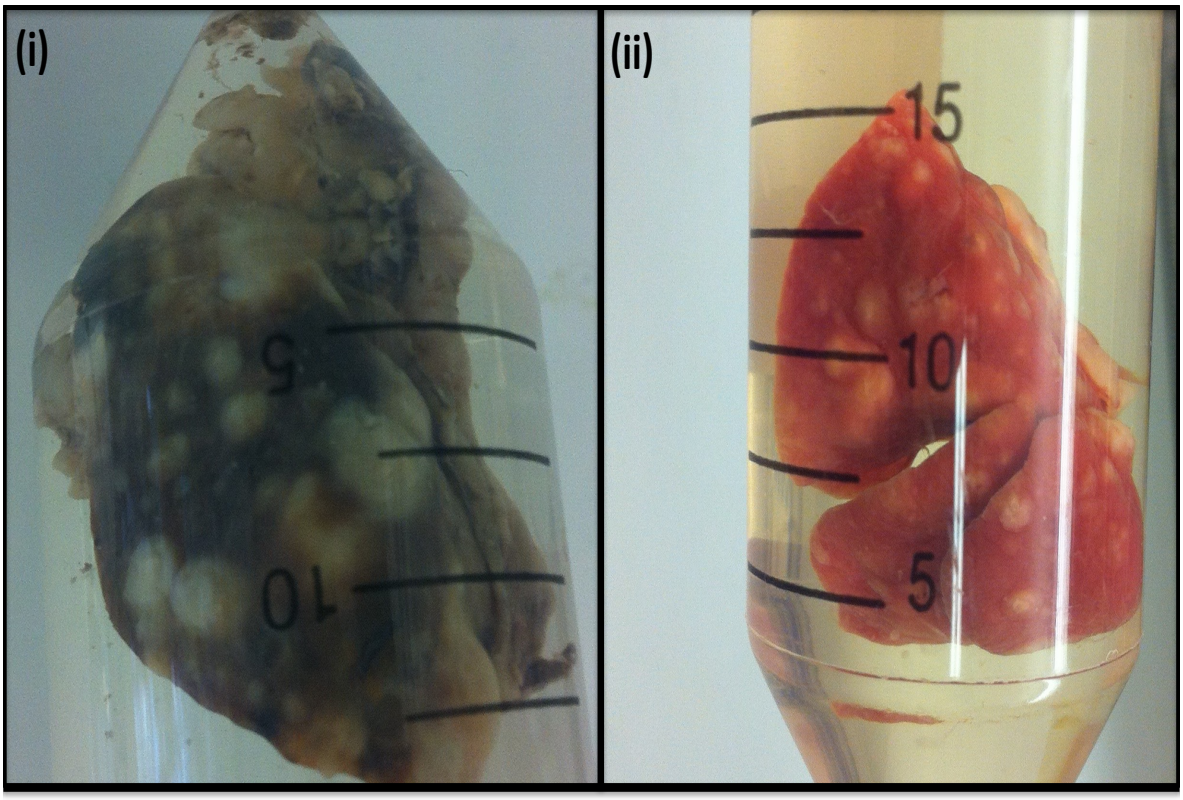


Figure 3.2: (i) Left lung and heart of AS/AGU rat found dead 35 days after Pathfinder injection.
(ii) Right lung of AS/AGU rat 44 days after Pathfinder injection.
Both lungs display evidence of disseminated malignancy in the form of multiple pulmonary nodules.

3.43 SERUM CREATININE

No difference in serum creatinine was noted between groups at any point, although a trend was noted towards increased serum creatinine in Pathfinder treated animals by Week 5. (See Figure 3.3).

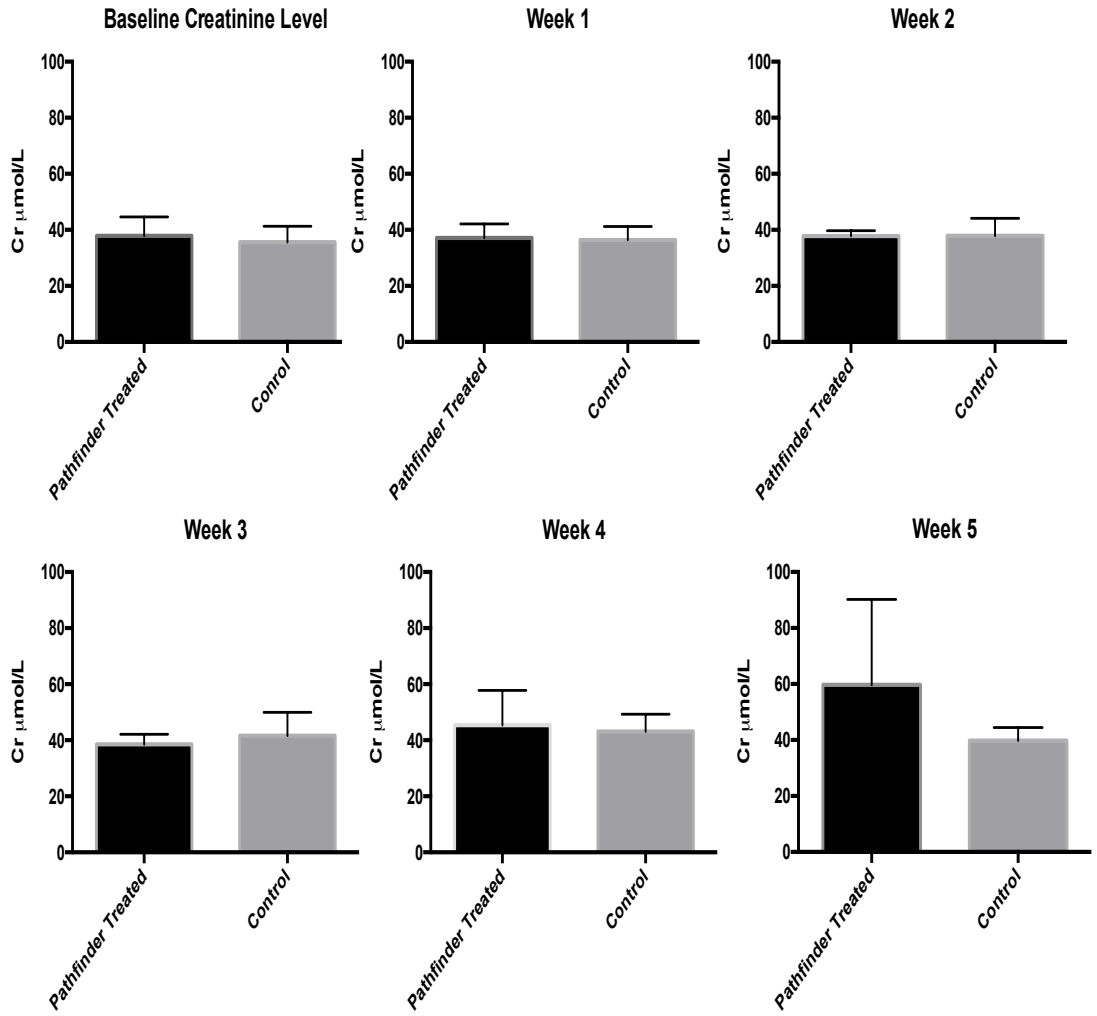


Figure 3.3: No difference was noted at any point in serum creatinine levels between Pathfinder treated and control animals. However, blood tests at the end of Week 5 did show a trend towards elevated serum creatinine in the Pathfinder treated group (p=0.089)

3.44 GLOMERULAR FILTRATION RATE

Only 6 Pathfinder treated animals underwent GFR studies, as one animal had been found deceased and another suffered a cardiac arrest under anaesthesia before steady state was reached. Furthermore, 2 Pathfinder treated animals were clearly unwell during GFR study and failed to pass urine, resulting in a GFR of zero. As a result, Pathfinder-treated animals were found to have a significantly lower GFR compared to control animals (p=0.0015). (See Figure 3.4)

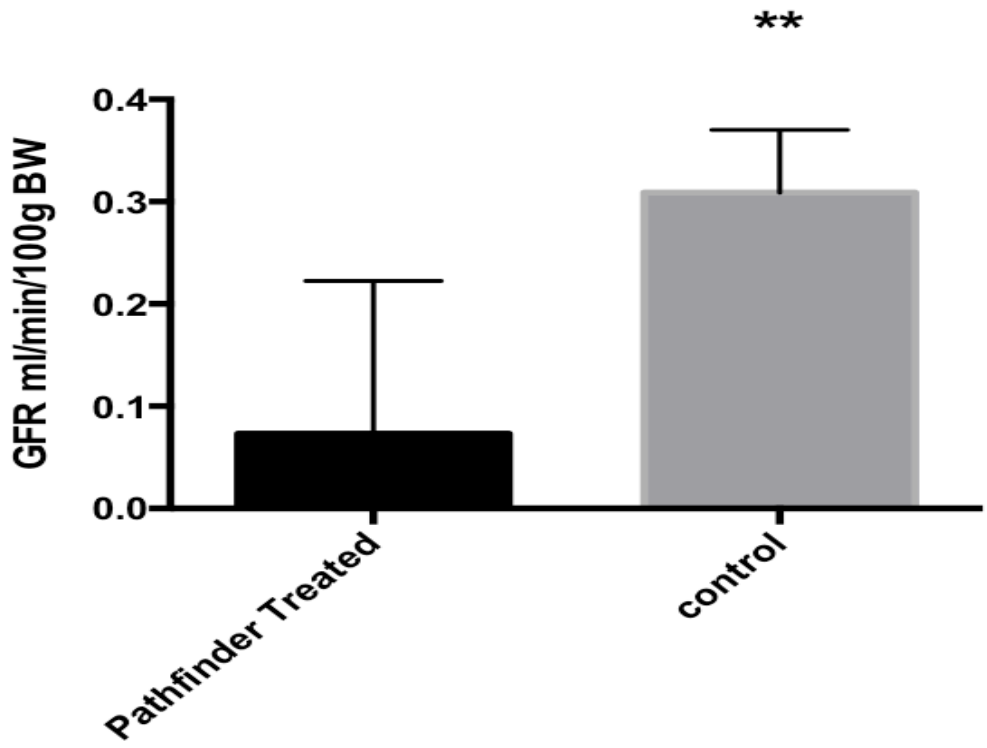


Figure 3.4: Pathfinder treated animals surviving to undergo GFR studies (n=6) had a significantly lower GFR compared to control animals (n=8). p =0.0015

3.5 DISCUSSION

In the field of regenerative medicine, the development of stem cell-based therapies are eagerly anticipated as promising tools to treat patients with a range of diseases. Such optimism is based on the ability of pluripotent and multipotent stem cells to differentiate into numerous cell lineages, with the potential to either replace damaged tissues directly by differentiation or to affect solid organ repair via a paracrine mechanism of action.

Due to the ethical issues surrounding the use of ESCs, research has tended to focus on the use of MSCs. These may be obtained from a number of different tissue types, although the use of BD-MSCs has predominated in most laboratories. However, to obtain sufficient numbers of cells to achieve efficacy in clinical protocols, extensive *ex vivo* expansion is required (Rosland et al., 2009). Unfortunately, accumulation of DNA damage through genomic instability, loss of cell cycle regulation and deregulation of epigenetic signature is more likely to arise during long-term, repeated culture under standard *in vitro* conditions (Maitra et al., 2005, Narva et al., 2010). This may eventually result in expanded stem-cell populations displaying tumorigenicity – defined as 'the capacity of a cell population inoculated into an animal model to produce a tumor by proliferation at the site of inoculation and /or at a distant site by metastasis (Organization., 2013). Therefore, the potential ability of stem-cell lines to undergo malignant transformation during repeated passage necessitates a stringent quality and assessment procedure in order to minimise the risk of negative, malignancy related outcomes to recipients (Yasuda and Sato, 2015).

Any normal cell in the body has the potential to undergo malignant transformation. Such cells usually exhibit changes in their morphology, growth kinetics, cell surface markers, genetic composition and tumorigenicity (Lye et al., 2016).

Pluripotent and multipotent stem cells, of both human and rodent origin, have been reported to undergo oncogenic transformation with the potential to cause disseminated malignancy as a result of their use in animal models. Passaged MSCs obtained from rodents have been reported to display abnormal morphology, high proliferation rates and aneuploidy (Miura et al., 2006, Furlani et al., 2009a). Evidence

of tumorigenicity has also been observed in rodent MSCs after only 3 passages, with MSCs undergoing malignant transformation and producing malignant tumors when injected into rats, rabbits and mice (Furlani et al., 2009a, Ahmadbeigi et al., 2011, Zhou et al., 2006).

The longer telomere lengths in rodents (30 – 100kb) versus humans (5 – 10kb) theoretically reduces the risk of spontaneous transformation in expanded cell lines of human origin (He et al., 2012, Smogorzewska and de Lange, 2002, Oeseburg et al., 2010). Nevertheless, malignant transformation during passage has been reported in human MSC cell lines (Rubio et al., 2005, Rosland et al., 2009). Subsequently, however, these findings were retracted as it was suspected that expanded human MSCs had been contaminated with tumour cell lines (Torsvik et al., 2010, de la Fuente et al., 2010). While some reports have stated categorically that cultured human MSCs do not undergo spontaneous transformation *in vitro* (Bernardo et al., 2007, Augello et al., 2010, Choumerianou et al., 2008), other researchers have recently indicated that such change does occur, while excluding the possibility of cell line contamination in their experiments (Pan et al., 2014, Wang et al., 2013a). Consequently, there is uncertainty regarding the ability of human MSC cell lines to display tumorigenicity after *ex vivo* expansion, although a reasonable body of evidence exists to suggest this phenomenon is at least a possibility (Lye et al., 2016, Yasuda and Sato, 2015).

While there are no previous reports of *ex vivo* cultured Pathfinder cells displaying tumorigenicity, there is little doubt that Pathfinder treated animals in this experiment developed malignant tumours as a result of their therapy. Furthermore, both the distribution of disease and the timing of onset of symptoms of tumour burden reported here mimic the results of a previous study where intravenous MSC therapy resulted in multiple pulmonary metastatic lesions (Rosland et al., 2009). It seems logical to conclude that the observed reduction in GFR results from dehydration and general illness that results from widespread metastatic disease that was universally seen in Pathfinder treated animals.

This finding serves to highlight the dangers associated with the use of passaged stem cell preparations. Patients undergoing renal transplantation are universally treated

with powerful immunosuppression medication in order to reduce the incidence of acute and chronic rejection episodes. However, immunosuppressed patients are known to be at increased risk of long-term malignancy as a consequence of this therapy. In addition, several studies in rodents have confirmed increasing teratoma formation rates with increasing levels of immune deficiency (Drukker et al., 2006, Dressel et al., 2008, Hentze et al., 2009).

Theoretically, it is possible that treatment with stem cell preparations in an attempt to improve long-term allograft function increases patient risk of adverse outcomes as a result of malignant stem cell transformation. That controversy exists regarding the ability of *ex vivo* expanded human MSC lines to display tumorigenicity is perhaps a moot point, as the potential for cell line contamination or spontaneous malignant transformation during passage will always exist – hence the clinical application of passaged cell preparations predisposes patients to unnecessary risk. When considering this fact, it is pertinent to recall the salient principle of clinical medicine *"Primum non nocere" – "First do no harm*".

It follows that to facilitate the implementation of novel stem cell-based therapies, the adoption of strategies that exclude *ex vivo* stem cell expansion are of paramount importance. Failing that, it would be highly desirable to implement stringent quality assurance procedures to exclude tumourogenesis, such as was observed here in immunocompetent animals. Two strategies with the potential to reduce the risk of malignant complications have been reported in the literature:

(1) The use of cell-free preparations, usually containing a biologically active fraction consisting of Extracellular secretory vesicles. (ECVs)
(2) The use of stem cell preparations requiring little or no ex vivo expansion. This may comprise cells freshly isolated and used immediately, or cells that are isolated and immediately cryopreserved, being stored for later 'off the shelf' use.

The negative findings of this chapter provided a persuasive argument to restrict the study of *ex vivo* expanded stem cell populations in animal models of renal failure. Indeed, the unexpected results reported above breeched the terms of the Project

Licence under which this experiment was conducted. The Home Office inspector stipulated that no further work should be undertaken with pathfinder cells which had been expanded *ex vivo* without prior discussion with the Home Office.

Consequently, due to the reduced potential for tumourogenesis, it was decided to focus investigation on the reno-protective abilities of both cell-free preparations and non-expanded stem cell preparations for the remainder of this thesis.

CHAPTER 4

INVESTIGATING THE EFFECTS OF PATHFINDER CELL-DERIVED MICROVESICLES IN AN IMMUNOCOMPETENT RAT MODEL OF RENAL ISCHEMIA/REPERFUSION

4.1 INTRODUCTION

Currently no therapies are in widespread clinical use for the treatment or prevention of acute injury to either native kidneys or renal allografts. This partly reflects the imperfect nature of the animal models in which novel treatments for renal injury are tested (Singh et al., 2012, Lieberthal and Nigam, 2000). *In vitro* models including renal cell cultures, isolated renal tubules and isolated perfused kidneys have been gainfully employed to research the pathophysiological mechanisms by which renal injury occurs at the cellular and molecular level (Ortiz et al., 2015). However, such models lack the physiological complexity to properly investigate the systemic effects of renal disease and the kidney's subsequent response to novel therapies (Lieberthal and Nigam, 2000). To draw meaningful conclusions regarding the efficacy and safety of cell-based treatments, *in vivo* models must be employed – a point highlighted by the findings of Chapter 3.

In *vivo* models of renal failure have been commonly described and utilized, with approximately 50% of experiments conducted in rats (Wei and Dong, 2012). For preliminary experiments, both rats and mice are considered favourable species as *in vivo* models for the investigation of renal disease. Rodents are widely available and inexpensive when compared to research using large animals species, although large animals exhibit the closest similarity with humans and are considered the 'gold standard' for testing novel therapies (Giraud et al., 2011). Nevertheless, rodents bear physiological and anatomical similarity with higher order mammals, and novel therapies may be screened using rodents before treatments are investigated further in large animal models.

Rodent models commonly utilized to investigate the pathological pathways that lead to renal damage include:

(1) Renal mass reduction by 5/6 nephrectomy
(2) Renal ischemia-reperfusion injury (IRI)
(3) Administration of nephrotoxic drugs to induce renal damage.

The 5/6 nephrectomy model is commonly used to create impaired renal function by ablation of renal mass. This produces proteinuria but does not mimic the pathological mechanisms seen in acute transplant-associated injury or the processes involved in the presentation of native renal disease (Perez-Ruiz et al., 2006).

Alternatively, prolonged warm renal IRI in rodents has been shown to cause significant tubular injury and an influx of inflammatory cells, leading to long-term renal fibrosis (Basile et al., 2001). Warm renal IRI is considered to be the main pathological process affecting renal allografts, and models using warm IRI are probably the best mimics of the clinically observed acute transplant injury (Lameire, 2005, Lameire et al., 2005). In addition, warm renal IRI may also negatively impact upon the function of native kidneys, and is often seen in patients subjected to cardiac and vascular surgery, trauma, and burns (Lameire and Vanholder, 2004). IRI is therefore a common and important pathology in which to study renal disease in an animal model.

Therefore, rodent IRI models are of clinical relevance and furthermore such models are useful for assessing the efficacy of potential treatments before testing in a more technically complex rodent transplant model. Here, a period of transplant cold storage is added to closely emulate the processes of involved during clinical transplantation (Wang et al., 2013b).

Established *in vivo* models of renal IRI may broadly be divided into two types: bilateral (B-IRI) or unilateral renal injury (U-IRI). (See figure 4.1)

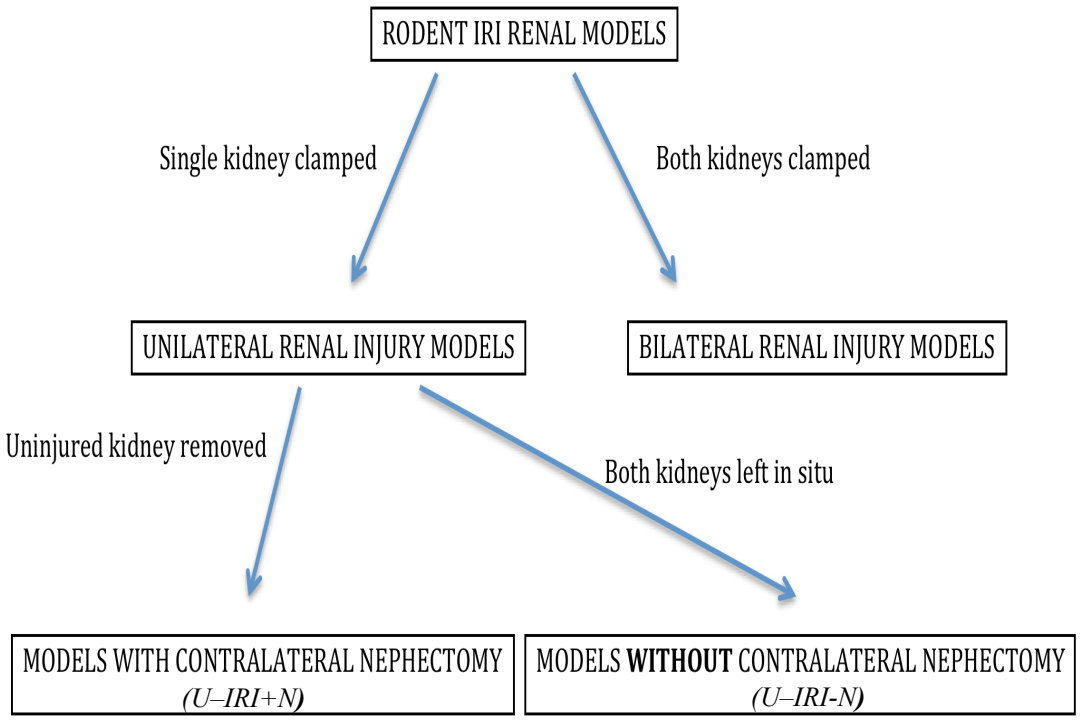


Figure 4.1: Commonly used rodent IRI models.

U-IRI may be further divided into models with or without a contralateral nephrectomy (Le Clef et al., 2016, Wei and Dong, 2012). Models using B-IRI or U-IRI with contralateral nephrectomy are the most commonly studied, probably because blood analysis may demonstrate trends in renal function throughout the experimental time course (Skrypnyk et al., 2013).

However, the metabolic and electrolyte disturbances resulting from a severe and prolonged acute kidney injury may lead to unacceptable numbers of postoperative animal deaths (Wang et al., 2012) (Jang et al., 2009) usually within 48 hours of surgery (Skrypnyk et al., 2013). As post-reperfusion renal function is known to be closely related to warm ischemic time, it is important to strike the correct balance between an acceptable number of post-procedure animal deaths and an ischemic period long enough to cause a permanent renal injury (Zager et al., 2011, Zager et al., 2013). Such a deficit in kidney function is necessary to demonstrate the long-term effects of novel therapies on renal recovery.

Most studies examining the use of renal IRI in rats report ischemic times of between 30 – 60 minutes (Le Clef et al., 2016, Wang et al., 2012, Singh et al., 2012, Jo et al., 2001, Jablonski et al., 1983, Nemoto et al., 2001, Ysebaert et al., 2000). Unfortunately, the majority of these experiments are concluded within a week of surgery and only a few studies applied to renal IRI report the long-term sequelae of an acute renal insult (Delbridge et al., 2007, Gueler et al., 2004, Kim and Padanilam, 2015). This may reflect the difficulty encountered when attempting to inflict a permanent renal injury using IRI without suffering unacceptable numbers of post surgical deaths from acute renal failure. For example, serum creatinine levels after 60 minutes of bilateral renal ischemia have been reported to return to sham levels after 7 days recovery and remain stable for 40 weeks subsequently (Basile et al., 2001). This duration of bilateral ischemia has been reported subsequently to result in ~ 50% mortality within 30 days post surgery (Wang et al., 2012).

Most IRI experiments are preformed using healthy young rodents. Clearly this does not mimic the clinical situation where organs from older donors are increasing used. The AS/AGU rat substrain displays premature renal dysfunction (Gingell-Littlejohn, 2014) and consequently may be a useful model in which to perform renal IRI experiments. Unfortunately, the available colony of AS/AGU rats was not large enough to power the planned IRI experiments, and so the Albino-Swiss strain was chosen for use in this experiment.

After review of the literature, it was decided to investigate the effects of a 30-minute period of unilateral warm ischemia in Albino-Swiss rats undergoing a simultaneous contralateral nephrectomy. The pathological course of unilateral ischemia with simultaneous contralateral nephrectomy model is expected to be quite similar to bilateral ischemia in that both models leave the animal with injured renal tissue only (Le Clef et al., 2016). This allowed the use of serum creatinine to monitor gross renal function over the course of the experiment. However, in view of the potential insensitivity of serum creatinine as a marker of rodent renal function, terminal inulin studies were performed after 14 days to characterize GFR in the most accurate way possible.

Furthermore, after the negative findings associated with the use of stem cell therapy described in Chapter 3, it was decided to investigate the use extracellular vesicles harvested from Pathfinder cells, as ESVs are thought to have a reduced malignant potential (Han et al., 2016, Vishnubhatla, 2014, Rani et al., 2015).

4.2 HYPOTHESIS

(1) 14 days after undergoing 30 minutes of warm renal IRI, changes in renal function, histology and molecular markers of damage will be evident versus controls not undergoing renal IRI.
(2) Pathfinder-derived microvesicles, given intravenously, will mitigate the effects of renal IRI, resulting in improved renal function and histology, while reducing molecular markers of damage.
(3) Microvesicles lack the ability to induce teratoma formation after intravenous administration.

4.3 METHODS

4.31 EXPERIMENTAL GROUPS

24 AS rats aged between 10 - 12 months were randomly assigned to 3 different groups. There were four males and four females, for a total of 8 rats in each group.

• Group 1 – Right nephrectomy + saline vehicle via tail vein injection.

• Group 2 – Right nephrectomy + left renal ischemia + saline vehicle via tail vein injection.

• Group 3 – Right nephrectomy + left renal ischemia + pathfinder derived microvesicle therapy via tail vein injection.

Group	Nephrectomy	Ischemia	MV Therapy
1	√	x	x
2	√	√	x
3	√	√	√

Table 4.1: Summary of experimental groups. Randomly assigned to each group were four male and four female Albino Swiss Rats.

Rodent husbandry, anaesthesia, positioning during surgery and post-surgical care, including venesection were conducted in standard fashion, as previously described in Sections 2.11 – 2.17.

Microvesicles were harvested from Pathfinder cells as outlined in section 2.61. Using data from studies where intravenous Pathfinder microvesicles had achieved normoglycemia in diabetic mice, microvesicle dose was calculated per gram of rodent body weight and adjusted for rats.

4.32 SURGICAL PROCEDURE: RIGHT NEPHRECTOMY +/- LEFT RENAL ISCHEMIA

A midline incision was made through the skin and subcutaneous fat using a 15 bladed scalpel, to expose the abdominal wall musculature. The linea alba was then incised with scissors from the xiphisternum and approximately 4cm inferiorly to provide excellent access to both kidneys. (See Figure 4.2) To expose the left renal pedicle, the small and large bowel were packed away as necessary using sterile gauze swabs soaked in warm saline. In all Groups, the left renal pedicle was dissected from the renal hilum proximal to the origin of the supra-adrenal vein, using a combination of sharp and blunt dissection. The renal artery, which lies supero-posteriorly to the renal vein on the left, was not dissected free of the vein. Once dissection had been achieved, the renal pedicle was clamped en mass using an atraumatic vascular clamp (B2-V) in animals belonging to Groups 2 and 3. The pedicle was not clamped in Group 1 animals. Visual inspection of the kidney at this point was undertaken, with the universal finding of a colour change, confirming correct application of the clamp.

A right nephrectomy was then carried out. The small and large bowel were repacked to provide exposure of the right kidney. A folded swab was also placed to retract the right lobe of the liver, which was carefully detached from the vena cava using sharp dissection. The right kidney was then mobilised from the renal bed, and the right ureter ligated using 8/0 nylon before being divided. Attention was then directed towards the right renal pedicle, which was dissected, double ligated en mass with 6/0 prolene and then divided. The right kidney was then removed, and hemostasis noted.

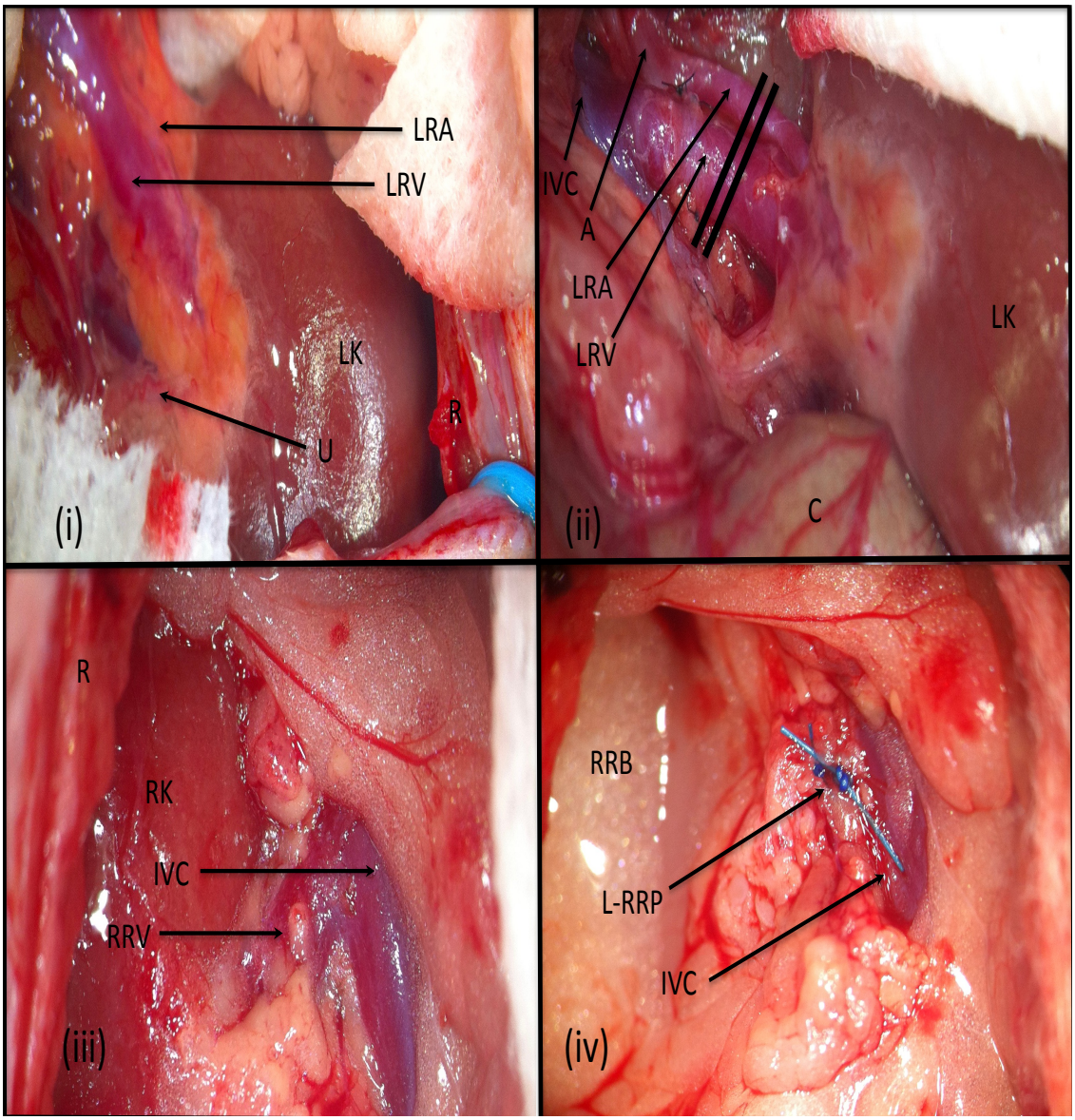


Figure 4.2: (i) Exposure of the left kidney (LK) via retraction of the rectus muscle (R). The left renal vein (LRV), left renal artery (LRA) and left ureter (U) are clearly visible. **(ii)** Extensive dissection of the left renal pedicle, to display relationship of relevant local anatomy. The inferior vena cava (IVC), aorta (A), left colon (C) are easily seen, as are the LRV and LRA. The vascular clamp (not pictured) was universally applied at the level of the double black line, just distal to the origin of the left gonadal vein. **(iii)** Retraction of the right rectus (R) to expose the right kidney (RK). The IVC and right renal vein (RRV) are seen. The right renal artery runs posterior to the RRV and is not visible. **(iv)** The right renal bed (RRB) after right nephrectomy. The doubly ligated right renal pedicle (L-RRP) is clearly seen.

In groups undergoing ischemia, the clamp was removed from the left renal pedicle, to provide exactly 30 minutes of warm ischemia.

The abdomen was closed en mass using a continuous 4/0 vicryl suture, with a subcuticular 4/0 un-dyed suture for the skin edges.

Groups 1 and two were given 800µl of 0.9% saline vehicle, intravenously via tail vein injection using a 30G insulin syringe. Group 3 animals received 800µl intravenous MV therapy via tail vein injection. Tail vein injection was given after abdominal closure, typically 20 minutes after unclamping of the renal pedicle.

Blood was taken from each animal for creatinine levels at days 2, 4, 7 and 10.

At day 14, the animals underwent terminal inulin clearance studies in order to accurately characterise the GFR of the remaining kidney, according to the standard protocol described in Section 2.2. After the animal was sacrificed, left kidneys were stored in 10% formalin and RNA later solution for later analysis.

Formalin preserved kidneys were paraffin embedded and sectioned as described in Section 2.3. Histological analysis and immunohistochemistry for P16, P21 and Ki 67 were carried out as described in Sections 2.3 and 2.4

4.4 RESULTS

4.41 GROUP WEIGHT LOSS COMPARED TO BASELINE WEIGHT

As shown in Figure 4.3 all animals suffered significant weight loss in response to the trauma of surgery. By day 14 animals undergoing nephrectomy alone (Group 1) had regained their baseline weight. In contrast, animals subjected to nephrectomy and ischemia (Groups 2 and 3), remained significantly below their baseline weight by Day 14.

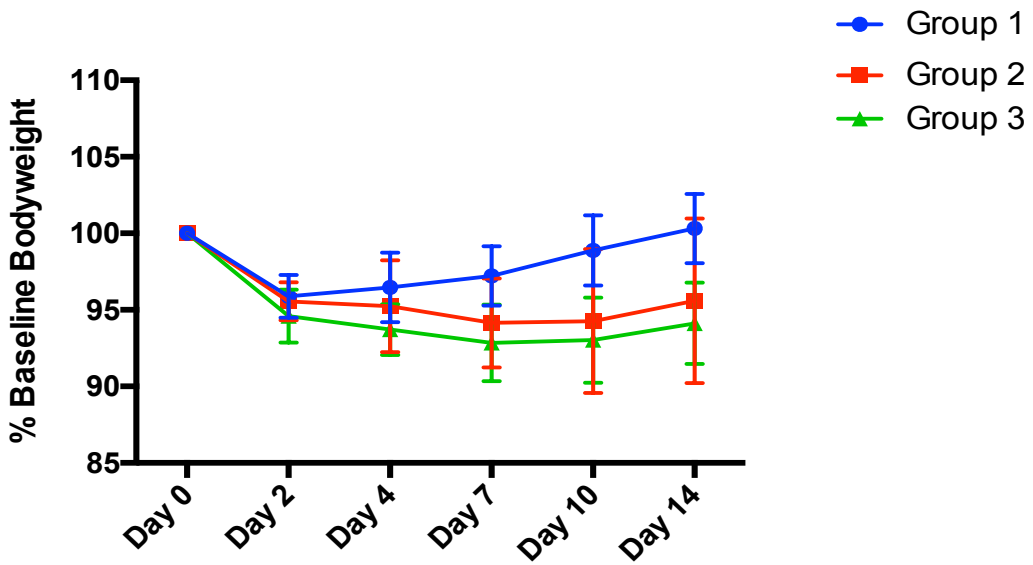


Fig 4.3: Compared to baseline, animals in Groups 1, 2 and 3 exhibited significant weight loss on Day 2 ($p = 0.0004$, 0.0003 and <0.0001 for Group 1, 2 and 3 respectively) There remained significant weight loss for all Groups on Day 4 ($p = 0.0006$, 0.0005, and <0.0001 respectively), Day 7 ($p = 0.0011$, <0.0001, and <0.0001) and day 10 ($p = 0.0006$, 0.0039 and <0.0001)

By day 14, animals undergoing nephrectomy alone (Group 1) had regained baseline weight ($p = 0.6980$). In contrast, animals subjected to nephrectomy and ischemia (Groups 2 and 3) failed regained their baseline weight by day 14 ($p = 0.0358$ and <0.0001 respectively).

Statistical analysis by unpaired Students t test.

4.42 COMPARISION OF PERCENTAGE WEIGHT LOSS FROM BASELINE BETWEEN GROUPS

Animals undergoing nephrectomy alone (Group 1) regained weight more quickly than animals subjected to nephrectomy and ischemia (Groups 2 and 3). No significant difference was observed at any point in weight loss compared to baseline, between Group 2 and Group 3. (Figures 4.4)

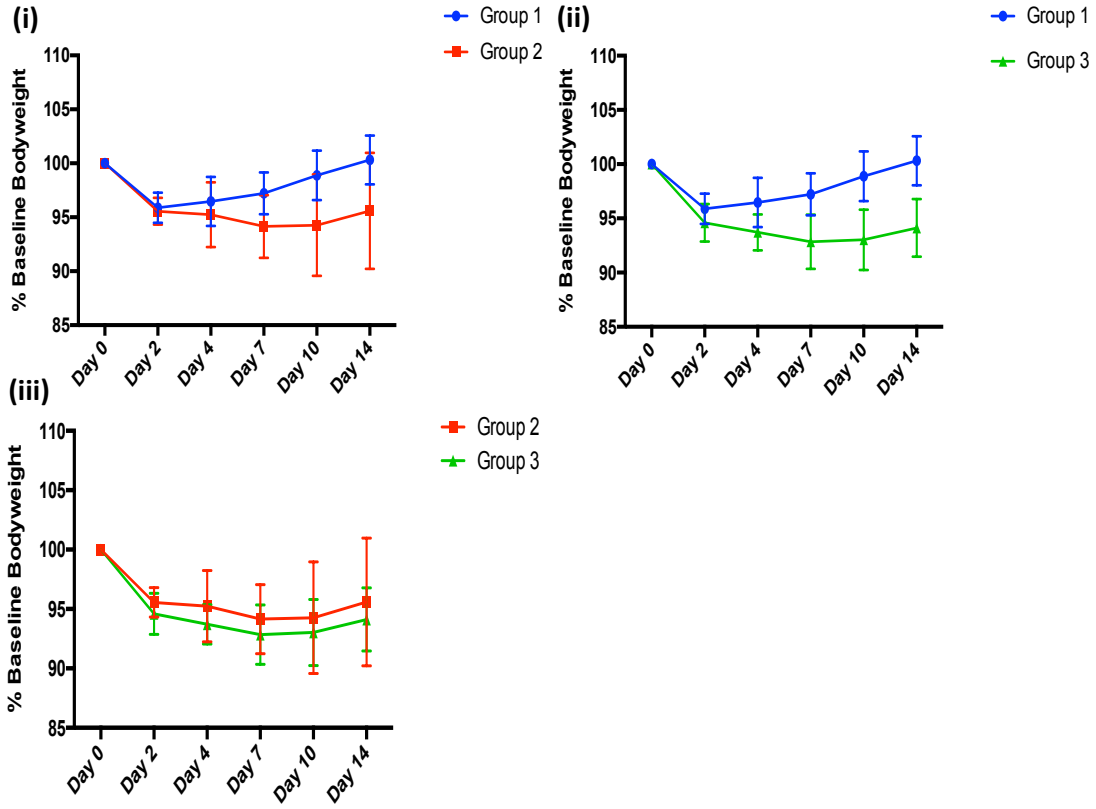


Figure 4.4(i) Group 1 animals were found to have lost significantly less of their baseline body weight at day 7 (p =0.0259), day 10 (p =, 0.0253) and day 14 (p= 0.0378) when compared to Group 2 animals. **(ii)** Group 1 animals lost significantly less of their baseline body weight compared to Group 3 animals at day 4 (p=0.0149), Day 7 (p=0.0015), Day 10 (p=0.0004) and Day 14 (p=0.0002) **(iii)** There was not significant difference in baseline body weight lost at any time point when comparing Groups 2 and 3. Statistical analysis by one-way ANOVA with Tukey multiple comparisons test

4.43 SERUM CREATININE LEVELS COMPARED TO BASELINE VALUES

After surgery, all groups displayed significantly elevated serum creatinine levels when compared to baseline for the duration of the experiment. (Figure 4.5).

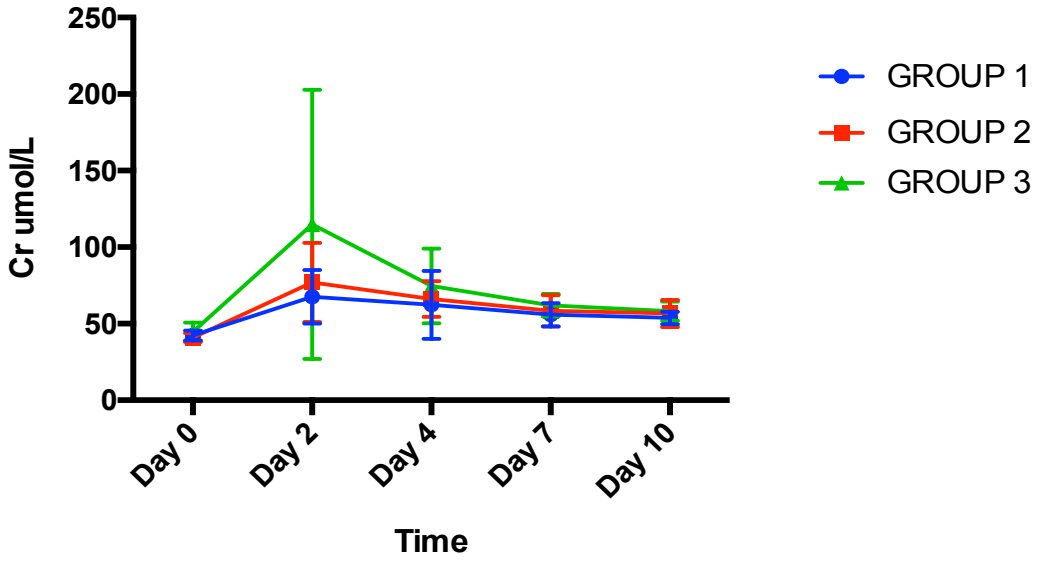


Figure 4.5: All groups displayed significantly elevated serum creatinine from baseline ($p<0.0001$), at all the experimental time points of Day 2, Day 4, Day 7 and Day 10.
Statistical analysis by one-way ANOVA with Tukey multiple comparisons test.

4.44 COMPARISION OF SERUM CREATININE LEVELS BETWEEN GROUPS

No significant difference in serum creatinine level was observed between any groups at any time point. (Figure 4.6).

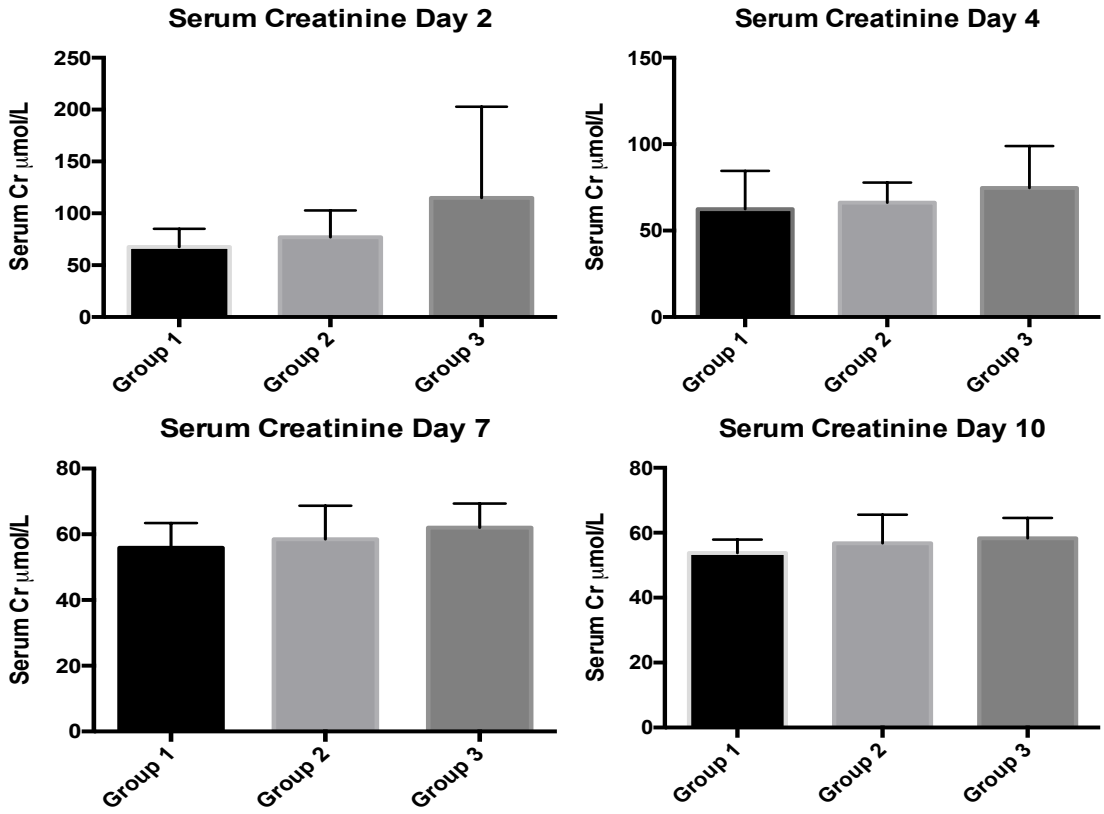


Figure 4.6: At all time points, Group 1 was found to have no significant difference in serum creatinine when compared to Group 2 (p = 0.9350, 0.9270, 0.8110 and 0.6478 at days 2, 4, 7 and 10 respectively) or Group 3 (p = 0.2093, 0.4583, 0.3365 and 0.3863 at days 2, 4, 7 and 10 respectively).
No significant difference in serum creatinine level between Group 2 and Group 3 was observed at any time point (p= 0.3359, 0.6815, 0.6912 and 0.8955 at days 2, 4, 7 and ten respectively). Statistical analysis by one-way ANOVA with Tukey multiple comparisons test.

4.45 GLOMERULAR FILTRATION RATE

All rats were culled on day 14 after undergoing terminal GFR experiments via continuous inulin infusion as described in Section 2.2.

No significant difference in GFR was observed between any of the groups. (See Figure 4.7)

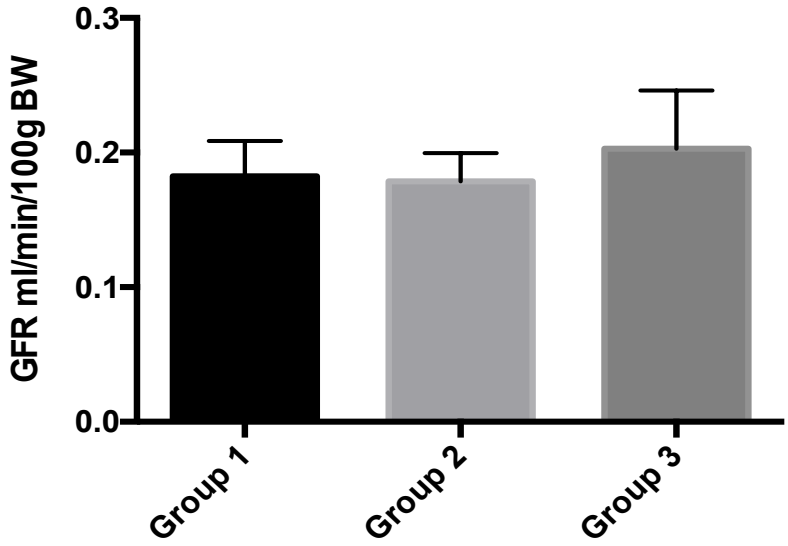


Figure 4.7: No significant differences were observed in GFRs between group 1 and 2 (p=0.9697), groups 2 and 3 (p=0.2976) and groups 1 and 3 (p=0.4143).
Statistical analysis by one-way ANOVA with Tukey multiple comparisons test.

4.46 RENAL HISTOLOGY SCORING

Histological examination of kidney tissue revealed no significant difference between Groups in either the number of epithelial breaks or hyaline casts per high-powered field. (Figure 4.8)

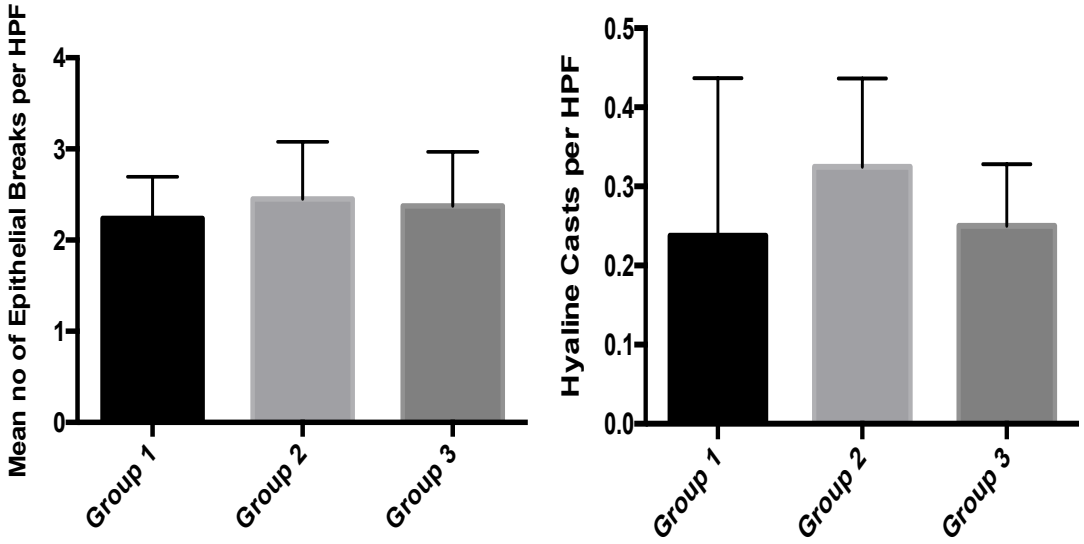


Figure 4.8: (i) No difference was observed in the number of epithelial breaks between Groups 1 and 2 (p =0.7353) or Groups 1 and 3 (p=0.8782). No difference in epithelial break formation was observed between Groups 2 and 3. (p=0.9619) **(ii)** No difference in hyaline cast formation was observed between Groups 1 and 2 (p=0.7663) or Groups 1 and 3 (p=0.9945). No difference in hyaline cast formation was found between Group 2 and 3 (p=0.8218)

Statistical analysis by one-way ANOVA with Tukey multiple comparisons test.

4.47 IMMUNOHISTOCHEMISTRY DATA

Histoscores for p16, p21 and Ki 67 were calculated by 2-blinded observers as described in Section 2.47.

No significant difference between groups was found for p16, p21 or Ki 67 (Figure 4.9).

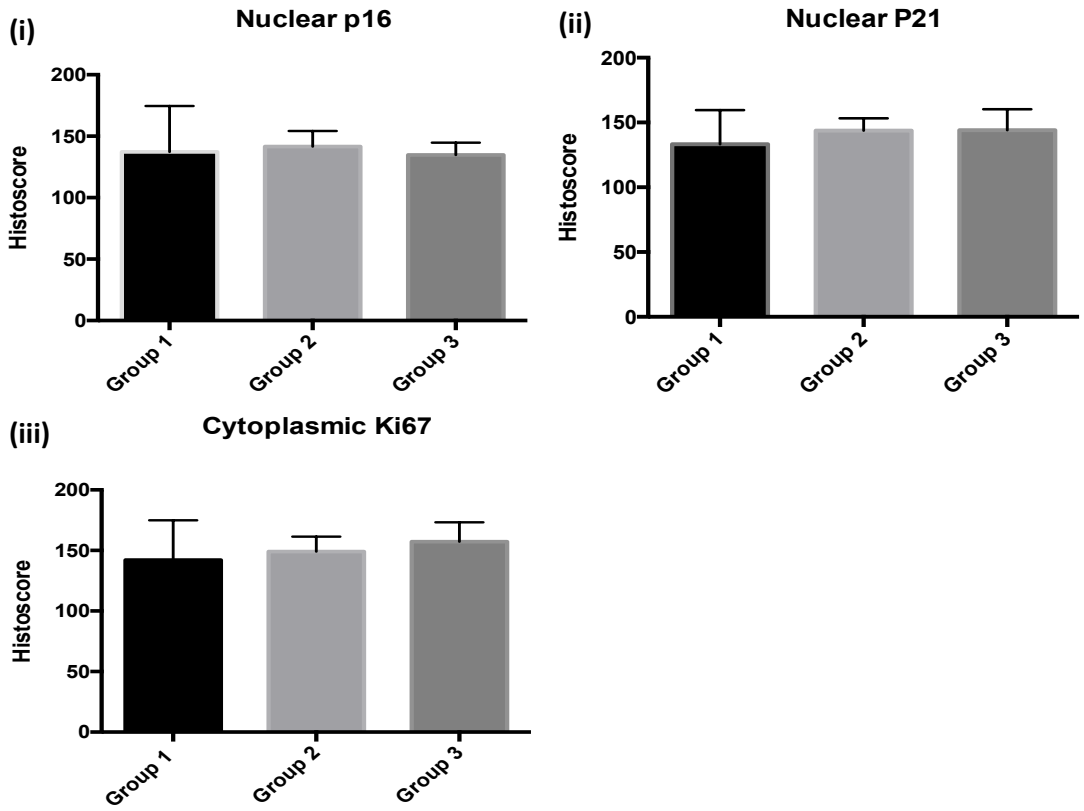


Figure 4.9: (i) No significant difference in cortical nuclear p16 expression was found between Group 1 and 2 (p=0.9644) or Groups 1 and 3 (p=0.9882). No difference was found between Groups 2 and 3 (p=0.9146). **(ii)** No significant difference in cortical nuclear p21 expression was found between Group 1 and 2 (p=0.8139) or Groups 1 and 3 (p=0.8060). No difference was found between Groups 2 and 3 (p=0.9999). **(iii)** No significant difference in cortical nuclear Ki67 expression was found between Group 1 and 2 (p=0.8313) or Groups 1 and 3 (p=0.4160. No difference was found between Groups 2 and 3 (p=0.7611).

Statistical analysis by one-way ANOVA with Tukey multiple comparisons test.

4.5 DISCUSSION

Home Office Legislation in the UK regarding animal work requires intra-experiment animal pain and post-procedural animal mortality to be kept to a minimum. Using conventional animal models, long periods of renal ischemia are therefore effectively prohibited as severe acute renal dysfunction is likely to cause severe metabolic and electrolyte disturbances that result in excessive numbers of post-procedural animal deaths (Skrypnyk et al., 2013, Wang et al., 2012).

The unilateral renal ischemia with contralateral nephrectomy rodent model has been utilised in a number of studies, although ischemic times applied to the remaining kidney have varied from study to study. Results from these studies indicate acceptable animal mortality rates with shorter periods of ischemia, but with increased mortality as ischemic times lengthen (Wang et al., 2012, Le Clef et al., 2016). Furthermore, the magnitude and duration of renal injury are clearly related to the length of the ischemic period (Grigoryev et al., 2008) with healthy rodents exhibiting considerable renal regeneration after ischemic renal injury (Ysebaert et al., 2000, Forbes et al., 2000). However, 30 - 60 minutes of ischemia has previously been reported in some studies to result in a long-standing functional renal deficit in rats, with associated changes in renal histology and increased molecular markers of injury (Gatti et al., 2011, Cantaluppi et al., 2012, Shih et al., 2013, Feng et al., 2010b, Cai et al., 2014, Chen et al., 2011).

30 minutes of ischemia was chosen for this experiment in the belief that this would produce a long-standing deficit in renal function with concomitant changes in renal histology and molecular markers of damage that would be detectable 14 days after injury occurring. By establishing a long-term renal injury in animals subjected to nephrectomy and ischemia, it was intended to investigate the protective and regenerative effects of stem cell derived ESV therapy. Also, it was hoped that animal mortality would be kept within the confines of the Home Office Project License.

Unfortunately, it is clear from this study in Albino Swiss rats undergoing a contralateral nephrectomy, 30 minutes of warm ischemia to the remaining kidney is not enough to produce a renal injury that is evident after 14 days recovery. While

body weight in animals undergoing nephrectomy alone (Group 1) recovered to baseline more quickly than those animals subjected to nephrectomy and ischemia (Groups 2 and 3), no markers of renal injury were significantly different between Group 1 and Group 2 after 14 days recovery and the GFR between groups was not statistically different.

Serum creatinine levels did not vary significantly between the Groups at any time. It may be that 30 minutes of warm unilateral ischemia in the Albino Swiss strain is not enough to produce an acute kidney injury resulting in renal dysfunction. However, another explanation is that serum creatinine lacks the sensitivity to accurately characterise renal function in rodents, especially when dysfunction is kept to a minimum (Finco and Duncan, 1976, Harvey and Malvin, 1965, Katayama et al., 2010, Meyer et al., 1985). This would explain the apparent lack of difference in serum creatine between Groups 1 and 2 during the initial post-operative period.

By day 14 the lack of significant difference between Groups in any of the functional, histological or molecular markers of damage, is an indication that 30 minutes of ischemia is not enough to cause a permanent renal injury in the Albino Swiss rat strain. The lack of injury negates the ability of such a model to detect any protective or regenerative effects of cellular therapy on the renal system, as these results suggest a healthy Albino Swiss rat has the ability to recover from a 30-minute period of ischemia within 14 days of ischemia occurring.

Consequently, it seemed impossible to continue to use this or similar models to investigate the long-term effects of stem cell-based therapies at mitigating renal ischemia. It was felt that longer periods of renal ischemia were necessary to produce a permanent renal injury, but that this would not be possible in conventional models without breaching the terms of the Home Office Project License. The natural conclusion was that in order to better test the medium and long-term effects of stem cell based therapies in rodents, a novel model was required.

CHAPTER 5

DEVELOPMENT OF A NOVEL RAT MODEL OF SEVERE RENAL ISCHEMIA-REPERFUSION INJURY

5.1 INTRODUCTION

Despite considerable research efforts to develop novel anti-ischemic compounds to combat renal IRI, there are no protective or regenerative agents in clinical use and supportive therapy remains the mainstay of treatment (Chatterjee, 2007, Chatterjee and Thiemermann, 2003).

The dearth of novel therapies in part reflects the lack of suitable animal models in which new medicines can be tested. The salient finding of Chapter 4 was the difficulty encountered when subjecting rats to an ischemic insult that causes a measurable permanent reduction in renal function without unacceptable numbers of animals dying first from acute kidney failure. Establishing a state of chronic renal failure is a necessary first step in order to investigate the long-term efficacy of emerging treatments (Lieberthal and Nigam, 2000).

The majority of renal IRI experiments have been conducted in rodents, which are considered favourable to larger animals due to their relatively low cost and wide availability (Ortiz et al., 2015). The most commonly studied rodent models utilise either bilateral renal ischemia (B-IRI), or unilateral ischemia (U-IRI) after performing a contralateral nephrectomy (U-IRI+N) (Wei and Dong, 2012). Serum creatinine and BUN are almost universally used in such models to monitor renal function (Wei and Dong, 2012). In both of these model types, ischemic times of 30 – 60 minutes have been shown to cause acute renal dysfunction (Jo et al., 2001, Nemoto et al., 2001, Ysebaert et al., 2000, Jablonski et al., 1983) although studies indicate this usually resolves within a week of surgery (Forbes et al., 2000, Ysebaert et al., 2000, Jablonski et al., 1983). Longer periods of warm ischemia are associated with unacceptable numbers of post-procedure animal deaths, often within 48 hours as a result of acute renal failure (Zager, 1987, Zager, 1991).

The majority of studies investigating the effects of novel therapies on renal IRI only report outcomes during the first few post-operative days, by which time rodent serum creatinine typically approaches baseline. Although a few longer-term studies have been published, most reports indicate that injured control kidneys return to a state of normal morphology within two weeks of injury. Serum creatinine and BUN levels

normalise by two weeks post procedure, remaining stable 40 weeks later indicating no long-term functional decay (Basile et al., 2001, Horbelt et al., 2007).

This highlights the shortcomings of conventional rodent models of renal IRI – namely the paradox of inflicting a severe enough injury to cause permanent renal damage while avoiding unacceptable numbers of post procedure animal deaths (Skrypnyk et al., 2013). Using conventional rodent models, it therefore appears difficult to inflict a longstanding renal injury, as rodent kidneys seem to regenerate to function normally after non-fatal periods of renal ischemia. The benefit of testing the long-term effects of novel therapies in conventional rodent models is therefore questionable (Wang et al., 2012).

U-IRI without contralateral nephrectomy (U-IRI-N) leaves animals with a healthy functioning kidney that serve as protection against acute renal failure and death (Zager et al., 2011). Consequently, such models allow the study of prolonged ischemic times well beyond the typical first few days of acute renal injury (Lech et al., 2009), with animals subjected to prolonged ischemia eventually developing chronic renal failure (Ascon et al., 2009). Ischemic times of up to in 60 minutes in mice (Adachi et al., 2013) and 190 minutes in rats (Craddock, 1976) have been reported. Such severe injury more closely resembles the nephropathology observed in the clinical setting (Salahudeen, 2004). However, U-IRI-N models do not allow functional elements of the ischemic injury to be studied using serial blood analysis, as renal function markers in such models are affected by the filtration of the uninjured kidney (Skrypnyk et al., 2013).

Nevertheless, while serial blood analysis may be useful to map renal function trends during the experimental time course, in rodents the accuracy of serum creatinine and BUN in assessing renal function is of dubious significance (Finco and Duncan, 1976, Harvey and Malvin, 1965, Katayama et al., 2010, Meyer et al., 1985, Namnum et al., 1983). With blood analysis unable to accurately determine both short and long-term rodent renal function in IRI experiments, any conclusion regarding the quantified efficacy of therapies based on blood analysis must be questionable, and more accurate methods of demonstrating renal function should be employed.

5.11 QUANTIFICATION OF RENAL FUNCTION

As described in Section 2.2, GFR as determined by inulin clearance is widely regarded as the most reliable and accurate method of quantifying renal function. Using inulin clearance, split renal function has been measured in dogs (Tsuji et al., 1993), pigs (Downey et al., 2001) and rats (Malis et al., 1983, Kassab et al., 2001) by separate cannulation of each ureter and accurate urine collection. Unfortunately, the size constraints when using a murine model make ureteric cannulation impractical. However, in rat models of U-IRI-N, inulin clearance with ureteric cannulation offers the most accurate method of quantifying split renal response to injury +/- intervention.

5.12 ROUTE OF DRUG DELIVERY

The optimum route of administration for stem cell therapy has yet to be clarified. Novel stem cell therapies have typically been delivered systemically during animal experiments investigating the mitigation of renal IRI. The most common route of systemic administration has been by intravenous injection, although other studies have given intra-aortic treatment via carotid cannulation (Feng et al., 2010b). However, while systemic delivery methods have been shown to mitigate renal ischemia, this requires larger numbers of cells to produce efficacy than with local administration (Shih et al., 2013). Moreover, systemic distribution results in the majority of administered cells being filtered by the pulmonary circulation (Fischer et al., 2009, Burst et al., 2010). This has been shown to produce undesirable effects such as pulmonary damage (Anjos-Afonso et al., 2004) and pulmonary vascular occlusion (Shih et al., 2013).

Direct renal intra-parenchymal injection of stem cells has been demonstrated to be an efficient route (Chen et al., 2011) and furthermore may increase the retention and survival of administered cells. However, a direct intra-parenchymal injection may result in local storage of stem cells and ultimately lead to renal damage (Mias et al., 2008).

Administration of therapy into the renal artery is the likely route of drug administration in clinical transplantation, where the availability of the renal artery allows easy *ex vivo* delivery prior to implantation. Intra-renal artery therapy injection

theoretically allows high local concentration of treatment without the potential side effects of systemic distribution. One study in an allogeneic rat renal transplant model has reported that intra-renal artery infusion of MSCs was superior to MSC treatment by intravenous injection for the prevention of acute rejection (Zonta et al., 2010).

However, in rodents, this is a technically demanding procedure requiring fluoroscopically guided renal artery catheterization in animal models where the renal artery has not been opened, or a direct arterial injection with subsequent vascular repair to prevent catastrophic haemorrhage if the artery is available. Nevertheless, to mimic the transplantation process as closely as possible, novel rodent models utilising intra-renal artery infusion of stem cell therapies are potentially of relevance.

5.2 HYPOTHESIS

(1) Rats subjected to 120 minutes of unilateral warm renal ischemia will exhibit long-term functional deficit in function of the damaged kidney, together with abnormal renal histology and elevated molecular markers of injury.

(2) The presence of a healthy contralateral kidney will reduce to an acceptable level the post procedure animal deaths that result from metabolic derangement as a result of acute renal dysfunction.

(3) Direct intra-renal artery injection of therapy is the route most likely employed during clinical transplantation. Animal models developed to investigate novel therapies should utilise this route.

5.3 METHODS

5.31 ANIMAL HOUSING AND HUSBANDRY

Male Fisher 344 rats, aged 12 - 14 weeks and weighing 225 – 250g were sourced from Harlan UK Ltd. Animals were housed in the JRF, University of Glasgow for at least two weeks prior to surgery to allow the animals to equilibrate with their new environment. Animals were housed and fed as described in Section 2.1. During equilibration prior to surgery, animals put on weight such that on the day of surgery, rats weighing 250 – 320g were used.

Post surgery, animals were weighed weekly and assessed on a daily basis for signs of distress or illness.

5.32 GROUP SELECTION

Animals were randomly assigned to sham operation (Group 1), 120 minutes of left renal IRI with two weeks recovery (Group 2), or 120 minutes of left renal IRI with six weeks recovery (Group 3). (See Table 5.1)

Group	120 minutes left renal ischemia	Recovery prior to Inulin Clearance	Intra-renal artery flush
1	X	2 weeks	None
2	√	2 weeks	Normal saline
3	√	6 weeks	Normal saline

Table 5.1: Summary of experimental groups. Six rats were randomly assigned to each group.

5.33 SURGICAL PROCEDURE

Rodent anaesthesia, positioning during surgery and post-surgical care, were conducted in standard fashion, as previously described in Sections 2.12 – 2.16.

Surgical Procedure: A high midline incision was made. The small bowel was wrapped in damp swabs, and retractors placed to enable access to the left renal pedicle. (Fig 5.1a) Mobilisation of the left colon was achieved by incising the lateral peritoneal attachments, thereby exposing the renal vasculature. The renal artery was separated from the renal vein using blunt dissection with cotton tips and/or forceps, to fully expose the length of the renal artery from its origin on the aorta to the renal hilum. (Fig 5.1b) This necessitated tying of the suprarenal vein. Once exposed, an atraumatic vascular clamp was placed on the on the artery, close to its junction on the aorta, signifying the start of the ischaemic period. A colour change in the kidney was quickly visible, confirming correct placement of the clamp.

Group 1 (sham operated animals) underwent dissection of the renal pedicle, but no clamping of the renal artery.

After 90 minutes of ischemia, an arteriotomy was made, and the kidney flushed with a 0.8ml intra-arterial injection of normal saline given via a 30G Ryecroft cannula. To facilitate this, a thin rubber sloop was placed around the cannula at the point of insertion and tension applied to prevent back-leak while an assistant gently injected the contents of the syringe. A colour change in the cortex of the kidney was universally observed, confirming infiltration of the kidney parenchyma with saline vehicle. (Fig 5.1d)

The artery was fully transected, then re-anastomosed in a standardised triangulated fashion, using 12 interrupted 10/0 nylon sutures. (Fig 5.1f-j) A frame clamp was used to secure the arterial ends during anastomosis, and background material placed to prevent damage to posterior structures. (See fig 5.1e) The arterial clamps were removed after exactly 120 minutes and reperfusion of the kidney confirmed visually.

Wound Closure: The abdominal musculature was closed en mass using a continuous 4/0 vicryl suture, with a subcuticular 4/0 suture applied to close skin edges. Using this closure, there were no wound infections or dehiscence, and only a single episode of an animal gnawing its skin sutures.

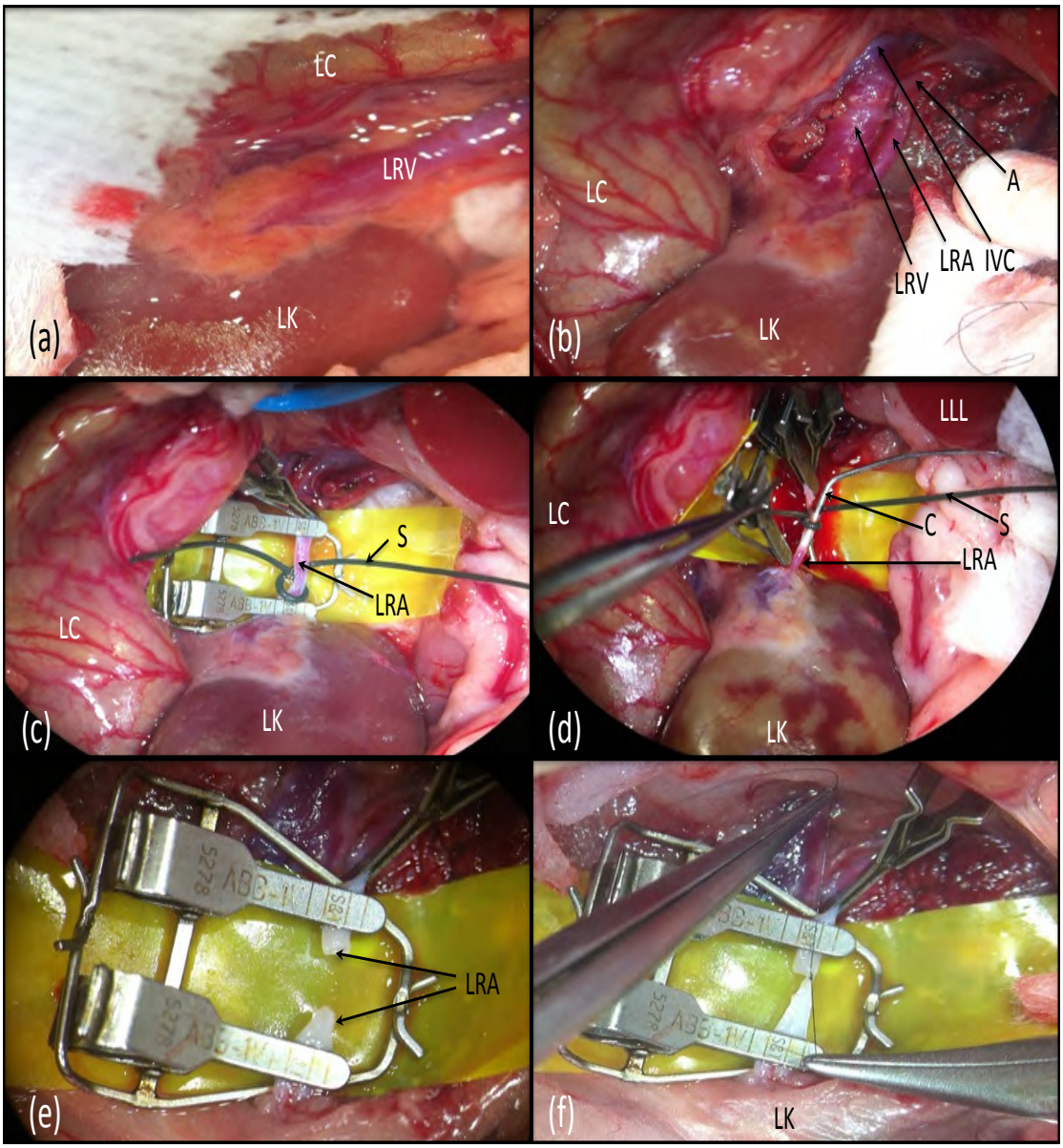


Figure 5.1: (a) View of the left renal pedicle before mobilisation of the left colon. **(b)** View after mobilising the left colon and dissection of the renal artery to its origin on the aorta. **(c)** Preparation of renal artery for intra-arterial injection. Background material has been inserted to protect posterior structures. **(d)** View during intra-arterial injection. Note colour change of renal cortex during perfusion. **(e)** Transected artery in frame clamp, ready for anastomosis. **(f)** Insertion of stay suture into the renal artery.

LC = left colon: LK = left kidney: LRV = left renal vein: RA = renal artery: IVC = inferior vena cava: A = aorta: LLL = left lobe of liver: S = rubber sloop: C = 30G cannula.

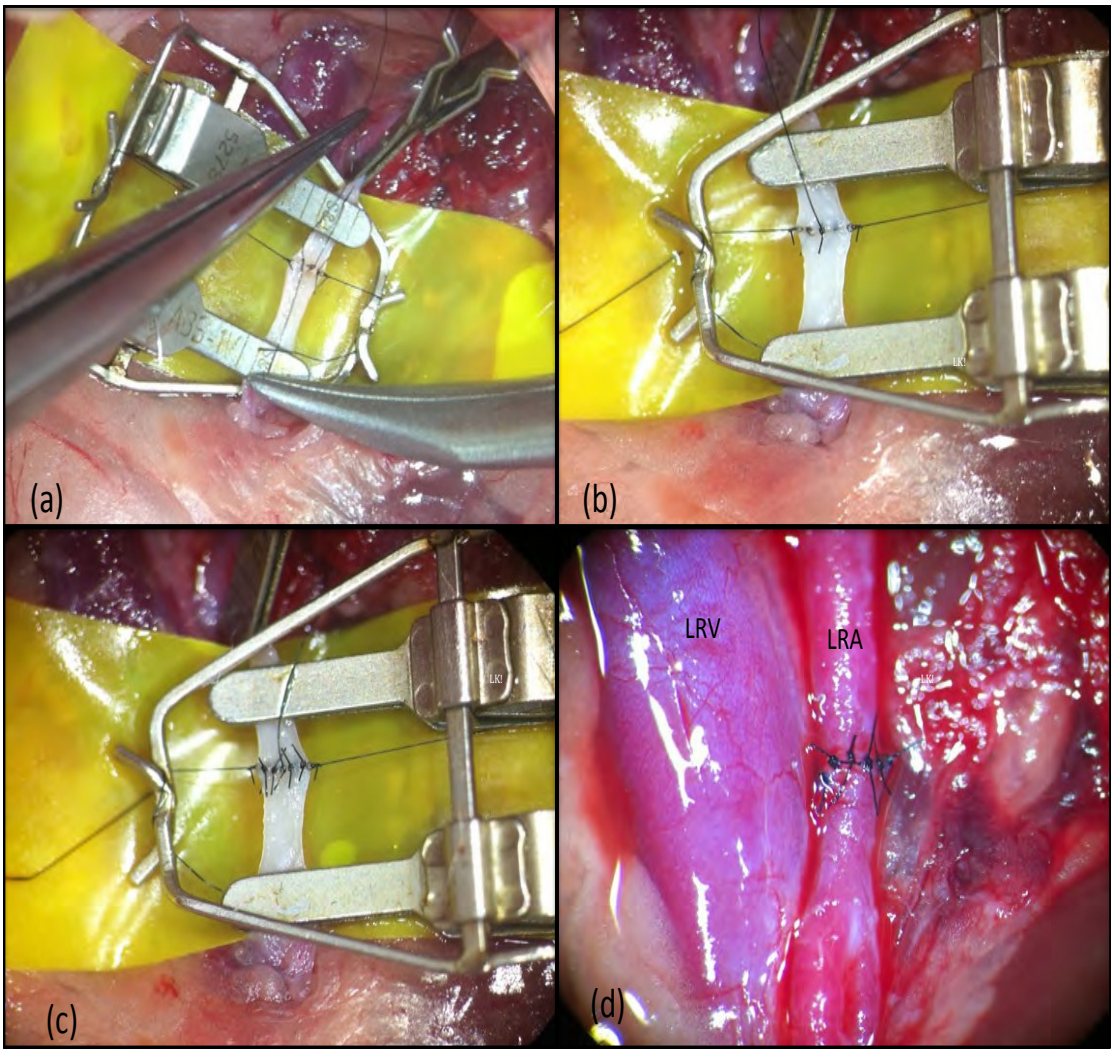


Figure 5.1(g) Completion of front arterial wall anastomosis. **(h)** The sixth suture inserted into the middle of the back wall. **(i)** Completed anastomosis prior to clamp removal**. (j)** Finished anastomosis post clamp removal. Note renal perfusion and bulging artery downstream of anastomosis indicating technical success.

5.34 POST-SURGICAL CARE

This was carried out in standard fashion as described in Section 2.16.

5.35 QUANTIFICATION OF RENAL FUNCTION AND RENAL INJURY

At day 14, Group 1 and Group 2 animals underwent inulin clearance studies to accurately characterise the function of the left kidney, according to the standard protocol described in Section 2.2. Group 3 animals underwent inulin clearance studies six weeks after ischemia, again according to standard protocol.
Left kidneys and right kidneys were weighed, before being stored in 10% formalin and RNA later solution for later analysis.

Formalin preserved kidneys were paraffin embedded and sectioned as described in Section 2.3 Histological analysis was carried out as described in Sections 2.3 and 2.4

5.4 RESULTS

5.41 POST-PROCEDURE MORTALITY RATES AND TECHNICAL COMPLICATIONS

In the 18 rats subjected to 120 minutes of unilateral warm renal ischemia, (and subsequently in more than 60 animals used to test stem cell-based therapies) there was only one post-procedure death.

Furthermore, there was one failed attempt to successfully anastomose the renal artery, indicating that for a trained microsurgeon, successful anastomosis of vessels ~ 0.6mm is technically feasible.

5.42 GROUP WEIGHT LOSS COMPARED TO BASELINE WEIGHT

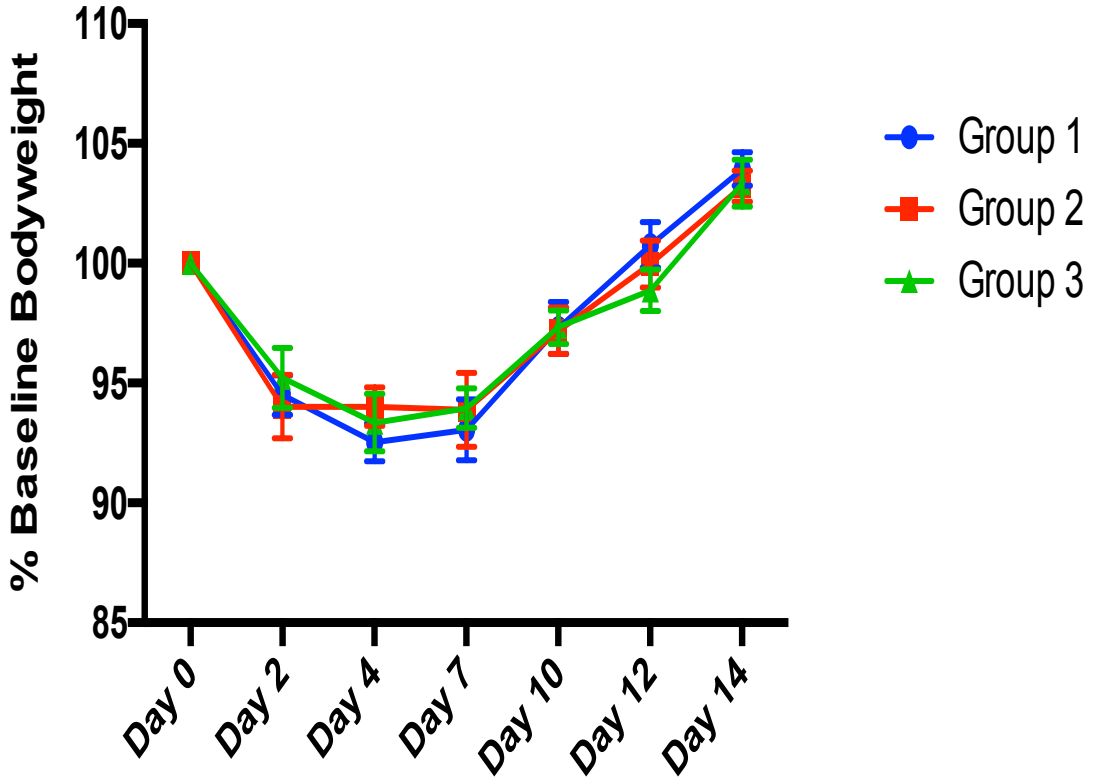


Fig 5.2: Compared to baseline, animals in Groups 1, 2 and 3 exhibited significant weight loss on Day 2 ($p < 0.0001$, 0.0011 and 0.0033 for Group 1, 2 and 3 respectively) There remained significant weight loss for all Groups on Day 4 ($p < 0.0001$, < 0.0001, and 0.0002 respectively), Day 7 ($p = 0.0003$, 0.0027, and 0.0001) and day 10 ($p = 0.0311$, 0.0157 and 0.0033). By day 12 animals weights in all groups had returned to baseline ($p = 0.4394$, 0.9734 and 0.2189 respectively).

By day 14, animals in all groups had put on weight in comparison to baseline ($p = 0.0002$, 0.0006 and 0.0066 respectively).

Statistical analysis by unpaired Student's t test.

5.43 COMPARISION OF PERCENTAGE WEIGHT LOSS FROM BASELINE BETWEEN GROUPS

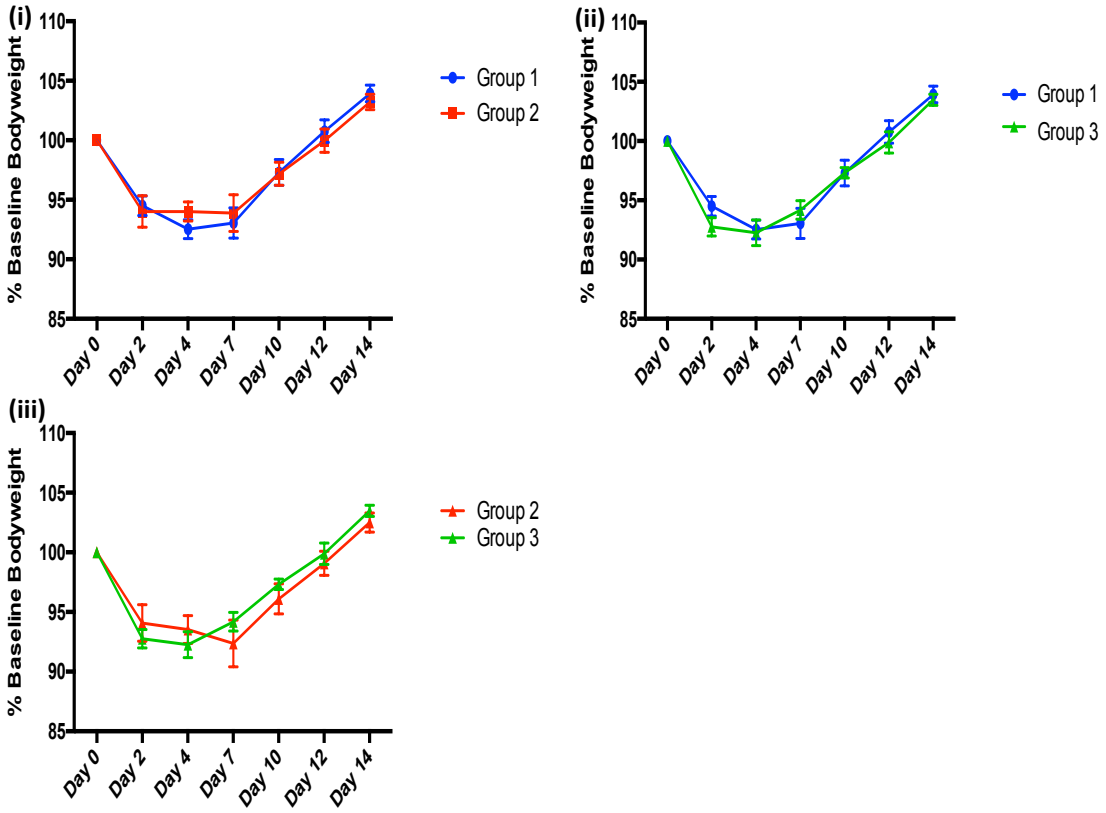


Figure 5.3(i) At no time point were Group 1 animals were found to have a significant weight difference as % of baseline weight, when compared to Group 2 animals. **(ii)** At no time point were Group 1 animals were found to have a significant weight difference as % of baseline weight when compared to Group 3 animals. **(iii)** At no time point were Group 2 animals were found to have a significant weight difference as % of baseline weight when compared to Group 3 animals. Statistical analysis by one-way ANOVA with Tukey multiple comparisons test.

5.44 GLOMERULAR FILTRATION RATE

120 minutes of renal ischemia was found to produce a permanent, severe and reproducible injury that resulted in a long-term GFR deficit. (See figure 5.4).

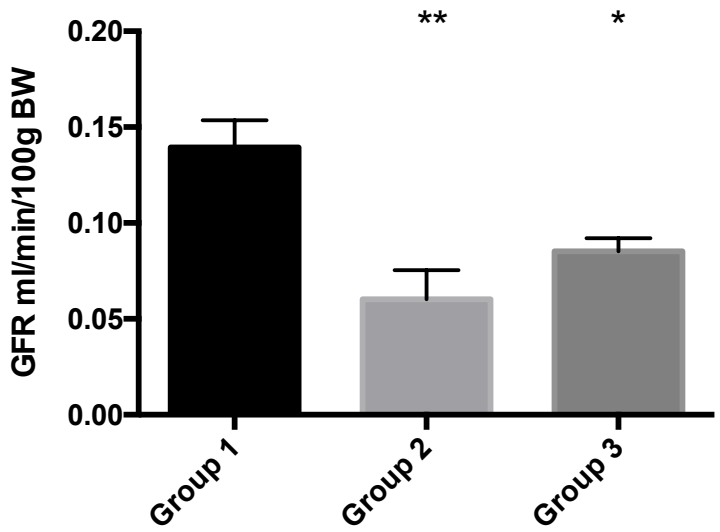


Figure 5.4: Left sided renal function in Group 1 (sham-operated), Group 2 (120 minutes IRI with two weeks recovery) and Group 3 (120 minutes IRI with six weeks recovery). Group 2 animals experience a severe reduction in GFR (~ 50%) compared to sham operated animals (p=0.0022). GFR is typically ~ 65% of sham values after six weeks recovery (p=0.0013).
Statistical analysis by one-way ANOVA with Tukey multiple comparisons test.

5.45 RENAL HISTOLOGY SCORING

Histological examination of kidney tissues as described above reveals significant disruption in renal architecture in those animals subjected to 120 minutes of warm ischemia. (See figure 5.5)

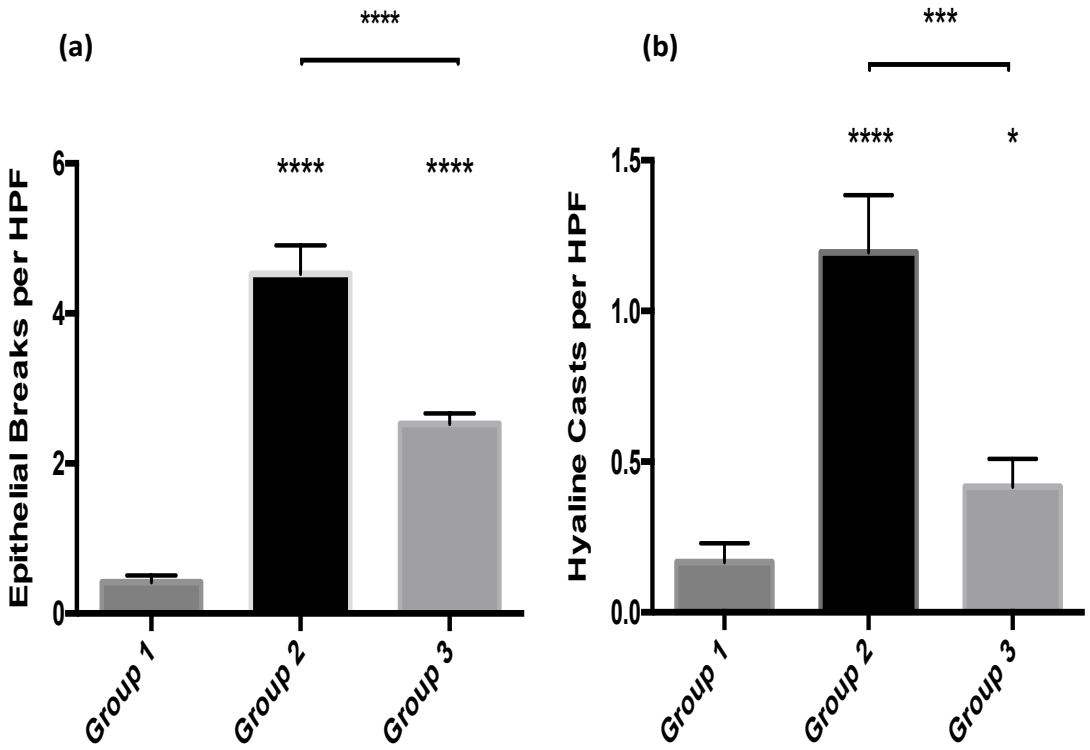


Figure 5.5: (a) Renal histology shows that Group 2 animals exhibit significantly more epithelial breaks than Group 1 animals ($p<0.0001$). Similarly, more epithelial cell breaks are observed in Group 3 animals than Group 1 ($p<0.0001$). Group 2 animals had more epithelial breaks than Group 3 animals ($p<0.0001$).

(b) More hyaline casts are observed in both Group 2 ($p<0.0001$) and Group 3 ($p=0.0285$) when compared Group 1. More hyaline casts are found in Group 2 animals compared to Group 3 animals ($p=0.0001$).

Statistical analysis by one-way ANOVA with Tukey multiple comparisons test.

5.5 DISCUSSION

Cell-based therapy has emerged as a potential treatment for acute renal injury affecting either native or transplanted kidneys. IRI is a particular problem in the field of transplantation where changing trends in organ donation have led clinicians to meet demand by utilising 'marginal' organs from the DCD category, or from older and less fit DBD donors (Schold et al., 2005, Tuttle-Newhall et al., 2009). Such kidneys, may be subjected to longer periods of warm ischemia during retrieval, and consequently are more susceptible to IRI during implantation (Port et al., 2002, Koffman and Gambaro, 2003, Metcalfe et al., 2001a, Metcalfe et al., 2001b). Developing strategies, including pharmacological interventions to improve the outcomes of these 'extended criteria' allografts is of the utmost importance.

The absence of interventions for the treatment of renal IRI reflects the lack of relevant animal models in which novel therapies may be easily studied. Many standard rodent models used to induce renal dysfunction do not rely on warm ischaemia as a mechanism of injury. These include models that utilise reduction of renal mass (5/6 nephrectomy), administration of nephrotoxins, or renal injury caused by ureteric obstruction. However, warm renal ischemia and the injury that follows reperfusion are the most common causes of renal transplant dysfunction encountered in clinical practice (Giraud et al., 2011, Jang et al., 2009). Establishing authentic animal models that mimic the pathological process of renal IRI is of vital importance in evaluating the feasibility and efficacy of novel therapeutic interventions. Unfortunately, commonly used rodent models of renal IRI lack the capacity to inflict a severe, long-standing renal injury without excessive post-operative animal deaths. To address this, Wang et al. investigated the use of prolonged warm ischemic times to create a severe and sustained renal injury model (Wang et al., 2012). They observed warm ischemic times of up to 90 minutes were associated with long-term disruption of renal architecture, increased levels of apoptosis and renal fibrosis. However, they reported prolonged ischemia resulted in almost 80% animal deaths at four weeks, although renal function as determined by serum creatinine, had normalised by this time in surviving animals.

Rodent kidneys subjected to prolonged periods of warm ischemia have been reported to display abnormal histology after several days recovery, while the serum creatinine/blood urea nitrogen (BUN) were found to be normal (Basile et al., 2001, Forbes et al., 2000, Wang et al., 2012, Chen et al., 2011, Jablonski et al., 1983, Marshall V, 1982). This paradox may be explained by the relative insensitivity creatinine and BUN at quantifying renal function in rodents (Finco and Duncan, 1976, Harvey and Malvin, 1965, Katayama et al., 2010, Meyer et al., 1985). While convenient, these surrogate markers of glomerular filtration do not become significantly elevated until 50 – 75% of kidney function is lost (Finco and Duncan, 1976). Also, creatinine secretion varies in acute renal failure. Creatinine clearance studies are reported to be more accurate than serum analysis alone, but necessitate the timed collection of urine in conjunction with serum analysis. This makes creatinine clearance an impractical tool for estimating renal function in postoperative rodents (Sturgeon et al., 1998). Furthermore, such studies have been shown to overestimate GFR by as much as 10% (Harvey and Malvin, 1965, Namnum et al., 1983).

With blood analysis unable to accurately determine both short and long-term renal function in IRI experiments, any conclusion regarding the efficacy of therapies based on such markers is questionable. To accurately quantify the effect of novel treatments, other methods of assessing renal function should be employed.

The model described here goes some way to addressing the problems encountered by Wang et al (Wang et al., 2012), and additionally has several advantages over commonly used *in vivo* models. This model can inflict a reproducible and severe renal IRI without excessive post-operative mortality. Cannulation of both ureters in a terminal procedure allows the accurate calculation the GFR of each kidney by inulin clearance. This is the most sensitive method of characterising the injury that results from a given ischemic time and also of assessing the renoprotective effect of any therapeutic intervention.

Given the severity of the ischemic insult inflicted, a major advantage of our model is the small number of post -operative animal deaths observed. This probably reflects the relative lack of metabolic disturbance that results from the filtration of the uninjured right kidney. It may be argued that this produces an artificial setting,

leaving an injured kidney to recover in a non-uraemic environment. However, a non-uraemic state after renal IRI has been reported to increase fibrosis in injured kidneys (Jablonski et al., 1985), hindering long-term renal recovery by arresting tubular epithelial cells in G2/M phase of the cell cycle (Yang et al., 2010). Indeed, one recent report hypothesised that U-IRI-N rodent models might offer greater reliability by allowing the assessment of the natural course of post-ischemic renal damage, without the anti-inflammatory effects of ischemia (Le Clef et al., 2016). There do not appear to be any studies that show U-IRI-N models to be unreliable.

Furthermore, this is one of the first descriptions of therapy given directly into the renal artery in an *in vivo* IRI animal model. Intra-renal artery infusion is a more technically challenging procedure than either intravenous injection or aortic injection via carotid cannulation, but with practice, it is possible to achieve arterial anastomotic patency rates approaching 100%. By using this route, the systemic drug distribution and the negative effects of pulmonary entrapment are minimised, while a high local concentration of therapy to the damaged kidney is provided. Importantly this method mimics the therapeutic route likely to be employed in clinical transplantation, where the renal artery is readily available for drug administration prior to implantation. This route could easily be utilised in a rodent transplant model. However, results obtained using our model are less variable as there is no need for venous and ureteric anastomosis. Hence, we can screen potential therapies' efficacy prior to their use in a more technically complex transplant model. This reduces animal numbers needed to test potential drugs for clinical transplantation and allows refinement of dosing schedules.

Finally, a recent paper using mice has described a U-IRI-N model similar to the rat model described here (Le Clef et al., 2016). That another group has identified many of the shortcomings of commonly used animal models partly validates the logic behind the development of this novel rat model. In recognition of this, findings of this Chapter were recently published (Whalen et al., 2016).

CHAPTER 6

INVESTIGATING THE RENO-PROTECTIVE EFFICACY OF SYNGENEIC CRYOPRESERVED ADIPOSE-DERIVED REGENERATIVE STEM CELLS IN A NOVEL RAT MODEL OF SEVERE RENAL ISCHEMIA-REPERFUSION INJURY

6.1 INTRODUCTION

As discussed previously, renal transplantation is established as the optimal treatment for ESRF with recognized benefits including reduced mortality, morbidity and cost when compared to remaining on dialysis (Laupacis et al., 1996, Wolfe et al., 1999, Zelmer, 2007). However, the increased demand for organs has not been matched by a concomitant rise in donation (Matas et al., 2013, Port, 2003) and clinicians have attempted to meet demand by utilizing organs from 'extended criteria donors' (ECD) or individuals who donate after circulatory death (DCD)(Port, 2003, Nathan et al., 2003). Ischemia-reperfusion injury (IRI) is magnified in these 'marginal' organs, resulting in worse short and long-term outcomes when compared to other categories of age-matched kidneys (Bagul et al., 2013, Neyrinck et al., 2013, Wadei et al., 2013).

IRI is an inevitable event during transplantation, characterized by an inflammatory reaction associated with the infiltration of monocytes and the production of reactive oxygen species (Bonventre and Yang, 2011, Eltzschig and Eckle, 2011). Furthermore, IRI is thought to be responsible for approximately 30% of delayed graft function (Kosieradzki and Rowinski, 2008). Such events, by increasing immunogenicity, predispose to acute rejection and early graft dysfunction (Bouma et al., 2009). Both of these are well recognized to be predictors of poor long-term outcomes (Joosten et al., 2004, Matas et al., 1994, Troppmann et al., 1995).

Therefore, the current trend towards increasing use of ECD and DCD kidneys necessitates the development of protective and regenerative novel therapies to mitigate IRI. Unfortunately, no such treatments are in widespread clinical use and current post-transplant management strategies remain largely supportive.

6.11 BONE DERIVED MSCs VERSUS ADIPOSE REGENERATIVE CELLS FOR THE TREATMENT OF RENAL ISCHEMIA REPERFUSION INJURY

Stem cell therapy has emerged as a potential approach to combat the early graft dysfunction and enhanced immunogenicity that follow IRI as a result of the transplantation process. Beneficial effects associated with the administration of mesenchymal stem cells (MSCs) have been reported in various rodent renal IRI models, where MSCs have been shown to act via anti-inflammatory and immunomodulatory mechanisms.

MSCs may be obtained from a variety of sources including bone marrow, umbilical cord blood and adipose tissue. Most studies investigating the effects of protective MSCs on organ ischemia have utilised bone marrow-derived mesenchymal stem cells (BD-MSCs). Whilst these are a convenient cell line in a laboratory setting, a number of potential problems exist with the application of BD-MSCs to the clinical environment.

Firstly, allogeneic MSCs have been demonstrated to be immunogenic (Nauta et al., 2006b) and the use of autologous or syngeneic MSCs is considered preferable in the transplant setting (Roemeling-van Rhijn et al., 2012). Unfortunately, extracting autologous BD-MSC preparations is invasive, and furthermore requires *ex vivo* cellular expansion. Achieving this within the time constraints associated with deceased kidney donation is likely to prove problematic. Moreover, the malignant potential of cells expanded *ex vivo* has raised safety concerns, especially when applied to immunosuppressed patients in the transplantation setting (Roemeling-van Rhijn et al., 2012, Lee et al., 2009, Mantovani, 2012, Ren et al., 2012).

Fortunately, there are other tissues sources from which to isolate stem cells for therapeutic application. Adipose tissue is abundant and in contrast to bone marrow can be obtained in a minimally invasive fashion. Large numbers of adipose-derived regenerative cells (ADRCs) can quickly be obtained from adipose tissue. It is important to note that the ADRC population is heterogeneous, containing mesenchymal stem cells, endothelial cells, endothelial progenitor cells and vascular smooth muscle cells (Lin et al., 2008).

No ethical concerns exist regarding the use of adipose tissue as a source of regenerative cells, and it is therefore an attractive option from which cells may be isolated for immediate use.

The yield of MSCs from adipose tissue is reported to be 40 times greater than from bone marrow (Kern et al., 2006). Hence, ADRCs can be isolated in large quantities by minimally invasive liposuction, and require no *ex vivo* expansion prior to storage or use. Therefore, ADRCs are an easily accessible and abundant source of autologous cells, which may be isolated and administered immediately, or cryogenically preserved for later use.

If the safety and efficacy of ADRCs can be demonstrated, it is feasible to envisage a scenario involving recipient liposuction at the same procedure as kidney transplantation. Cell isolation would yield an autologous ADRC preparation that could be delivered directly into the renal artery *ex vivo*, thereby providing a high local cell concentration, whilst minimizing systemic distribution. A study confirming that ADRC function and phenotype is not affected by the uremic conditions suffered by ESRF patients has important implications for autologous ADRCs use in transplant recipients (Roemeling-van Rhijn et al., 2012).

However, for logistical reasons, ADRC isolation may not be possible at the time of transplantation. Here, it is feasible that ADRCs could be harvested from a patient soon after they were placed on a transplant waiting list. Cryogenic freezing of the ADRCs at the transplant center would allow cells to be ready for use in the event of that individual being offered a kidney, although the efficacy of cryogenic ADRCs would need to be demonstrated in studies beforehand.

6.12 ROUTE OF DELIVERY

Several studies have reported the safety and efficacy of ADRCs in the treatment of renal IRI rat models. Initial studies, using fresh and cryogenically frozen cells (either syngeneic or autologous), used carotid artery cannulation (Feng et al., 2010b) and intra-venous (Chen et al., 2011) delivery methods that resulted in systemic ADRC distribution. Whilst systemic delivery methods have been shown to mitigate renal ischemia, this requires larger numbers of cells to produce efficacy than with local

administration (Shih et al., 2013). Moreover systemic distribution results in the majority of MSCs remaining in the lungs, where the large size of MSCs (15 -19μm) results in MSC entrapment by pulmonary filtration (Fischer et al., 2009, Burst et al., 2010, Schrepfer et al., 2007). This has been shown to produce undesirable effects such as pulmonary infarction (Anjos-Afonso et al., 2004) and pulmonary vascular occlusion (Shih et al., 2013). Local administration of therapy into the renal artery is therefore preferable, and has been shown to be a more effective route at ameliorating IRI in rodents treated with MSCs, undergoing renal transplantation (Zonta et al., 2010, Iwai et al., 2014). Furthermore, it is the likely route of drug administration in clinical transplantation, where the availability of the renal artery allows easy *ex vivo* delivery prior to implantation.

6.13 CHOICE OF ANIMAL MODEL FOR TESTING ADRCs

Current studies in rat renal IRI models have not convincingly demonstrated a long-standing benefit of MSCs on renal function (Wang et al., 2012). In part, this is due to shortcomings in conventional models that usually subject animals to 30 - 60 minutes of warm ischemia (Jo et al., 2001, Ysebaert et al., 2000, Nemoto et al., 2001, Jablonski et al., 1983). This typically only results in impaired renal function for the first postoperative week (Forbes et al., 2000, Ysebaert et al., 2000, Jablonski et al., 1983, Marshall V, 1982).

The recognized shortcomings of commonly used rodent models led to the development of the novel model described in Chapter 5. In this chapter, the effects of uncultured, cryopreserved syngeneic ADRCs in renal IRI in the novel rodent model are investigated, utilizing an intra-renal artery infusion. Renal function was examined after a 2-week recovery period.

6.14 RATIONALE FOR DOSES OF ADRC

In order to reduce experimental time and animal numbers, the authors of a study (Feng et al., 2010b) reporting the effects of intra-arterial ADRCs on renal IRI were contacted for advice regarding initial dosing protocols. A starting dose of 1×10^6 ADRCs was recommended. After 6 animals were treated with 1×10^6 ADRCs, it was decided to increase the dose to 1.3×10^6 to investigate if this affected efficacy. As it

was quickly apparent that 1.3×10^6 ADRCs had a negative effect on renal perfusion, a group of 6 animals were treated with 7×10^5 ADRCs.

6.2 HYPOTHESIS

(1) Syngeneic, cryopreserved ADRCs may protect long-term renal function from severe renal ischemia-reperfusion injury

(2) The effects of ADRCs may occur in a dose-dependent fashion.

6.3 METHODS

6.31 ANIMAL HOUSING AND HUSBANDRY

This was conducted in standard fashion, as previously described in section 5.31. Briefly, male Fisher 344 rats, aged 12 - 14 weeks and weighing 225 – 250g were allowed to equilibrate for at least 2 weeks prior to surgery. Animals were housed and fed as described in Section 2.1. During equilibration prior to surgery, animals put on weight such that on the day of surgery, rats weighing 250 – 320g were used.

Post surgery, animals were weighed weekly and assessed on a daily basis for signs of distress or illness.

6.32 GROUP SELECTION

Control Groups

Animals were randomly assigned to sham operation (Group 1, n=6) or 120 minutes of left renal IRI and renal artery injection with normal saline vehicle (Group 2, n=6). Animals were then recovered for 2 weeks.

Adipose Derived Mesenchymal Stem Cell Groups

Group 3 animals (n=6) were treated with 1 x 10^6 cryopreserved ADRC, given via the renal artery and recovered for 2 weeks. Group 4 animals (n=4) were treated with 1.3 x 10^6 ADRC and recovered for 2 weeks. Group 5 animals (n=6) were treated with 7 x 10^5 ADRC and recovered for 2 weeks.
(See Table 6.1)

Group	120 minutes left renal ischemia	Recovery prior to Inulin Clearance	Intra-renal artery Injection (0.9ml)
1	X	2 weeks	None
2	√	2 weeks	Normal saline
3	√	2 weeks	1 x 10^6 ADRC
4	√	2 weeks	1.3 x 10^6 ADRC
5	√	2 weeks	7 x 10^5 ADRC

Table 6.1: Summary of experimental groups. 6 rats were randomly assigned to each group.

6.33 SURGICAL PROCEDURE

Rodent anesthesia, positioning during surgery and post-surgical care, including were conducted in standard fashion, as previously described in Sections 2.12 – 2.16.

Surgery was carried out was described in Section 5.33. Treatment groups received intra-renal artery therapy as outlined in Table 6.1. All intra-renal artery infusions were made up to a total volume of 0.9ml in normal saline immediately prior to use.

6.34 POST-SURGICAL CARE

This was carried out in standard fashion as described in Section 2.16.

6.35 QUANTIFICATION OF RENAL FUNCTION AND RENAL INJURY

At day 14, animals underwent inulin clearance studies in order to accurately characterize the function of the both kidneys, according to the standard protocol described in Section 2.2.

Left kidneys and right kidneys were weighed, before being stored in 10% formalin and RNA later® solution for later analysis.

Formalin preserved kidneys were paraffin embedded and sectioned as described in Section 2.3 Histological analysis and immunohistochemistry for P16, P21 and Ki 67 were carried out as described in Sections 2.3 and 2.4

6.36 DISECTION OF RAT INGUINAL FAT PAD

(1) A male Fisher 344 rat, weighing 250 – 320g was euthanized by placing in a pre-filled CO_2 chamber until motionless.

(2) 1 minute was allowed to pass from the animal's last movement until the animal was removed from the chamber.

(3) The abdomen and groins of the rat were shaved, and the animal pegged out on a cork board, cleaned with 2% chlorhexidine and covered with a surgical drape.

(4) Horizontal incisions in both groin creases were made to allow exposure of the groin adipose fat pads, which lies between the skin and muscles of the femoral triangle.

(5) Lymph nodes in both fat pads were excised and discarded. Likewise, any visible vessels were excised.

(6) The fat pads were dissected free and weighted. Typically ~ 4g of fat was obtained from each rat.

(7) Fat was placed in sterile Hartmann's solution ((Baxter Healthcare, Thetford UK)

(8) At this point, fat was transferred to the British Heart Foundation Building, University of Glasgow for ADRC isolation to begin.

6.37 ADRC ISOLATION FROM INGUINAL FAT PAD

ADRCs were isolated from the inguinal fat pad of freshly culled Fisher 344 rats (200 – 320g) as previously described (Feng et al., 2010b, Schenke-Layland et al., 2009), but with minor modifications. ADRC isolation work was done solely by Dr Diane Hardie Ph.D.

(1) 5ml of Hartmann's solution was added to a vial containing 35mg of Celase (a type of collagenase) reagent. (Cytori Therapeutics, San Diego, CA, USA) This produced a concentration of ~ 30 units/ml.
(2) Homogenization of adipose tissue was achieved using large, and then small sterile scissors in a sterile, dry Petri dish. Homogenization was judged complete when pieces were <4mm in diameter.
(3) Homogenized adipose tissue was placed in a 50ml conical tube with 5x volume of collagenase solution.
(4) The conical tube was agitated for 30 minutes, whilst being incubated at 37^{o}C.
(5) The tube was shaken violently to break up the largest remaining tissue pieces and agitated for a further 15 minutes at 37^{o}C.
(6) The ADRC fraction was separated by centrifugation at 600 g for 5 min.
(7) The resulting pellet was washed in sterile PBS, centrifuged at 400g for 5 mins and repeated.
(8) Incubation with Intravase™ (Cytori Therapeutics, San Diego, CA, USA) for 10 min was followed by more PBS washing.
(9) The cells were then passed through 100- and 40-µm Falcon™ cell strainers (BD Biosciences, San Jose, CA, USA), sequentially.
(10) ADRCs were counted and prepared for cryopreservation.

6.38 ADRC CRYOPRESERVATION

Cryopreservation of ADRCs was as described in a previous study (Feng et al., 2010b).

Briefly, ADRCs were isolated and frozen in 10% serum from Fisher 344 rats and 10% dimethyl sulphoxide in lactated Ringer's solution at a concentration of 3 x 10^6 cells/ml. Cells were stored in liquid nitrogen until required.

Prior to infusion, cells were thawed rapidly and re-suspended in 10× volume of PBS. The cells were centrifuged at 400 g for 10 min, washed in PBS and re-suspended in a minimum volume. Counting of viable cells was carried out to ensure that the desired number of viable cells was obtained. Immediately prior to injection, cells were diluted with normal saline to give a total volume of 0.9ml.

6.39 CHARACTERISTICS OF CELLULAR PREPARATION OBTAINED FROM ADIPOSE TISSUE

Flow cytometric studies were not performed on adipose tissue-derived cell preparations, isolated as part of these experiments. However, previous studies utilizing the same protocols for ADRC extraction have performed flow cytometric analysis o*f* fresh and cryopreserved cell extracts obtained from rat adipose tissue (Feng et al., 2010b).

This revealed that ~ 44% of cells were $CD45^+$, most of which were monocytes, neutrophils and tissue macrophages. The majority of $CD45^-$ cells were found to be $CD73^+$ and $CD90^+$ and considered to be of mesenchymal origin.

Hence ~ 50% of cells obtained using this protocol were considered MSCs, with the remainder thought to be blood and tissue derived leucocytes (~ 44%) and endothelial cells (~3.4%).

Epitope expression was retained following cryopreservation, with a slight reduction in the frequency of $CD45^+/CD11b^+$ cells, and an increase in $CD45^-/CD31^+$ cells. This probably represents a fall in neutrophil presence in the samples, which is consistent with the known sensitivity of neutrophils to freezing and thawing (Feng et al., 2010b).

6.4 RESULTS

6.41 GROUP WEIGHT LOSS COMPARED TO BASELINE WEIGHT

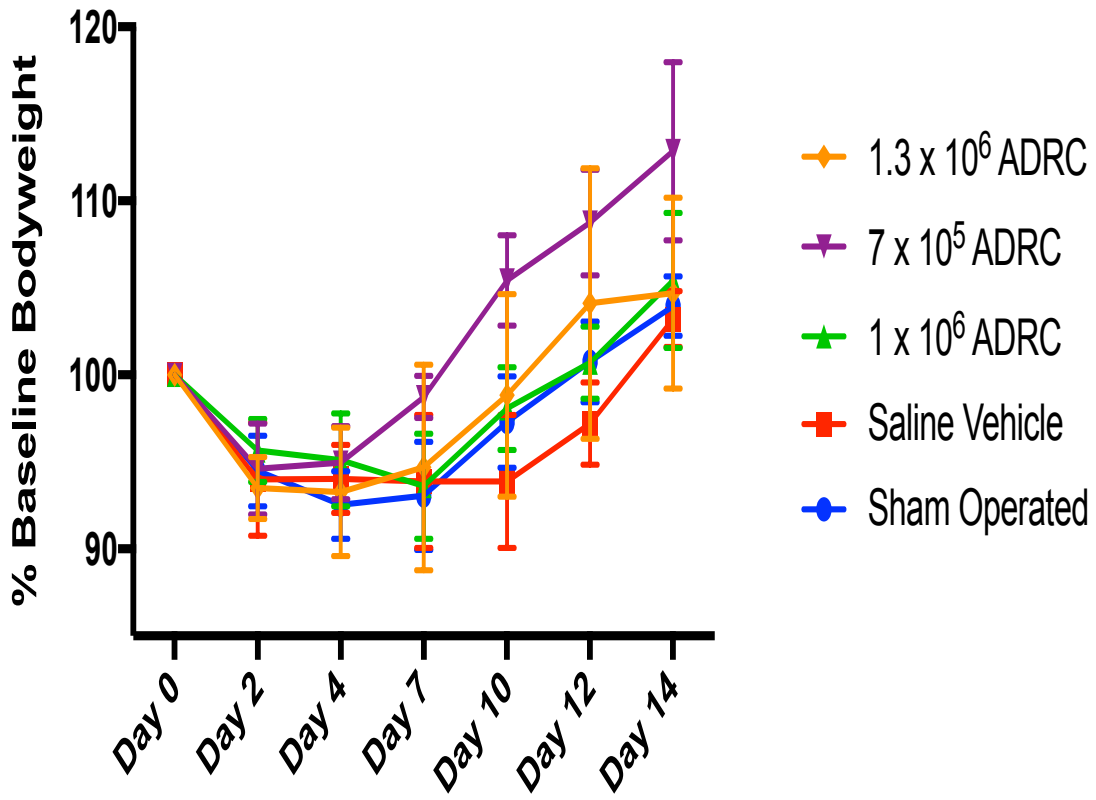


Fig 6.1: Compared to baseline, sham-operated animals, those treated with saline vehicle, those treated with 1 x 10^6 ADRC and those treated with 1.3 x 10^6 ADRC exhibited significant weight loss on Day 2 ($p < 0.0001$, 0.0011, 0.0001 and $p < 0.0001$ respectively). There remained significant weight loss for these Groups on Day 4 ($p < 0.0001$, <0.0001, 0.0012 and 0.0019 respectively), Day 7 (p=0.0003, 0.0027, 0.0004 and 0.0490). By day 10, sham-operated animals, saline treated animals and those given 1 x 10^6 ADRC were still below their baseline weight (p=0.0311, 0.0157 and 0.0740), whilst animals treated with 1.3 x 10^6 ADRC weights were not significantly below baseline (p=0.6106).

By day 12, animals in the aforementioned groups had regained baseline weight (p=0.4394, 0.9734, 0.4232 and 0.2038 respectively).

By day 14, animals the aforementioned groups had put on weight in comparison to baseline (p =0.0002, 0.0006 and 0.0064 and 0.0573 respectively).

Animals treated with 7 x 10^5 ADRC lost weight on day 2 (p=0.0005), day 4 (p=0.0002) and day 7 (p=0.0278). By day 10, the weight of animals treated with 7 x 10^5 ADRC had risen above baseline (p=0.0005) and remained so at day 12 ($p<0.0001$) and at day 14 (p=0.0001).

Statistical analysis by unpaired Students t test.

6.42 COMPARISION OF PERCENTAGE WEIGHT LOSS FROM BASELINE BETWEEN GROUPS

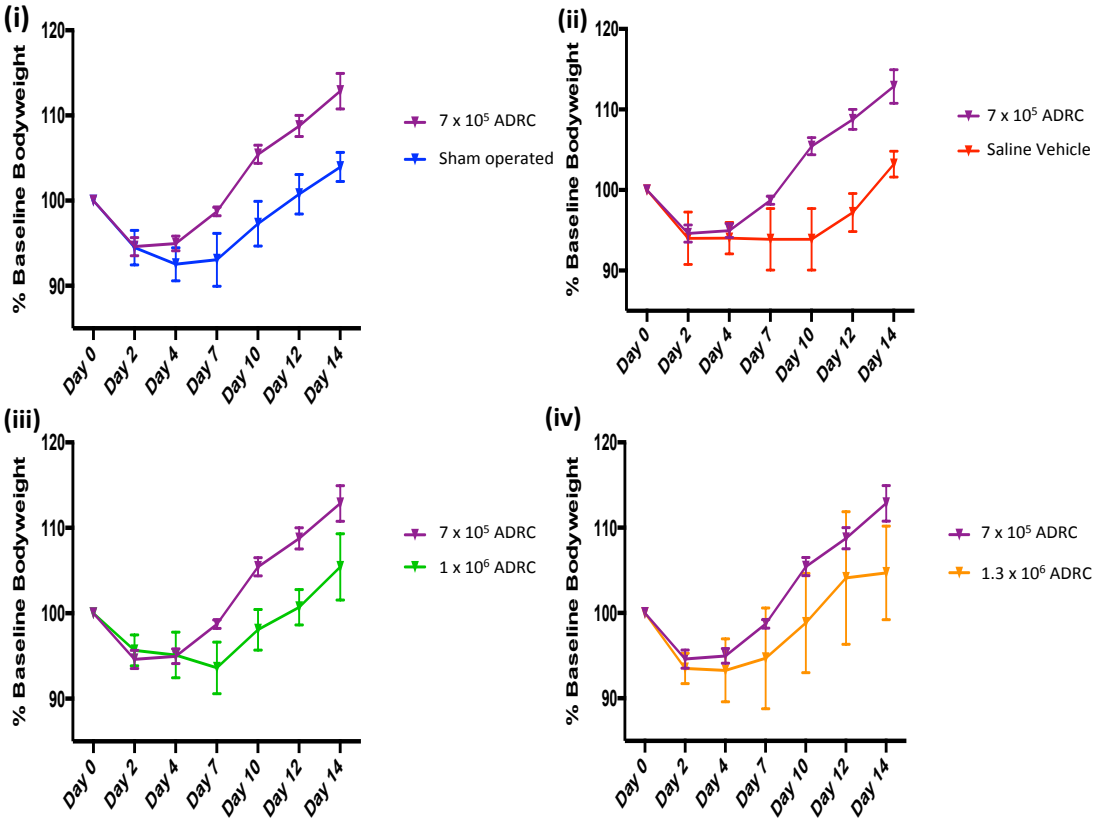


Figure 6.2: (i) There was no difference in weight trends between sham-operated animals and animals treated with 7 x 10^5 ADRC up to day 7. By day 10, the 7 x 10^5 ADRC group were observed to have gained weight faster than sham-operated animals (p=0.0011). This trend continued at day 12 (p=0.0038) and day 14 (p=0.0028). **(ii)** There was no difference in weight trends between animals treated with saline vehicle and those treated with 7 x 10^5 ADRC up to day 7. By day 10, the 7 x 10^5 ADRC group were observed to have gained weight faster than the saline group (p=0.0009). This trend continued at day 12 (p=0.0013) and day 14 (p=0.0012).

(iii) There was no difference in weight trends between animals treated with 7 x 10^5 ADRC and those treated with 1 x 10^7 ADRC up to day 7. By day 10, the 7 x 10^5 ADRC group were observed to have gained weight faster than the 1 x 10^7 ADRC group (p=0.0034). This trend continued at day 12 (p=0.0034) and day 14 (p=0.0165). **(iv)** There was no difference in weight trends between animals treated with 7 x 10^5 ADRC and those treated with 1.3 x 10^6 ADRC up to day 12. By day 14, the 7 x 10^5 ADRC group were observed to have gained weight faster than the 1.3 x 10^6 ADRC group (p=0.0009).

No significant difference was observed between the % baseline weights of sham-operated animals, saline-treated animals, 1 x 10^6 ADRC or 1.3 x 10^6 ADRC, at any time point.

Statistical analysis by 1 way ANOVA with Tukey multiple comparisons test.

6.43 GLOMERULAR FILTRATION RATE

Effect on GFR after ADRC Infusion

ADRCs were found to protect GFR in a dose-dependent manner.

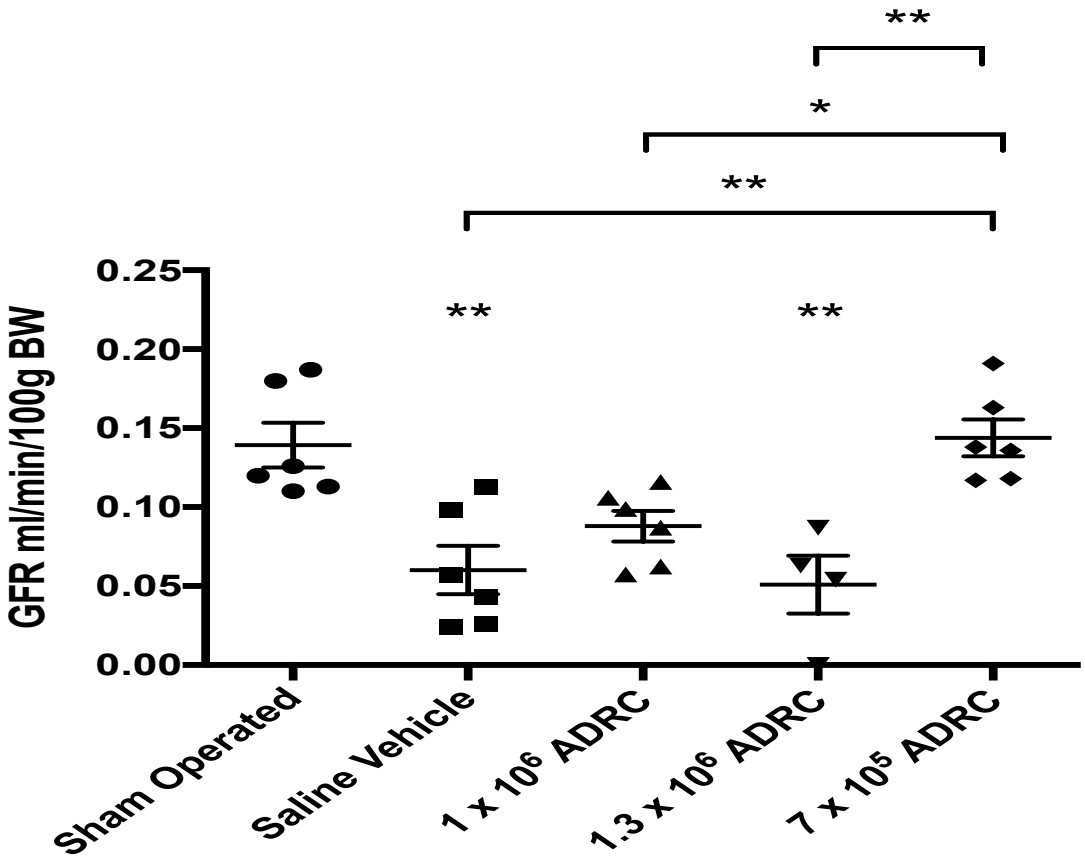


Figure 6.3: Compared to saline vehicle alone, a dose of 7 x 10^5 ADRCs was found to significantly protect GFR versus saline treated animals (p= 0.0014). Indeed, the GFR of sham-operated animals and animals treated with 7 x 10^5 ADRCs were not significantly different (p= 0.9992).
7 x 10^5 treated animals were observed to have significantly higher GFR versus animals treated with 1 x 10^6 ADRC (p=0.0457) and those treated with 1.3 x 10^6 ADRC (p=0.0015). A larger dose of 1 x 10^6 ADRCs was not found to improve GFR versus saline treated animals (p=0.5793).
Statistical analysis by 1 way ANOVA with Tukey multiple comparisons test.

6.44 RENAL HISTOLOGY SCORING

Epithelial Breaks

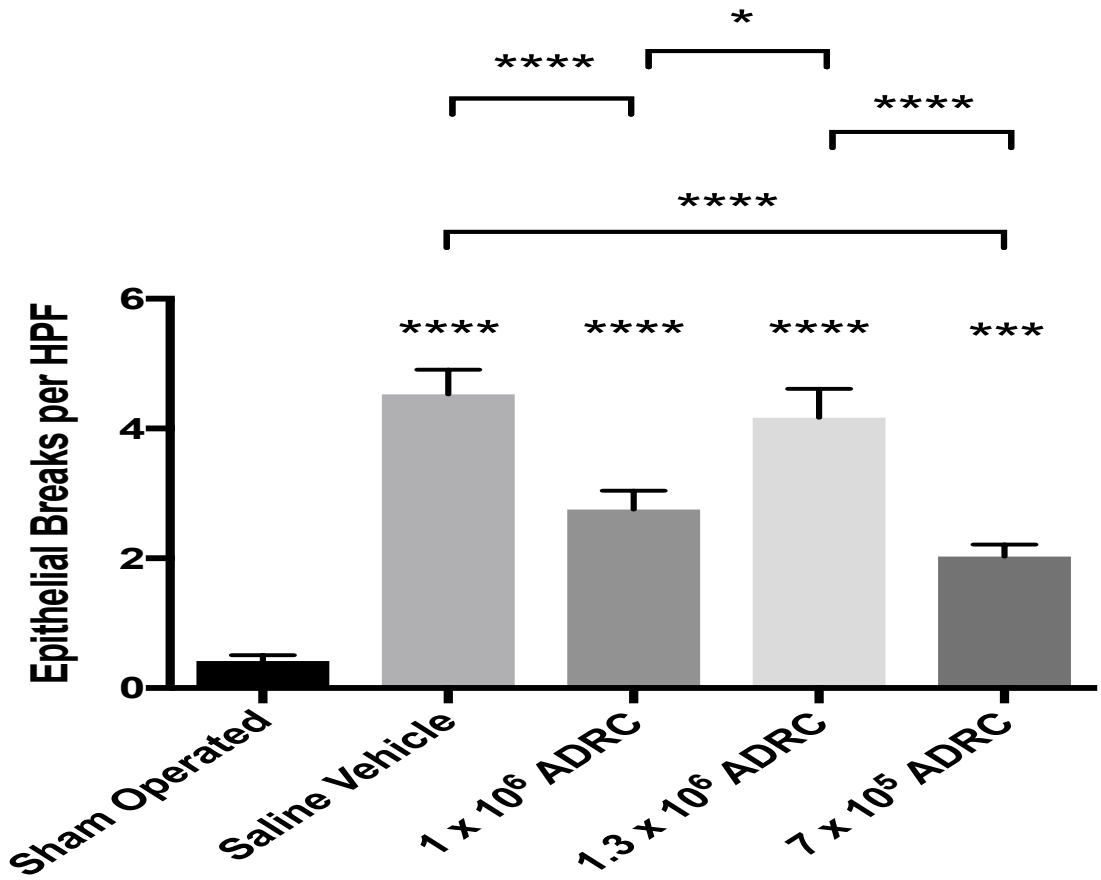


Figure 6.4: Sham-operated animals were found to have significantly fewer epithelial breaks compared to animals treated with saline ($p<0.0001$), 1 x 10^6 ADRC ($p<0.0001$), and 1.3 x 10^6 ADRC ($p<0.0001$). Similarly, sham operated animals were found to have fewer epithelial breaks than animals treated with 7 x 10^5 ADRC ($p=0.0003$).

Animals treated with 7 x 10^5 ADRC were found to have fewer epithelial breaks compared to animals treated with saline ($p<0.0001$), and animals treated with 1.3 x 10^6 ADRC ($p<0.0001$).

Animals treated with 1 x 10^6 ADRC had fewer breaks compared to animals treated with saline ($p<0.0001$), and those treated with 1.3 x 10^6 ADRC ($p=0.0216$).

Statistical analysis by 1 way ANOVA with Tukey multiple comparisons test.

Hyaline Cast Formation

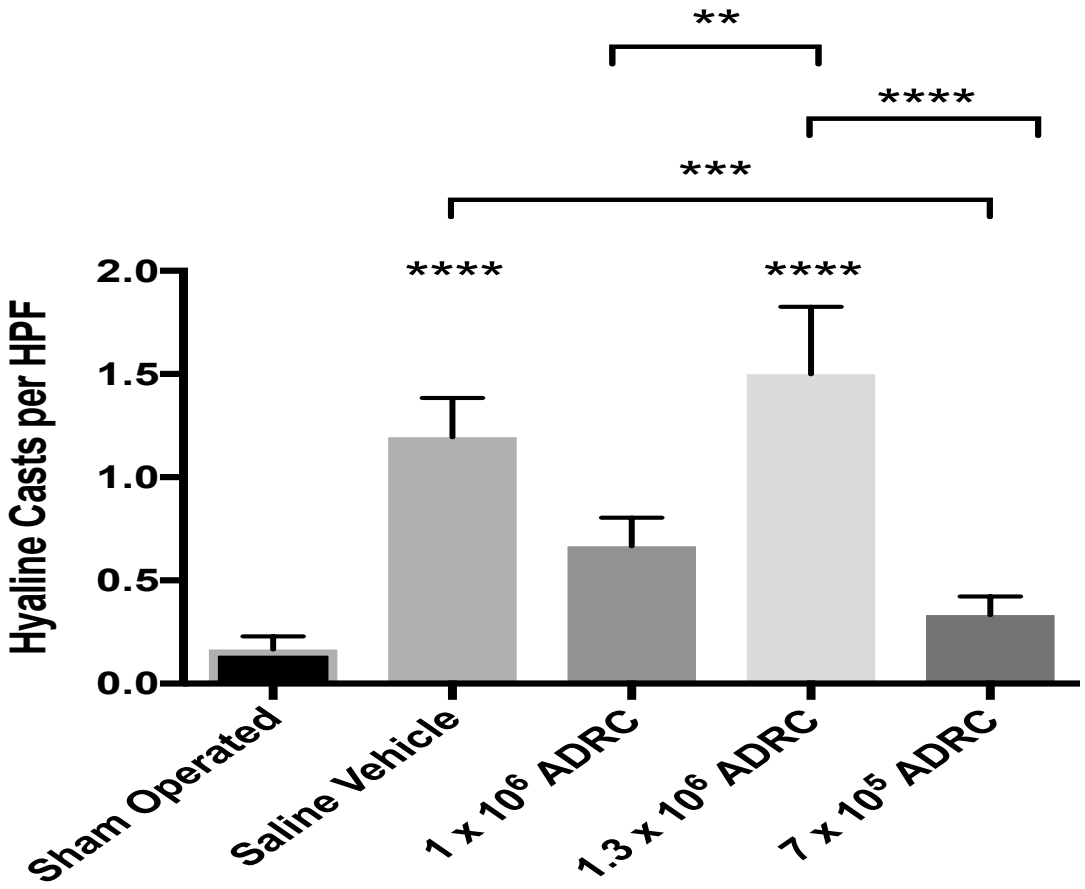


Figure 6.5: Sham-operated animals were found to have fewer hyaline casts than saline treated animals ($p<0.0001$) and those treated with 1.3 x 10^6 ADRC ($p<0.0001$).
Animals treated with 7 x 10^5 ADRC were found to have fewer hyaline casts than those treated with saline ($p=0.0004$) and those treated with 1.3 x 10^6 ADRC ($p<0.0001$).
Fewer casts were found in animals treated with 1 x 10^6 vs 1.3 x 10^6 ADRC ($p=0.0091$).
Statistical analysis by 1 way ANOVA with Tukey multiple comparisons test.

Furthermore, 1.3 x 10^6 ADRCs were universally observed to result in renal artery 'clogging' post clamp removal, resulting in renal hypoperfusion post clamp removal (see figure 6.7). Experiments with 1.3 x 10^6 ADRCs were therefore halted after recovering 4 animals, with a further 2 animals culled intra-operatively.

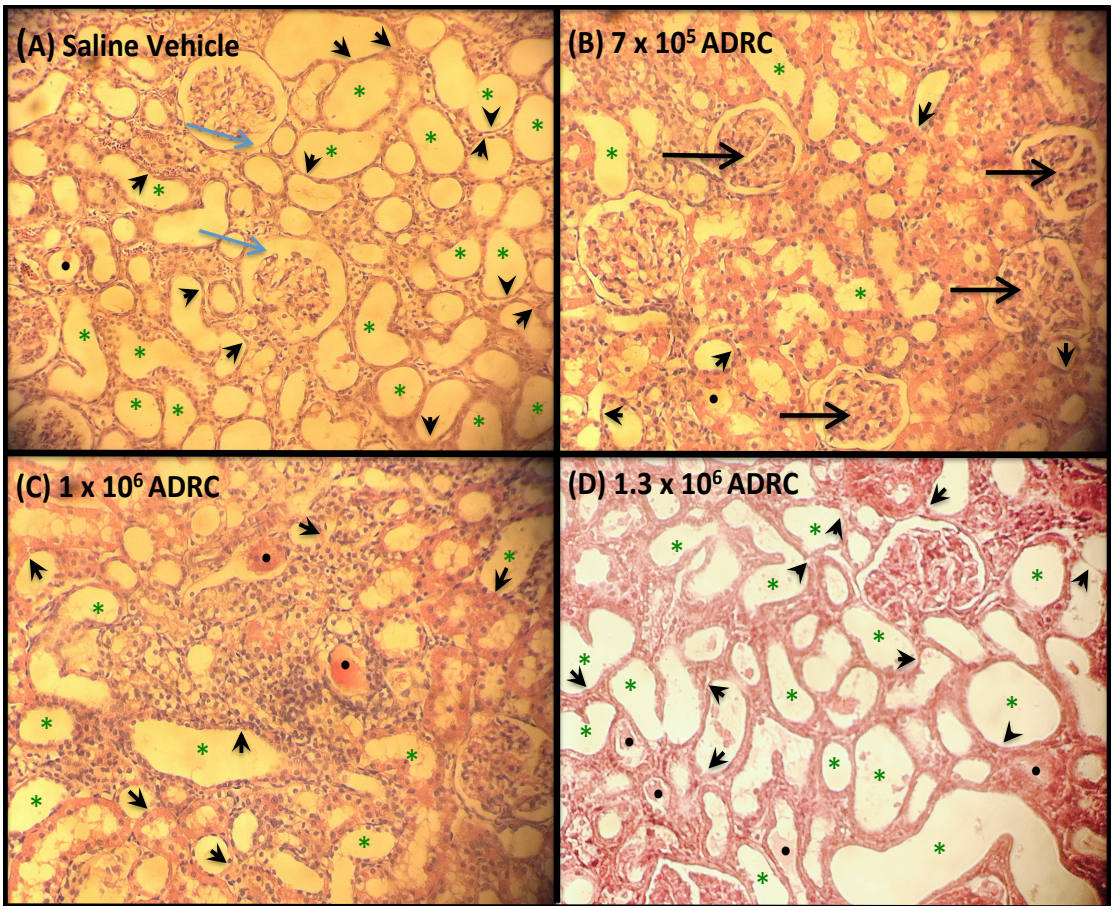


Figure 6.6: (a) Haematoxylin and eosin stained kidney sections from rats 2 weeks post surgery. Kidneys from saline treated animals show marked tubular dilation (green asterisks) and epithelial breaks (black arrowheads). Hyaline casts are also present (black dots). Dilation of Bowman's space is also present (blue arrows). **(b)** These changes are less marked in animals treated with 7 x 10^5 ADRC, which display healthy glomeruli (large black arrows). **(c)** Animals treated with 1 x 10^6 ADRC have fewer markers of damage compared to saline treated animals. **(d)** Animals treated with 1.3 x 10^6 ADRC have grossly abnormal renal histology, similar to those animals treated with saline alone.

6.5 DISCUSSION

The current trend towards transplanting 'marginal' kidneys necessitates the development of novel therapies to combat peri-transplantation injury and IRI in particular. Stem cell therapy has great potential in this regard, with investigators actively testing the efficacy of cellular preparations to reduce injury affecting both native and transplanted kidneys.

Various stem cells have been investigated for the treatment of renal IRI, including BD-MSCs, ADRCs and hematopoietic stem cells. The ideal characteristics of a stem cell source include widespread availability with minimally invasive harvesting, thereby allowing isolation of fresh or cryopreserved preparations, without the need for *ex vivo* cellular expansion.

Here, ADRCs were selected for further investigation as ADRCs fulfill many requirements that are necessary to apply a stem cell source to the clinical environment. Also, the paracrine mechanisms of ADRCs have been shown to be different from those of BD-MSCs, showing more potent anti-inflammatory and immunomodulatory function (Banas et al., 2008).

One weakness of this experiment is the lack of molecular data to support the mechanisms by which ADRCs may produce their effects. However, it is known that the renal tubular epithelium releases pro-inflammatory mediators including TNF- α, IL-6 and IL-8 and transforming growth factor β in response to IRI. Numerous studies examining the effects of ADRCs in renal ischemia have demonstrated that ADRC administration is associated with down-regulation of the inflammatory response. Chen et al demonstrated that ADRC therapy protected renal function by suppressing oxidative stress and the inflammatory response by reducing both cytokine production and expression of cell surface adhesion molecules (Chen et al., 2011). Feng et al observed ADRC therapy to be associated with reduced macrophage infiltration as a result of reduced CXCL2 and IL-6 expression. The authors concluded the increased tubular epithelial cell proliferation they observed was the result of a dampened inflammatory response that helped to restore renal architecture and preserve renal function (Feng et al., 2010b). In a mouse model of renal IRI, Furuichi et al observed

treatment with ADRCs caused the suppression of IL-1β and TNF-α, leading to anti-inflammatory activity and alleviation of tubular necrosis (Furuichi et al., 2012). More recent studies, examining the effects of local ADRC administration on rat renal IRI have also reported a reduced inflammatory response after ADRC therapy (Shih et al., 2013, Wang et al., 2013b). It is possible that molecular data from this study would be in agreement with the above findings.

Here, one possible route of administration in clinic transplantation was mimicked in order assess the feasibility of intra- renal artery administration. Furthermore, intra-renal artery administration avoids the potentially detrimental effects of systemic stem cell delivery that have been reported with administration via the intravenous route. Other studies report local arterial administration of cell therapy is superior to other administration methods (Iwai et al., 2014, Zonta et al., 2010). However, at the outset of this experiment, there were apparently no published studies using ADRCs via the renal artery route in renal IRI models. Therefore the authors of a previous study administering ADRCs via carotid cannulation were contacted for advice regarding ADRC dose (Feng et al., 2010b). Following discussion, a starting dose of 1×10^6 ADRCs was decided upon.

Utilizing the intra-renal artery route in our rate model, a dose-dependent renoprotective effect of ADRCs was observed. The lowest dose 7×10^5 ADRCs were found to significantly protect GFR and renal histology from severe IRI, whilst a higher dose of 1×10^6 ADRC preserved renal histology but did not protect GFR.

The highest dose of 1.3×10^6 ADRCs was associated negative outcomes when compared to controls. This finding may be explained by the universal intra-operative observation of macroscopic occlusion of the terminal branches of the renal artery when injecting 1.3×10^6 ADRCs (see figure 6.7). Despite relieving this by downstream massage of the occluding cell 'plug', the kidney failed re-perfuse normally. As ADRCs have a larger diameter than tissue capillaries (Furlani et al., 2009b, Vulliet et al., 2004), it is likely that higher doses of cells cause occlusion of the macro and/or microscopic renal circulation. The subsequent hypoperfusion is likely to result in negative effects on long-term kidney structure and function.

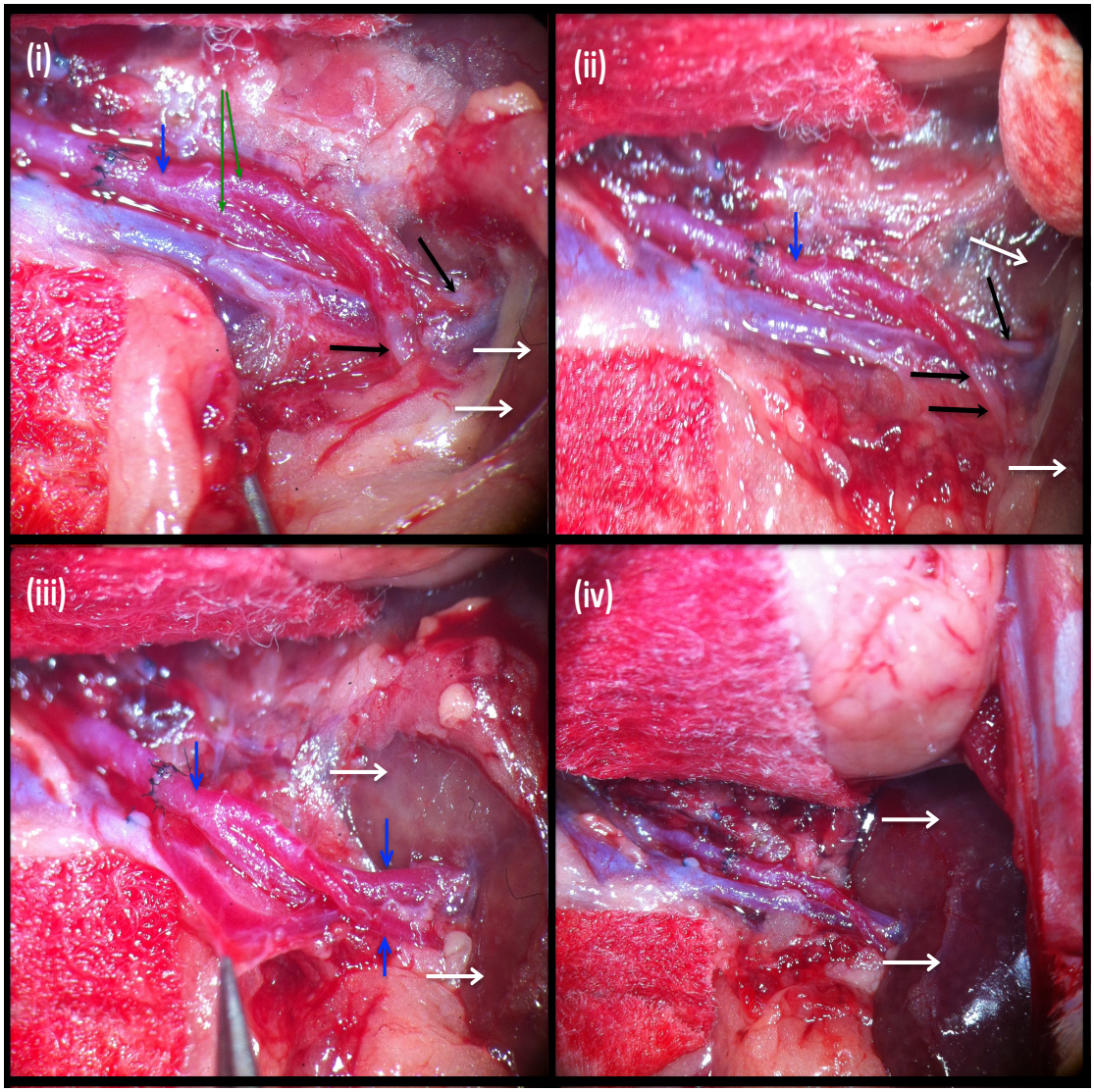


Figure 6.7: (i) Dissected renal pedicle after attempted reperfusion of left kidney post administration of 1.3 million ADSC. Despite the patent anastomosis, and a clear pulse distal to this (blue arrow), the kidney is poorly perfused (white arrows). The renal artery bifurcation is also patent (green arrows). However, there is obvious renal artery occlusion due to viscous ADRC preparation causing arterial plugging (black arrows). **(ii)** Further dissection of the hilar branches of the renal artery reveals the majority of the distal branches are occluded (black arrows). **(iii)** After clearing the renal artery of cell preparation by gentle downstream 'milking' with forceps, the kidney still perfused poorly (white arrows). A pulse remains in the artery distal to the anastomosis and is now present in the hilar branches (blue arrows). **(iv)** The kidney perfuses poorly and becomes discolored, indicating ongoing ischemia. (white arrows)

Prior to this experiment, studies investigating organ ischemia, utilizing both systemic and local administration of BD-MSCs had reported the dose-dependent phenomenon we observe (Furlani et al., 2009b, Freyman et al., 2006). Freyman et al used 5 x 10^7 BD-MSCs and reported adverse effects of intracoronary infusion of MSCs in a porcine model of myocardial infarction (Freyman et al., 2006). They concluded that the reduced myocardial blood flow might have been due to occlusion of the cardiac microcirculation (Freyman et al., 2006).

Subsequent to the conclusion of this experiment, studies observing dose dependence in rat models of renal IRI, utilizing renal artery administration of both BD-MSCs and ADRCs were published (Cai et al., 2014, Shih et al., 2013, Lee et al., 2012). The findings of these studies are in agreement with the observation here that excessive doses of locally administered MSCs may produce negative effects via blockage of the tissue microcirculation.

In rat models of renal IRI, laser Doppler has demonstrated reduced renal blood flow after administration of 5 x $10^7$of ADRCs (Lee et al., 2012) or 1 x 10^6 BD-MSCs (Cai et al., 2014) directly into the renal artery. Subsequently, using quantum dot-labeled ADRCs, renal vessel occlusion by ADRCs was reported after 1 x 10^7 and 5 x 10^7 doses of cells were given via the renal artery (Shih et al., 2013). These reports are in keeping with the findings here that local administration of 1.3 x10^6 ADRCs was associated with negative long-term outcomes, and that a lower dose of 1 x 10^6 ADRC did not improve GFR in comparison with saline vehicle controls.

Nevertheless, here a dose of 7 x 10^5 ADRCs is associated with significant reno-protection, when compared to saline vehicle controls. The optimal dose of locally administered ADRCs here is broadly in keeping with the findings of previous studies, as is the observation that ADRCs can protect long-term renal histology from IRI damage (Shih et al., 2013, Lee et al., 2012, Feng et al., 2010b, Villanueva et al., 2013). However, in contrast to the other studies, here it is reported that ischemia causing long-term reduction in renal function is significantly mitigated by intra-renal artery ADRCs therapy at the correct dose.

The post-operative weight recovery of the animals treated with 7 x 10^5 ADRC was significantly better than the other experimental groups, including sham animals. This observation may reflect more 'healthy' animals in the 7 x 10^5 ADRC group when compared to other animals subjected to ischemia. However, the superior weight of 7 x 10^5 ADRC treated animals versus the sham group is difficult to interpret. Theoretically, sham animals ought to regain their weight faster postoperatively than animals subjected to ischemia, and this finding may simply reflect a slower than expected weight gain in the sham group. As the age and starting weights of animals in all groups was not significantly different, the reason for the apparent slow weight gain in the sham group, or the faster weight gain of the 7 x 10^5 group, is unclear.

Translation of ADRCs therapy into routine clinical practice requires further investigation, with emphasis on clarifying the initial safe doses of ADRCs and the long-term effects in immunosuppressed transplant patients. Establishing a rodent transplant model, utilizing both cold and warm ischemia whilst administering immunosuppression post-operative closely mimics the clinical transplant process. Such a model, again subjecting the injured kidney to prolonged ischemia might enable the effects of intra-renal artery ADRCs to be studied in a safe environment. After this*, ex vivo* models of porcine kidney reperfusion circuits could also be used to test initial ADRCs dosing schedules prior to efficacy testing in porcine renal transplant models.

CHAPTER 7

THE MEDIUM AND LONG-TERM EFFICACY OF PATHFINDER DERIVED MICROVESICLES IN A NOVEL RAT MODEL OF SEVERE RENAL ISCHEMIA-REPERFUSION INJURY

7.1 INTRODUCTION

Stem-cell therapy has been reported to ameliorate both acute and chronic dysfunction of cardiac, respiratory, and renal systems in numerous animal models. As described in Section 1.37 – 1.38, debate continues regarding the likely mechanism by which stem cells act. However, general consensus accepts that the reported beneficial effects of stem cell therapy are likely to occur via a paracrine mechanism (Abreu et al., 2016), and this especially appears to be the case in the kidney (Wise and Ricardo, 2012).

It is clear that cell-to-cell communication plays a vital role in the regulation of tissue and organ function in multicellular organisms. Extracellular vesicles (ECVs) have been implicated as important mediators of intercellular communication, appearing to act via ancestral mechanisms amongst cells and tissues (Valadi et al., 2007).

The content of ECVs consists primarily of proteins, lipids and genetic material including messenger-RNA (mRNA) and micro-RNA (miRNA) (Bruno et al., 2016, Raposo and Stoorvogel, 2013, Witwer et al., 2013). In general, the content of ECVs reflects the content of the parent cell (Quesenberry et al., 2014). Recent data suggest that ECVs released from stem cells mediate paracrine effect by shuttling their content into target cells (Lai et al., 2011, Yeo et al., 2013, Lai et al., 2013). The transfer of miRNA by ECVs is thought to be particularly important in regulating cell processes, as miRNAs have been shown to modify recipient cell gene expression and hence protein production (Raposo and Stoorvogel, 2013, Ratajczak et al., 2006b, Anthony and Shiels, 2013). The transfer of genetic material and consequent cell stimulation may therefore modify target cells and reprogram their biological processes, resulting in long-lasting changes to cell phenotype, function and fate (Biancone et al., 2012, Bruno and Camussi, 2013, Deregibus et al., 2007, Ratajczak et al., 2006a, Valadi et al., 2007).

The ability of ECVs to produce epigenetic changes in their recipients depends on upon the phenotype of the parent cell (Katsuda et al., 2013). Experimental studies have shown that ECVs derived from MSCs emulate the effect of their parent cells in various experimental models of visceral injury, notably stimulating cell proliferation and enhancing tissue repair (Bruno et al., 2012, Herrera et al., 2010, Bruno et al.,

2009b). Data indicate such 'cell-free' therapies may have similar therapeutic efficacy compared to cell-based therapy, but are postulated to hold a number of advantages over the use of stem cells themselves.
As a result, there is active research in the development of ECVs as novel treatments of organ damage.

Potential advantages of cell-free therapies include:

(1) Cell-free therapy is thought to carry significantly less risk of cell maldifferentiation and malignancy (Bruno et al., 2016, Kunter et al., 2007). The risk of this occurring was highlighted by the findings of Chapter 3.

(2) The biologically active factors in cell-free therapy are smaller and more soluble than stem cells. Theoretically, therapeutic doses of cell-free ECV therapy carried reduced risk of arterial or capillary occlusion (Rani et al., 2015). The risk of occlusion was a significant finding in experiments using ADRCs, described in Chapter 6.

(3) Identification of the factors responsible for producing beneficial effects may subsequently allow synthetic manufacture packaged in vesicle form. This would ensure quality control of biologically active contents (Vishnubhatla, 2014, Taylor and Shah, 2015).

(4) Cell-free preparations are easier to store, transport and use in clinical settings (Vishnubhatla, 2014, Kordelas et al., 2014, Yeo et al., 2013).

7.11 PROBLEMS IDENTIFYING THE BENEFICIAL BIOLOGICAL FACTORS IN THE STEM CELL SECRETOME

ECVs are a heterogeneous population of small, spherical, membrane-bound particles that originate from different sites in parent cells. Consequently, ECVs differ in cell surface markers and crucially in size. As highlighted in Section 1.4, this research field is still in its infancy and as yet no ECV classification system has been applied universally. However, classification based on size and origin is now gaining widespread acceptance. In part, this is due to the recognition that it is vital to identify

the specific beneficial ECV subtypes, thereby allowing the biologically active content to be characterized prior to manufacture for therapeutic application.

ECVs may be considered to consist of 3 separate subtypes or 'fractions' (Anthony and Shiels, 2013):

(1) Exosomes: 40 – 100nm in diameter, and of endosomal origin. Exosomes are stored prior to their release, which occurs after fusion with multivesicular bodies. (See figure 1.5)

(2) Microvesicles: 100nm - 1µm in diameter, are formed after budding directly from the plasma membrane in an energy-dependent process, reliant on calcium influx and cytoskeleton reorganization (Cocucci et al., 2009). (See figure 1.5)

(3) Apoptotic bodies (also termed 'Shedding vesicles') – 1 - 5µm sized particles released upon fragmentation of the plasma membrane that occurs during apoptosis.

Each ECV subtype is normally present within the *in vitro* cultured cell medium and body fluids from which ECVs are typically obtained prior to experimental use. Isolation of a specific ECV fraction may be achieved either by differential ultracentrifugation, ultrafiltration and/or immunoprecipitation with the use of antibody loaded magnetic cell beads (Fierabracci et al., 2015). Failure to adhere to a strict ECV isolation protocol will lead to preparations contaminated with different ECV types prior to use.

This is important as the proteins, lipids and genetic material contained within each of the ECV fractions has been shown to differ in type and quantity (Quesenberry et al., 2014, Keerthikumar et al., 2016). Additionally, the content of ECVs from any particular cell type may be influenced by physical or chemical stressors, such as hypoxia, oxidative stress and inflammation (Robbins and Morelli, 2014). Consequently, each ECV fraction from the same parent cell may modulate protein and gene expression to a different extent in recipient cells. Logically, therefore, identifying the ECV fraction which produces beneficial cellular effects is a crucial

step in characterizing protective/regenerative factors contained within that fraction, prior to manufacture of a synthetic drug.

Unfortunately, the inconsistent use of terms in a number of reports has resulted in misleading titles and conclusions regarding the efficacious ECV subtype.
For example, early and extensive work by Camussi et al used 'microvesicles' as a universal term to include each of the 3 separate ECV types (Gatti et al., 2011, Cantaluppi et al., 2012, Bruno and Camussi, 2013, Camussi et al., 2013, Camussi et al., 2011). The group concluded that 'microvesicles' conferred beneficial effects after renal injury, although ECVs were harvested by using undifferentiated 100,000g centrifugation forces, probably resulting in a preparation containing exosomes, microvesicles and apoptotic bodies (Gatti et al., 2011, Cantaluppi et al., 2012, Bruno et al., 2009b). Indeed, using analysis of therapeutic ECV preparation by transmission electron microscopy and Nanosight analysis in one study reported 90% of administered ECVs to range in size from 60 – 160nm (ie seemed to contain both exosomes and microvesicles) (Cantaluppi et al., 2012). A more recent paper (Bruno et al., 2016) by the same authors replaced the term 'microvesicles' with 'extracellular vesicles' - perhaps after recommendation by the International Society for Extracellular Vesicles that ECV should be used as an umbrella term for each of the 3 ECV types (Katsuda et al., 2013). Whilst it now appears the classification system used in this thesis (Section 1.4) is gaining acceptance, it remains necessary to examine the centrifugal spin protocols to determine the identity of the vesicles administered in any particular study.

7.12 PATHFINDER DERIVED EXTRACELLULAR VESICLES

Pathfinder cell administration has been shown to restore normoglycaemia with the recovery of normal tissue function in a murine model of streptozotocin-induced diabetes (Stevenson et al., 2011). Furthermore, Pathfinder cells have been shown to restore normal renal function and architecture in a murine model of renal IRI, whilst also significantly reducing P16 levels in treated animals (McGlynn et al., 2013). In both studies, Pathfinder cells were only faintly detected in damaged organs, with the majority of tissue restoration occurring with host cells. This implies Pathfinder cells also act via a paracrine mechanism.

A recent study by the same group has demonstrated that ECVs derived from Pathfinder cells were able to replicate the findings of previous experiments in the streptozotocin-induced diabetic murine model (McGuinness et al., 2016). Importantly, by using differential centrifugation spin rates, exosomes enriched fractions were obtained and found to be ineffectual at repairing streptozotocin-induced damage. The microvesicle enriched fraction however had equivalent efficacy at repairing damage when compared to Pathfinder cells (McGuinness et al., 2016).

This finding implies that the microvesicles may be the paracrine entity by which Pathfinder cells produce their beneficial effects and consequently may have the ability to mitigate renal IRI. This finding is contrary to previous reports, which have concluded that exosomes are more physiologically relevant and powerful components of stem cell secretome (Kordelas et al., 2014). However, whilst it is possible MSCs produce their effects via exosomes, this does not appear to be the case with ECVs derived from pathfinder cells. Therefore, we decided to investigate the effects of pathfinder-derived microvesicles in our novel model of rodent renal ischemia.

7.2 HYPOTHESIS

(1) Pathfinder-derived microvesicles may protect kidney function and architecture in both the medium and long-term in a novel rodent model of severe renal IRI.

7.3 METHODS

7.31 ANIMAL HOUSING AND HUSBANDRY

This was conducted in standard fashion, as previously described in section 2.1
Post surgery, animals were weighed weekly and assessed on a daily basis for signs of distress or illness.

7.32 GROUP SELECTION

Control Groups

Animals were randomly assigned each group. Sham operation animals (Group 1, n=6), underwent laparotomy and dissection of the left renal pedicle only.
After 120 minutes of left renal IRI, Group 2 animals (n=6) were treated with 0.9ml of intra-renal artery normal saline and 2 weeks recovery. Group 3 animals (n=6) were likewise treated with 0.9ml saline vehicle after 120 minutes of left renal IRI, but with 6 weeks recovery.

Pathfinder Derived Microvesicle Treatment Group

400ng of Pathfinder-derived microvesicles (PF-MV) were thawed and dissolved in 0.9ml of normal saline immediately prior to use. After 120 minutes of left sided renal IRI, Group 4 animals (n=6) were treated with 0.9ml of intra-renal artery PF-MVs, before being allowed to recovery for 2 weeks. Group 5 animals (n=6) were likewise treated with 0.9ml of intra-renal artery PF-MVs after 120 minutes of IRI but were recovered for 6 weeks.

Group (n=6)	120 minutes left renal ischemia	Recovery prior to Inulin Clearance	Intra-renal artery Injection (0.9ml)
1	X	2 weeks	None
2	√	2 weeks	Normal saline
3	√	6 weeks	Normal saline
4	√	2 weeks	400ng PF-MV
5	√	6 weeks	400ng PF-MV

Table 7.1: Summary of experimental groups. 6 rats were randomly assigned to each group.

7.33 SURGICAL PROCEDURE

Rodent anesthesia and positioning during surgery were conducted in standard fashion, as previously described in Sections 2.12 – 2.16.

Surgery was carried out as described in Section 5.33. Treatment groups received intra-renal artery therapy as outlined in Table 7.1. All intra-renal artery infusions were made up to a total volume of 0.9ml immediately prior to use.

7.34 POST-SURGICAL CARE

This was carried out in standard fashion as described in Section 2.16.

7.35 QUANTIFICATION OF RENAL FUNCTION AND RENAL INJURY

At day 14, animals in Groups 1, 2, and 4 underwent inulin clearance studies in order accurately to characterize the function of the remaining kidney, according to the standard protocol described in Section 2.2. Group 3 and Group 5 animals underwent inulin clearance studies 6 weeks after ischemia, again according to standard protocol. Left kidneys and right kidneys were weighed, before being stored in 10% formalin and RNA later solution for later analysis.

Formalin preserved kidneys were paraffin embedded and sectioned as described in Section 2.3 Histological analysis and immunohistochemistry for P16, P21 and Ki 67 were carried out as described in Sections 2.3 and 2.4

7.36 PATHFINDER DERIVED MICROVESICLE ISOLATION

This was achieved in a standard fashion as described in Section 2.6, to give an aliquot prepared in a minimal volume containing ~ 400ng of microvesicles. This sample was made up to 0.9ml in normal saline immediately prior to intra-renal artery infusion.

7.4 RESULTS

7.41 ANIMAL DATA AFTER 2 WEEKS RECOVERY POST SURGERY

GROUP WEIGHT LOSS COMPARED TO BASELINE WEIGHT

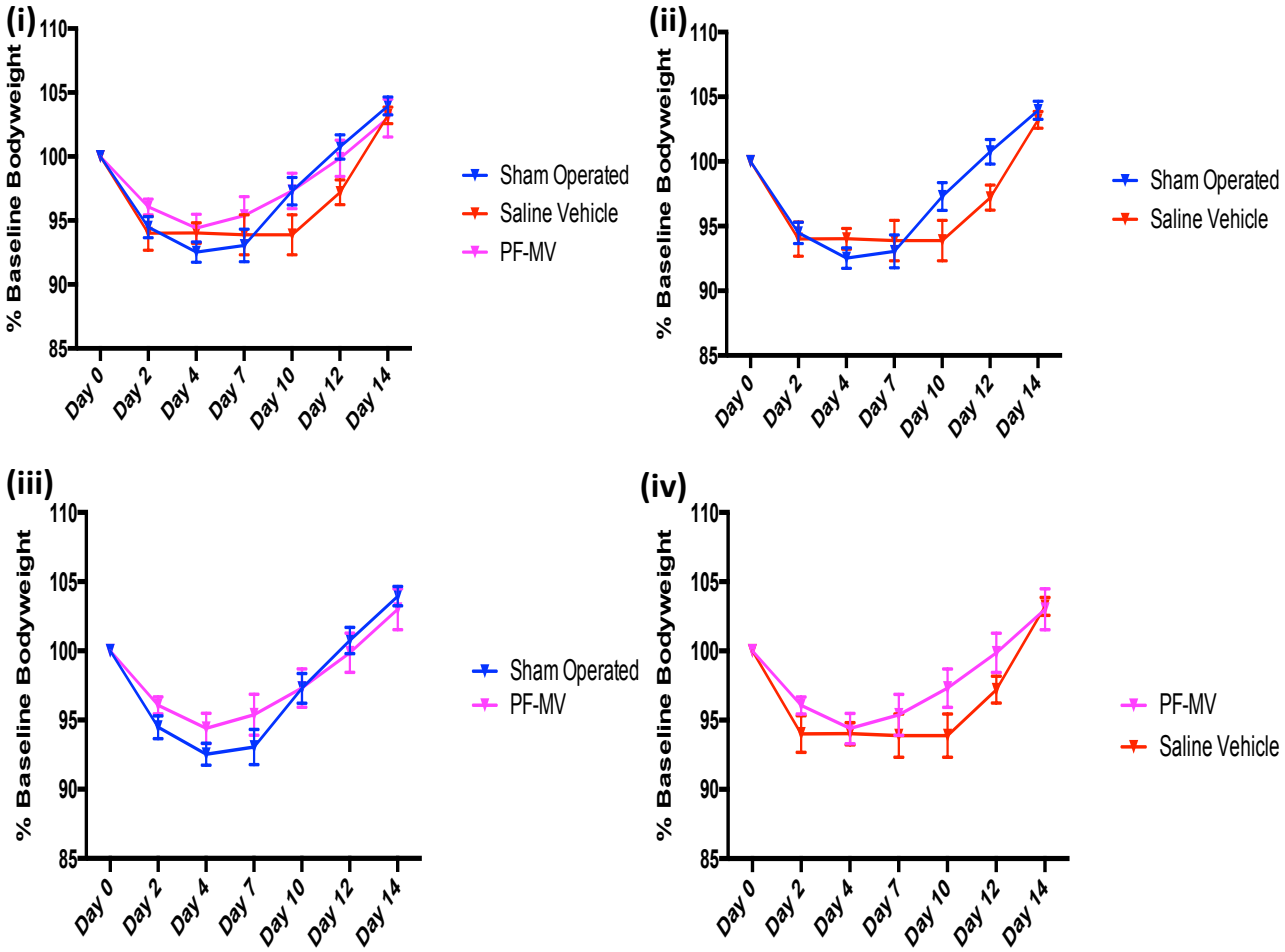


Figure 7.1: There were no significant differences in % baseline body weight loss between sham-operated, saline treated or PF-MV treated animals at any time point postoperatively.
Statistical analysis by 1 way ANOVA with Tukey multiple comparisons test

7.42 GLOMERULAR FILTRATION RATE

Effect on GFR after PF-MV Administration after 2 weeks recovery

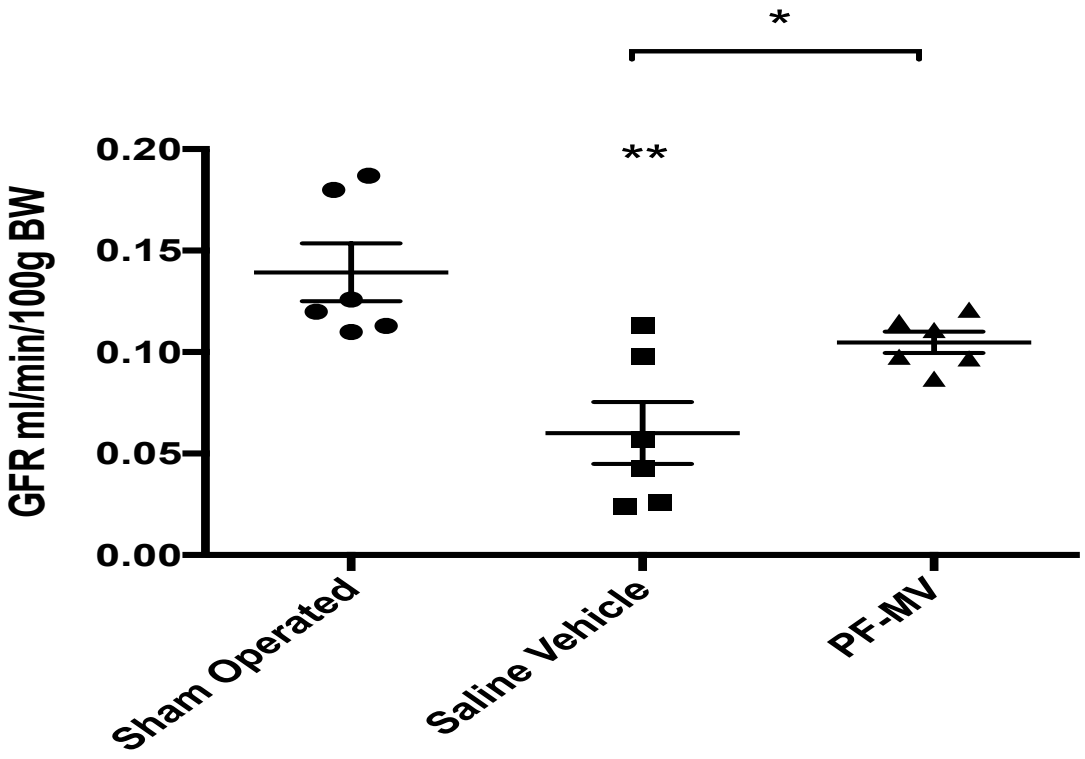


Figure 7.2: Sham-operated animals had a higher GFR than animals subjected to 120 minutes of ischemia and treated with saline (p=0.0022). A trend towards higher GFR in sham-operated animals vs PF-MV was observed, although this was not statistically significant (p=0.0623).
Treatment with PF-MVs protected GFR from ischemia when compared to treatment with saline alone (p=0.0201).
Statistical analysis by 1 way ANOVA with Tukey multiple comparisons test.

7.43 RENAL HISTOLOGY

7.431 Epithelial Breaks after 2 weeks recovery

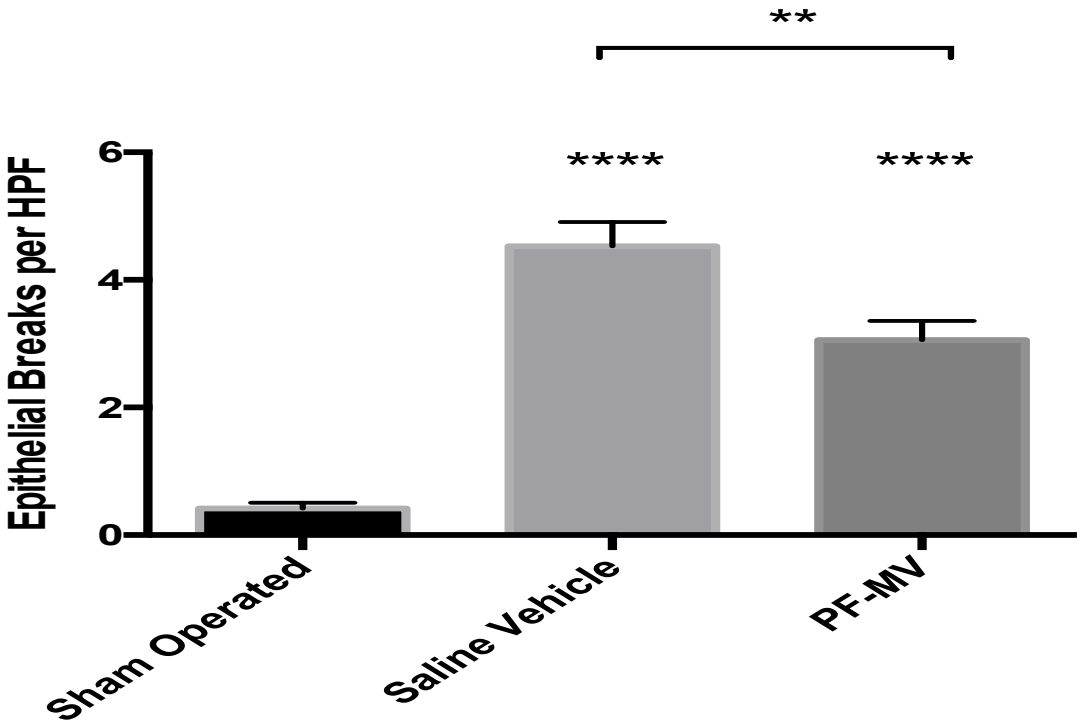


Figure 7.3: After 2 weeks recovery, sham-operated animals had significantly fewer epithelial breaks than animals subjected to 120 minutes of ischemia and treated with saline ($p<0.0001$) or PF-MV ($p<0.0001$).

Animals treated with PF-MV had fewer epithelial breaks than animals treated with saline alone ($p = 0.0012$).

Statistical analysis by 1 way ANOVA with Tukey multiple comparisons test.

7.432 Hyaline Cast Formation after 2 weeks recovery

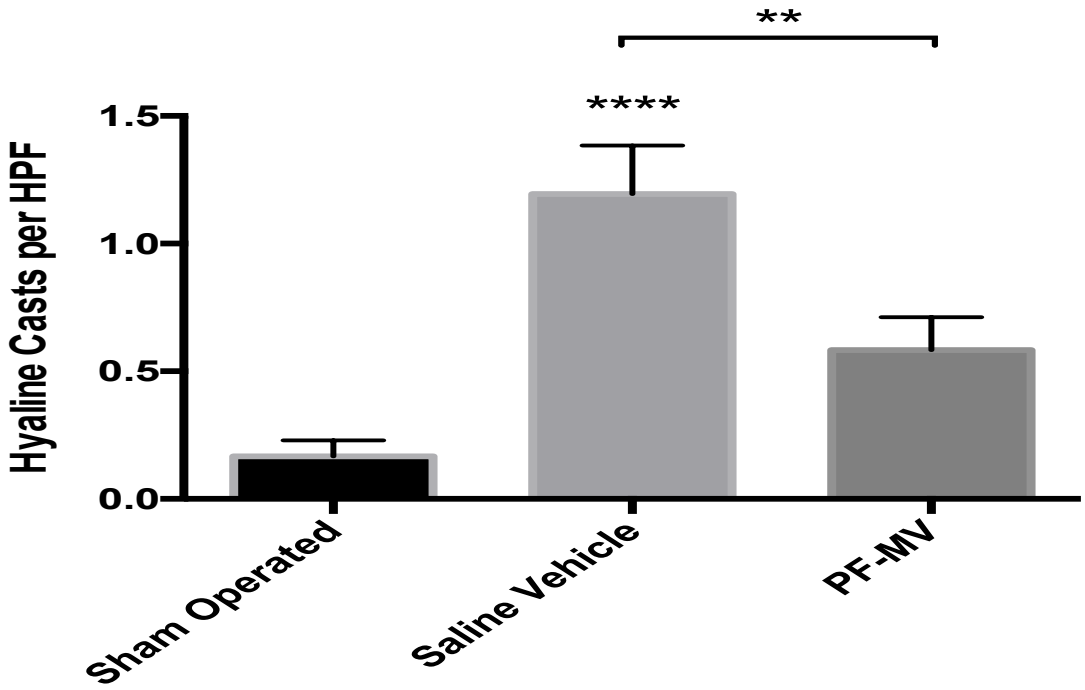


Figure7.4: Sham-operated animals had fewer hyaline casts vs animals treated with saline ($p<0.0001$). Although a trend towards fewer casts in sham-operated animals vs PF-MV treated animals was observed, this was not statistically significant ($p=0.0860$).
PF-MV treated animals had fewer hyaline casts than those treated with saline alone ($p=0.0061$). Statistical analysis by 1 way ANOVA with Tukey multiple comparisons test.

7.44 ANIMAL DATA AFTER 6 WEEKS RECOVERY POST SURGERY GROUP WEIGHT LOSS COMPARED TO BASELINE WEIGHT

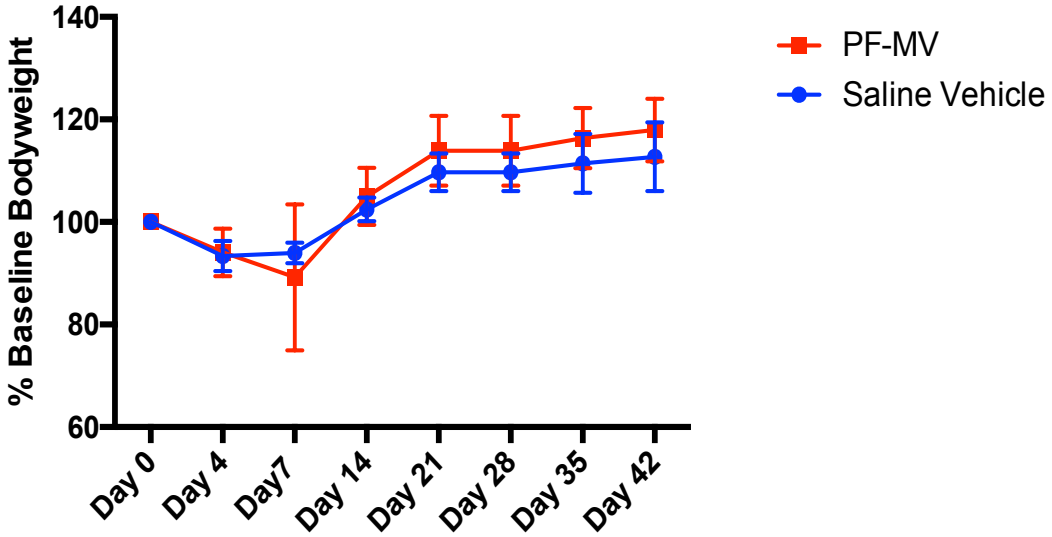


Figure 7.5: Both saline and PF-MV treated animals lost significant weight from baseline in the first week following surgery. Whilst saline treated animals remained below baseline weight at Day 7 ($p<0.0001$) and Day 10 ($p=0.033$), PF-MV treated animals had regained baseline weight by Day 7 ($p=0.0935$). Saline-treated animals regained baseline weight by Day 12 and thereafter both groups continued to gain weight until the experiment was terminated.
Statistical analysis by unpaired Students t test.

7.45 COMPARISION OF PERCENTAGE WEIGHT LOSS FROM BASELINE BETWEEN GROUPS

There was no significant difference between the weights of saline and PF-MV treated animals at any time point during the experiment (see figure 7.5).

7.46 GLOMERULAR FILTRATION RATE

Effect on GFR after PF-MV after 6 weeks recovery

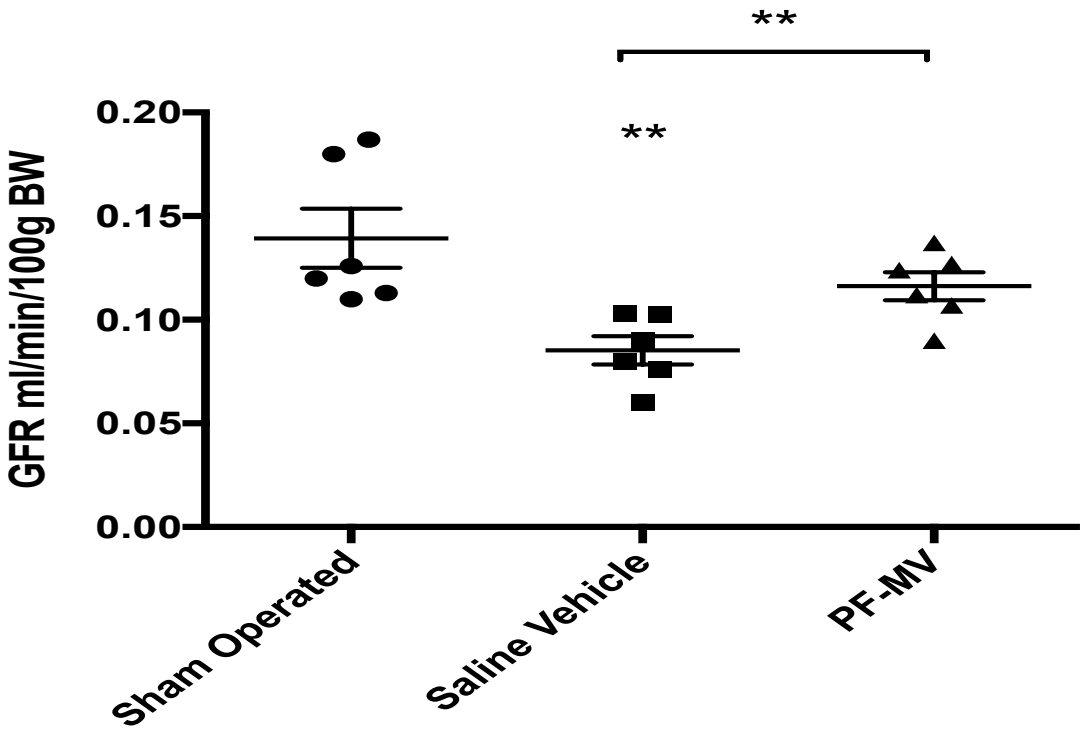


Figure 7.6: Sham-operated animals were found to have a higher left sided GFR than those animals subjected to 120 minutes of ischemia and treated with saline. (p=0.0041) There was no difference in the GFR of sham animals, and those treated with PF-MVs, after 6 weeks recovery (p=0.2541).
6 weeks post surgery, PF-MV treated animals had a significantly higher GFR compared to saline treated animals (p=0.0093).
Statistical analysis by 1 way ANOVA with Tukey multiple comparisons test.

7.47 RENAL HISTOLOGY SCORING

7.471 Epithelial Breaks after 6 weeks recovery

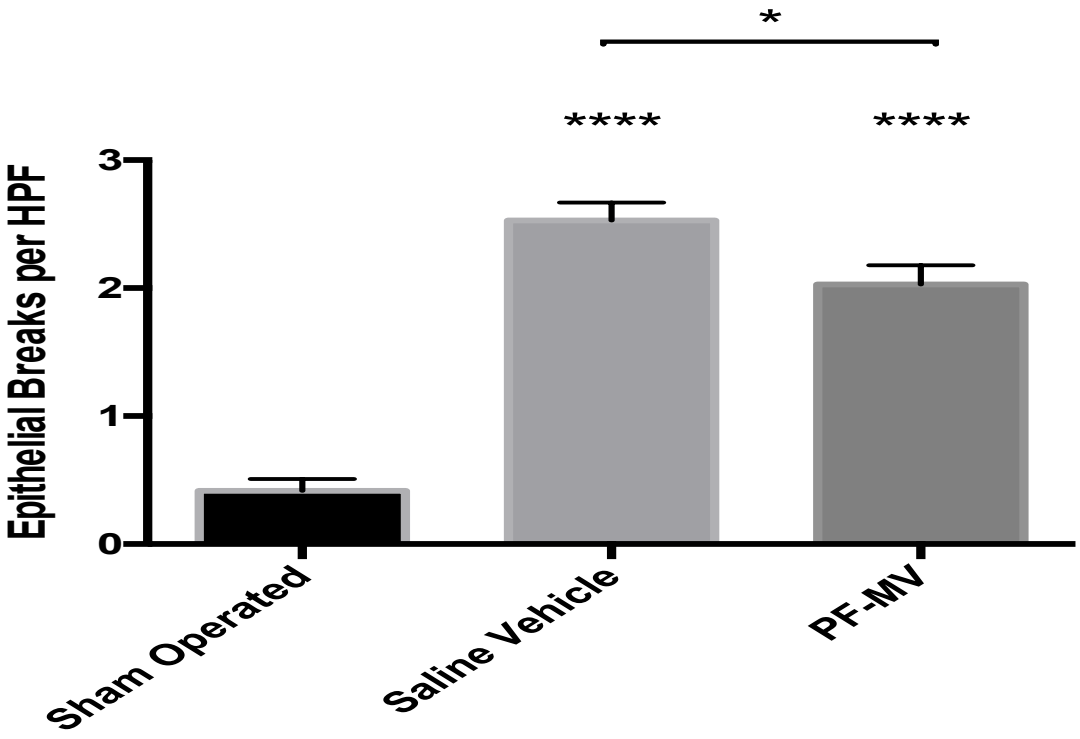


Figure 7.7: Sham-operated animals had few epithelial breaks compared to animals treated with saline ($p<0.0001$) and PF-MV ($p<0.0001$), 6 weeks after surgery.
PF-MV animals had fewer epithelial breaks than those animals treated with saline ($p=0.0217$).
Statistical analysis by 1 way ANOVA with Tukey multiple comparisons test.

7.472 Hyaline Cast Formation after 6 weeks recovery

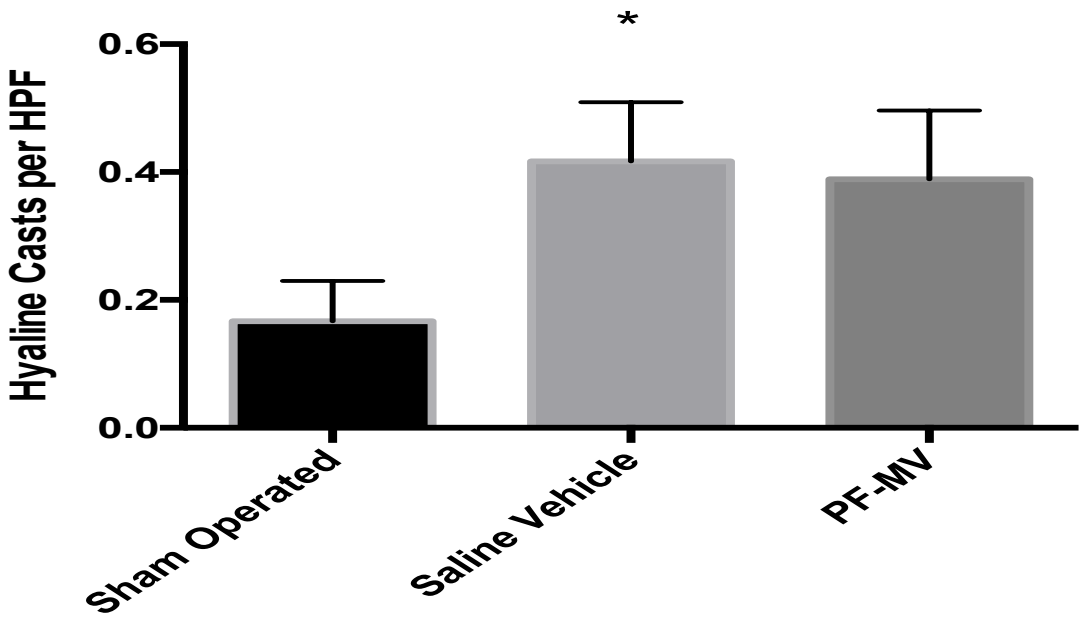


Figure 7.8: 6 weeks post surgery, sham-operated animals had fewer hyaline casts than animals treated with saline (p=0.0285). A trend was observed towards fewer casts in sham animals than those treated with PF-MV, although this was not statistically significant (p=0.0788).
6 weeks post surgery, there was no difference in hyaline cast number between animals treated with saline and those treated with PF-MV (p=0.8452).
Statistical analysis by 1 way ANOVA with Tukey multiple comparisons test.

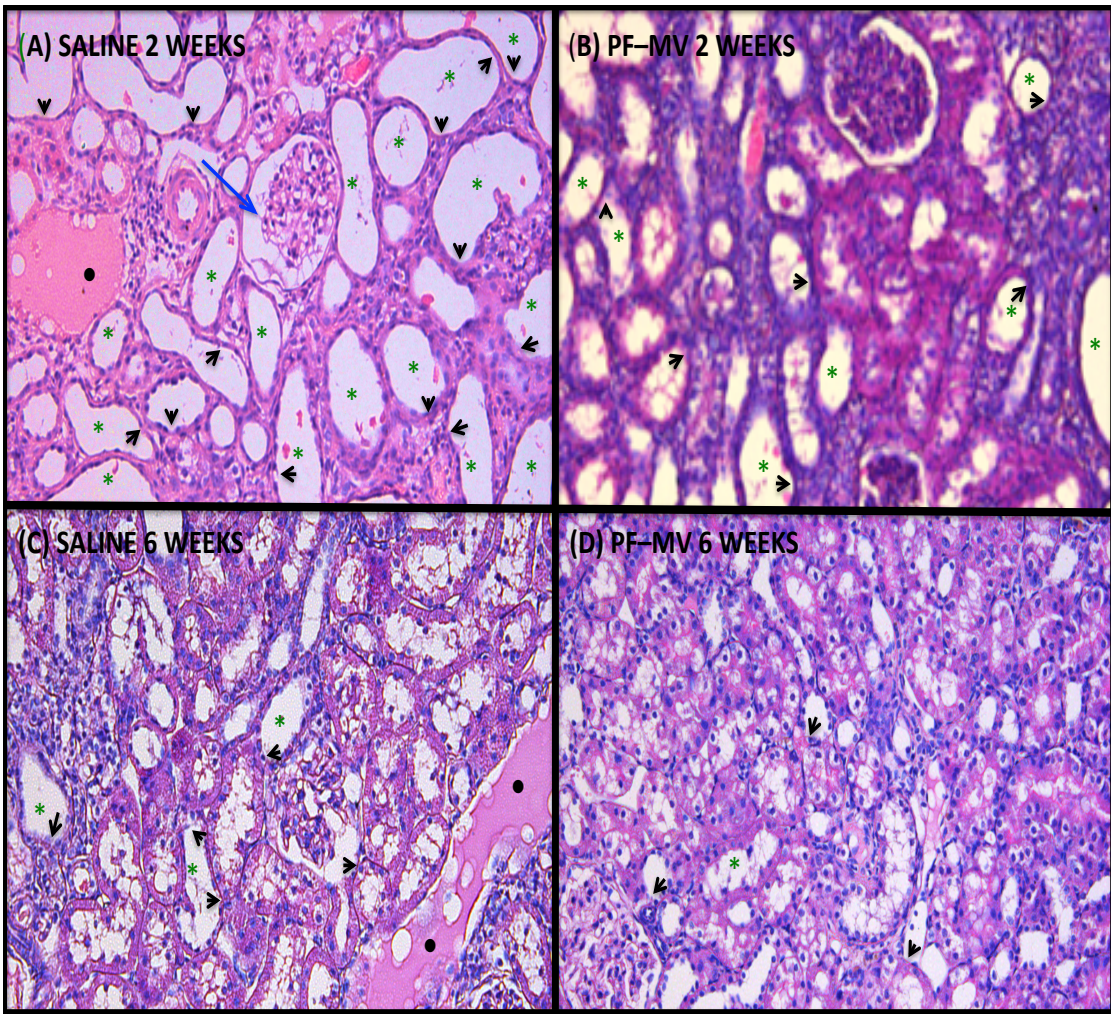


Figure 7.9: **(a)** Histology from a saline-treated rat subjected to 120 minutes of warm ischemia, 2 weeks post surgery. There is marked tubular dilation (green asterisk), with epithelial thinning and breaks (black arrowheads). A glomerulus with an increase in Bowman's space is marked with a blue arrow. Hyaline casts are marked with black dots. (b) Tissue from a PF-MV treated rat 2 weeks after ischemia. There is less disruption to renal histology compared to saline treated animals. **(c)** Tissue from a rat subjected to 120 minutes of warm ischemia, 6 weeks post surgery. Tubular dilation and epithelial breaks are less pronounced than 2 weeks post surgery, and there are fewer hyaline casts present. **(d)** Tissue from a rat treated with PF-MV, 6 weeks post surgery. Compared to saline treated animals, tubular dilation and epithelial breaks are less marked.

7.5 DISCUSSION

7.51 Stem Cell versus Cell-free therapy

Stem cell therapy, whilst potentially conferring a considerable benefit as a novel treatment for the protection and rejuvenation of damaged organs, remains associated with a number of serious possible side effects. In this thesis, the findings of Chapter 3, where disseminated malignancy as a result of cellular therapy, and Chapter 6 where supra-therapeutic doses of cells produced arterial occlusion, highlight some of the concerns raised previously regarding stem cells as a novel treatment. Cell-free therapy theoretically has a reduced risk of inducing malignancy in recipients, and due to the small size of ECVs, microcirculation occlusion in organs treated with local arterial infusion is thought to be extremely unlikely (Rani et al., 2015). Furthermore, if the active and beneficial components contained within ECVs can be identified, manufacture of a synthetic and pure drug may be possible. However, in order to achieve this, it is necessary to demonstrate convincingly that ECV therapy produces long-term benefit, whilst identifying the biologically active components of the ECV fraction that are responsible for this.

The experiment described here demonstrates that cell-free therapy has the potential to ameliorate severe renal IRI, with the preservation of renal morphology ultimately associated with improved long-term renal function as determined by GFR. While the results of ECV therapy in renal IRI have been described by previous studies, this is the first account describing the effect of Pathfinder-derived ECVs on renal IRI. Furthermore, for the treatment of renal IRI, this appears to be the first application of a preparation containing a truly 'purified' microvesicle fraction, obtained from any particular stem cell source.

ECVs derived from BD-MSCs were the first cell-free preparations to be tested in models of acute kidney injury. Bruno et al showed a single intravenous injection of BD-MSC derived ECVs were able to accelerate structural and functional recovery in a murine model of glycerol-induced AKI (Bruno et al., 2009b). Importantly, the effects observed were comparable to those obtained with the cells of origin, indicating that ECVs might replicate the beneficial effects reported with MSC therapy. Gatti et al would later report that a single intravenous dose of ECVs, again derived from BD-

MSC, protected the renal function and morphology of rats subjected to contralateral nephrectomy and 45 minutes of unilateral ischemia (Gatti et al., 2011). Molecular data presented suggests that shortly after injury, treated animals renal histology exhibit reduced leucocyte infiltration, a down-regulated inflammatory response leading to less tubular epithelial cell apoptosis and proliferation in response to injury. Long-term structural changes in renal morphology, such as glomerulosclerosis and tubulointerstitial were consequently reduced with the protection of long-term renal function. They also demonstrated that treatment of ECVs with RNAase prior to injection abolished ECV efficacy, implying that the RNA cargo played a vital role in conferring the beneficial effects seen with ECVs.

In a similar rat model of renal IRI, after administering ECVs derived from endothelial progenitor cells, Cantaluppi et al observed preserved renal function and histology as a result of ECV therapy, with progression to chronic renal failure prevented in treated animals (Cantaluppi et al., 2012). The observed effects were again abolished by RNAase treatment.

The lack of efficacy reported with ECVs subjected to RNAase treatment suggests a crucial role of RNA in the mechanism by which ECV convey renoprotection. When compared to the parent cell RNA content, MSC-derived ECVs have been shown to be enriched in small non-coding RNAs, but also contain 239 mRNAs that are involved in cell differentiation, transcription, cell proliferation and immune regulation (Eirin et al., 2014, Bruno et al., 2009b).

Furthermore, ECVs contain concentrated levels of microRNAs (miRNAs). miRNAs are non-coding RNA molecules that typically consist of 22 nucleotides. They are highly conserved, negative regulators of gene expression that act on the post-transcriptional level by binding to complementary sequences of mRNA in recipient cells. This inhibits the translation of mRNA and promotes mRNA degradation (Bartel, 2004). It is postulated that by silencing mRNA translation, the horizontal transfer of miRNA by ECVs may down-regulate recipient cell injury response, and in particular the inflammatory cascade.

The importance of miRNA in the renoprotective effect of ECVs was demonstrated by Cantaluppi et al (Cantaluppi et al., 2012), and this finding was later replicated by Collina et al (Collino et al., 2015). Dicer and Drosha are miRNA processing enzymes that play a crucial role in the maturation of miRNAs (Kuehbacher et al., 2007). Application of either Dicer (Cantaluppi et al., 2012) or Dosha (Collino et al., 2015) knock-down in progenitor cells from which ECVs were harvested, resulted in the abolition of the renoprotection previously observed with ECVs from normal progenitor cells. In addition, depletion of specific miRNAs from ECVs characterized the importance of the proangiogenic miRNA-296 and miRNA-126 (Cantaluppi et al., 2012). These results support the assertion that via horizontal transfer within ECVs, miRNAs play a crucial role in reprogramming damaged resident renal cells to a regenerative state.

7.52 Which Extracellular Vesicle Type protects the Kidney - Exosomes or Microvesicles?

The results of this study corroborate the findings of McGuiness et al, who reported that within the ECV population derived from Pathfinder cells, it is the MV fraction that contains the biologically active cargo responsible for the protective/regenerative effects on injured cells (McGuinness et al., 2016). Prior to this experiment, the finding of McGuiness et al appears to be the only study that demonstrated therapeutic efficacy of a properly characterized population of microvesicles, whilst reporting the separated exosome fraction lacked biological effect (McGuinness et al., 2016). It is worth re-iterating that as described in Section 7.11, a number of previous studies have reported the protective effects of administering stem cell-derived 'microvesicles' to injured organs (Cantaluppi et al., 2012, Bruno et al., 2009b, Bruno et al., 2012, Gatti et al., 2011, Bonventre, 2009). However the protocols used in those studies is likely to have resulted in a mixed exosomes/microvesicle preparation according to the now widely accepted definition of ECVs used in this thesis, and it is impossible to conclude that the content of microvesicles alone was responsible for the observed protective effects.

The result of the current experiment – namely that 'true' microvesicles alone may ameliorate acute organ injury – disagrees with the previous reports in published literature. The general consensus is that exosomes are likely to contain the factors by

which stem cells produce their paracrine effects. The majority of reports have investigated ECVs obtained from MSCs that are of either a bone marrow or adipose origin (Han et al., 2016). Those studies that have attempted to differentiate between the effects of individual ECV fractions have concluded that MSC-derived exosomes are able to promote angiogenesis in ischemic tissue and ameliorate tissue damage after ischemic injury (Arslan et al., 2013, Bian et al., 2014, Xin et al., 2013, Hu et al., 2015, Lin et al., 2016).

Work investigating the effects of ECVs from other stem cell classes also report the exosome fraction conveys the observed beneficial effects of parent stem cells. Berger et al reported that exosomes derived from endothelial colony forming cells protected against renal IRI in a murine model (Burger et al., 2015). Furthermore, the same group recently reported that this effect appears to be mediated by the horizontal transfer of miRNA -486-5p, which they observe to be selectively enriched within the exosomes fraction (Vinas et al., 2016).

The apparent discrepancy regarding the efficacy of exosomes versus microvesicles may be explained by the parent stem cells source. It is well recognized that the content of ECVs is dependent upon the type of stem cell from which they originate, and the conditions in which those stem cells were kept prior to ECV harvest. For example, ECVs derived from fibroblasts contain little biologically active genetic material, and confer no organ protection compared to saline control in animals models of renal IRI (Quesenberry et al., 2014). Logically, it seems possible that pathfinder cells may produce their effects via the transfer of genetic material contained within microvesicles, but that other stem cell types produce their therapeutic effects predominantly via exosomes. Whilst studies have characterized a least some of the active miRNA classes within ECV fraction (Cantaluppi et al., 2012, Vinas et al., 2016), work is on-going to identify the responsible factors within the PF-MV preparation (McGuinness et al., 2016).

One clear weakness in this study is a lack of molecular data, which may have provided some insight into the mechanisms by which PF-MVs act.

Nevertheless, other studies using Pathfinder cells or PF-MVs have published molecular data that indicate the mechanisms by which the beneficial biological effects are obtained. McGlynn et al reported the reduced renal expression of $p16^{ink4a}$ in mice treated with Pathfinder cells 2 weeks after IRI to be associated with improved renal function and morphology (McGlynn et al., 2013). No difference in apoptosis, as measured by TUNEL staining was observed between treated and untreated animals, probably because the apoptotic process would have been completed 14 days post injury. Furthermore, Pathfinder cells, as demonstrated by FISH, were notable by their absence in treated kidneys. The authors concluded that via a paracrine mechanism, Pathfinder cells reduced cellular senescence in response to injury (McGlynn et al., 2013). This finding was in keeping with previous studies using Pathfinder cells, where reduced cellular senescence in damaged organs was been noted in Pathfinder treated animals, in the absence of Pathfinder engraftment (Stevenson et al., 2011). The authors concluded that Pathfinder cells, probably via material contained within microvesicles encourage organ repair through cell regeneration. In the kidney, this may be via up-regulated mechanisms including the de-differentiation of surviving tubular epithelial cells followed by proliferation to repopulate damaged tubules, as suggested by Bonventre et al (Bonventre, 2003).
Several different sources of ECVs have been reported to have similar efficacy at mitigating acute renal injury, culminating in the ability to reduce kidney fibrosis as a long-term outcome. However, this effect cannot yet be assigned to a particular molecular mechanism. This is perhaps unsurprising when considering the content of ECVs reflects the phenotype of the parent cell and consequently, the cargo within ECVs of different origin is may be markedly different (Quesenberry et al., 2014). There is no evidence to suggest that Pathfinder mediated protection is produced by an anti-inflammatory action. This is in stark contrast to the postulated action of MSCs, where there is now a wealth of evidence to support the anti-inflammatory and immune-modulatory properties of MSCs, which appear to be mediated by ECVs in a paracrine fashion.

Therefore, it is possible that MSCs and Pathfinder cells aid long-term recovery of organs after acute injury via different mechanisms. There is clear evidence that Pathfinder cells do this by the transfer of material in microvesicles, and that this reprograms cells to enter regenerative pathways and avoid a senescent state. In

contrast, MSCs appear to provide initial protection against injury by dampening local inflammatory responses and that this effect is primarily achieved via the horizontal transfer of genetic material contained within exosomes. However, a recent study observed that a combination of ADRCs *and* ADRC derived exosomes provided additional protection against renal IRI when compared to either therapy alone (Lin et al., 2016). This suggests that ADRC derived microvesicles act synergistically with ADRC derived exosomes to confer additional protection.

In conclusion, these experiments strongly suggest that ECV treatment has the potential to mitigate the unavoidable renal IRI that occurs during the transplantation process. Characterization of PF-MV content and obtaining molecular data to gain insight into the mechanisms by which PF-MV content produces effects are important next steps. Furthermore, experiments to investigate any possible synergistic effect of PF-MVs and ECVs derived from MSCs are warranted.

CHAPTER 8

THE EFFECT OF PATHFINDER DERIVED MICROVESICLES ON PROLONGED COLD AND WARM ISCHEMIC TIMES IN A NOVEL RAT MODEL OF RENAL TRANSPLANTATION

8.1 INTRODUCTION

There are few, if any, animal models that accurately reproduce the insults a renal allograft encounters during the journey from donor to recipient. Although animal models are a valuable tool for understanding the pathophysiological processes involved during renal IRI, the clinical translation of experimental results have several limitations, usually due to the anatomical and physiological differences between species. Nevertheless, animal models have accentuated our understanding of the insults transplanted kidneys encounter, and may yet a play a significant role in the search for therapeutic intervention (Becker and Hewitson, 2013).

Animal research requires investigators to continually refine experimental protocols so that the likelihood of gaining the knowledge sought is maximised, while animal suffering and a total number of laboratory animals used are reduced (Kobayashi et al., 2012, 2011b). Some of the shortcomings of commonly used animal models have been highlighted in this thesis – indeed these failings led to the development of a novel rat model of renal IRI described in Chapter 5. However, while IRI remains the leading cause of injury to transplanted kidneys and is accentuated in the increasingly used ECD and DCD kidneys, other acute insults combine to negatively influence long-term transplant outcomes (Jang et al., 2009). Such processes include hypoxia during cold storage, acute rejection, and calcineurin toxicity in the short and long-term (De Rosa et al., 2016).

Most preclinical renal IRI animal models use ischemia caused by occlusion of the renal artery to produce an acute kidney injury. While this does not exactly replicate the clinical situation, several pathways are up-regulated that are important participants in acute allograft injury. These include activation of the coagulation system (Thuillier et al., 2010), leukocyte infiltration (Versteilen et al., 2011), endothelium (Kwon et al., 2009) with overexpression of adhesion molecules (Kato et al., 2009), cytokine release, intrarenal vasoconstriction pathway and apoptosis (Saikumar and Venkatachalam, 2003). Nevertheless, these ‘traditional’ models of ischemia-reperfusion do not expose animals to immunological or pharmacological insults, nor do they subject injured kidneys to cold storage. In order to mimic this process and to investigate the ability of stem cell-based therapies to ameliorate renal injury that

results from such insults, animal models of kidney transplantation need to be employed (Becker and Hewitson, 2013, Lieberthal and Nigam, 2000, Saat et al., 2016, Masoud et al., 2012). However, renal transplantation is a technically more demanding exercise than warm renal IRI, introducing more variables into both control and experimental groups. It therefore seems reasonable to screen novel therapies for efficacy in IRI models, before progressing to experiments in models of transplantation.

8.11 WARM VERSUS COLD RENAL ISCHEMIA

Static cold storage is traditionally used to preserve kidneys *ex vivo* during the transplantation process. Throughout cold storage, the kidney is placed in a hypothermic solution designed to cool the kidney and by reducing cellular metabolism, the effect of on-going ischemia is minimised. Organs are usually cooled to 4^{o}C, as it has been shown that each 10^{o}C drop in temperature results in a 2-fold reduction in cellular metabolism (Lee and Mangino, 2009).

The decrease in free radicle formation encountered as a result of reduced cellular metabolism during static cold storage reduces cell depolarization and membrane destabilisation. However, while static cold perfusion lessens the degree of cell injury caused by ischemia, cold storage does not completely prevent it (Ponticelli, 2015). Reduced ATP production leads to dysfunction of the Na/K ATPase pump and calcium influx that eventually results in cell swelling and cell cycle arrest (Ponticelli, 2015). Also, ECD and DCD allografts tolerate cold anoxia poorly, with the detrimental impact of hypothermia demonstrated in several studies (Hamed et al., 2015, Kayler et al., 2011, Summers et al., 2013). Furthermore, the abrupt temperature shift from hypothermia to normothermia that occurs during reperfusion has been shown to result in mitochondrial dysfunction and up-regulate pro-apoptotic signal transduction (Minor et al., 2013).

Warm and cold ischemia are thought to produce structural injury in different areas of the kidney by distinct pathological processes. Warm ischemic injury, characterised by an interruption in renal blood flow, produces tissue hypoxia as a principle insult. The resulting alteration in the medullary microcirculation causes endothelial

dysfunction and proximal tubular injury, principally in the cortico-medually junction (also referred to as the outer stripe of the medulla) (Heyman SN, 2010).

The recognised mechanism of cold ischemic injury is also related to the disturbance in osmoregulation, energetics, and aerobic metabolism. Dysfunction of the Na/K ATPase pump results in higher intracellular sodium concentrations and cell swelling. Ongoing anaerobic respiration results in lactic acid production and intracellular acidosis (Salahudeen, 2004). However, cold ischemia has been shown to produce preservation of the proximal tubules, although distal nephron injury is more prominent upon reperfusion. This produces an injury that is principally located in the inner medullary stripe, although damage to the outer medually stripe is observed if the period of cold ischemia is prolonged (Heyman SN, 2010). For this reason, cold-storage induced injury is a pivotal contributing factor to early graft dysfunction in renal transplant recipients, predisposing to DGF and the subsequent adverse outcomes that are associated with its occurrence (De Rosa et al., 2016).

Wang et al. examined the effect of ADRC administration in a rat model of cold renal IRI (Wang et al., 2013b). Using intra-renal and intravenous autologous ADRC injections after a 30-minute period of cold ischemia, protection of renal function and morphology was reported to be associated with a down-regulated inflammatory response in ADRC treated animals after 24 hours recovery (Wang et al., 2013b).

Interestingly, telomere-independent cellular senescence, as shown by elevated p16 expression has been associated with poor long-term renal allograft outcomes in humans and rodents (McGlynn et al., 2009, Koppelstaetter et al., 2008, Melk et al., 2005, Serrano et al., 1996). Braun et al reported that murine kidney transplants from donors lacking the $p16^{INKK4a}$ locus had reduced interstitial fibrosis and tubular atrophy compared to transplanted kidneys from wild-type controls (Braun et al., 2012). This suggests that cellular senescence as a result of IRI/immunological rejection leads to premature chronic allograft nephropathy and ultimately allograft failure, implying that therapy that reduces senescence may benefit transplant outcomes. McGlynn et al have previously demonstrated Pathfinder cell therapy to be associated with a reduction in p16 expression and serum creatinine levels in a murine model of renal IRI (McGlynn et al., 2013).

The results of experiments in the novel rat model of renal IRI suggest that PF-MVs and ADRCs protect renal function and structure against prolonged warm ischemia. In order to mimic the process of clinical transplantation, it was decided to investigate the ability of stem cell-based therapies to protect against both cold and warm ischemia by developing an animal model of transplantation.

8.12 CHOICE OF SPECIES IN ANIMAL MODELS OF RENAL TRANSPLANTATION

The first major decision during the development of an animal model is the selection of species involved. Essentially, smaller animal species, such as rodents, are easier to house and handle than larger animals but are anatomically distinct from humans, processing unilobular and unipapillary kidneys (Simmons et al., 2008). Unfortunately, the different vascular properties of the renal medulla contribute to variability in the pathophysiology of ischemic AKI between species. When novel therapies are applied to large animal models, replicating the findings from rodent experiments may consequently be difficult. This hampers clinical translation of new treatments. Rodent models of renal transplantation are therefore limited in the relevant information they provide by physiological and anatomical differences. Furthermore, the size constraints associated with rodent renal transplantation requires a skilled and experienced surgeon to provide consistent results and avoid multiple surgical failings (Khalifeh et al., 2015). Nevertheless, the relatively low-cost and ease of licensing compared to large animals makes rodent transplant models more practical. However, rodent IRI renal models remain more popular still, probably because conventional IRI models lack the requirement for vascular or ureteric anastomosis, and hence require less surgical expertise.

Larger animals more closely resemble human anatomy and physiology but are more expensive, labour intensive, and their use in experimental research is limited by ethical debate. Nevertheless, three large animal models have been used extensively in models of renal transplantation: dogs, pigs, and non-human primates. Of these, the pig has the significant advantage in that the public has little resentment for porcine research, leading to fewer ethical and legal restrictions on porcine studies (Dehoux and Gianello, 2007). Furthermore, pigs are reasonably similar to humans in size,

metabolism, immunology and renal anatomy (Kirk, 2003, Giraud et al., 2011, Rothkotter, 2009). While housing and husbandry of standard pigs are expensive and time-consuming, the advent of genetically modifiable mini-swine may change this situation (Lunney, 2007). Finally, pigs are easily bred, quickly bled and it is possible to repeatedly obtain biopsies during the experimental time course. This establishes the pig as the 'gold-standard' in renal transplantation research (Lunney, 2007). However, due to the expense, strict legislation and relative logistical difficulty of working with pigs, the numbers of reported studies using porcine models of renal transplantation are scarce when compared to those using rodents.

While large animal studies are essential before moving to clinical trials, this was not feasible with the research facilities available. With a view to the reducing the total number of animals experimented upon, it was decided to test novel therapies in a rodent transplant model after therapeutic effectiveness had been demonstrated in the novel rodent IRI model. It was hypothesised this might provide efficacy data, prior testing in porcine models with a view to clinical translation in pilot studies.

8.13 RODENT MODELS OF RENAL TRANSPLANTATION

With the purpose of investigating the ability of stem cell-based therapies to combat both cold and warm renal ischemia, we decided to develop a rat model of renal transplantation. Previous models of rodent transplantation have tended to use allografts from young healthy donors, exposed to only minimal cold and warm ischemic times. This is clearly not representative of the human population undergoing kidney transplantation. Furthermore, as described in Section 5.1, rat kidneys have the ability to recover long-term function from near fatal levels of ischemic injury. In order to investigate the long-term effects of novel treatments on severely injured kidneys, it was decided to subject the transplanted kidneys to prolonged periods of cold and warm ischemia prior to reperfusion. This has not been investigated previously, probably because prolonged transplant ischemic times are associated with high numbers of post-procedural animal deaths. It was therefore decided to leave the rat with a functioning native kidney to prevent death from acute renal failure and to calculate GFR by inulin clearance with ureteric cannulation as described in Chapter 2.

Rat kidney transplantation became established in the 1960s, and subsequently, several studies have reported surgical techniques that make the procedure easier to perform (Pahlavan et al., 2006, Schumacher et al., 2003, Spanjol et al., 2011). 2 methods of transplantation have been routinely described:

(1) *Heterotophic transplantation:* End to side anastomosis of the renal artery and vein onto the infra-renal abdominal aorta and inferior vena cava respectively, with clamps applied superiorly and inferiorly to the incision in the recipient vessel. Ureteric implantation is achieved via ureterovesical anastomosis. The transplanted kidney is left within the peritoneal cavity. (See Figure 8.1)

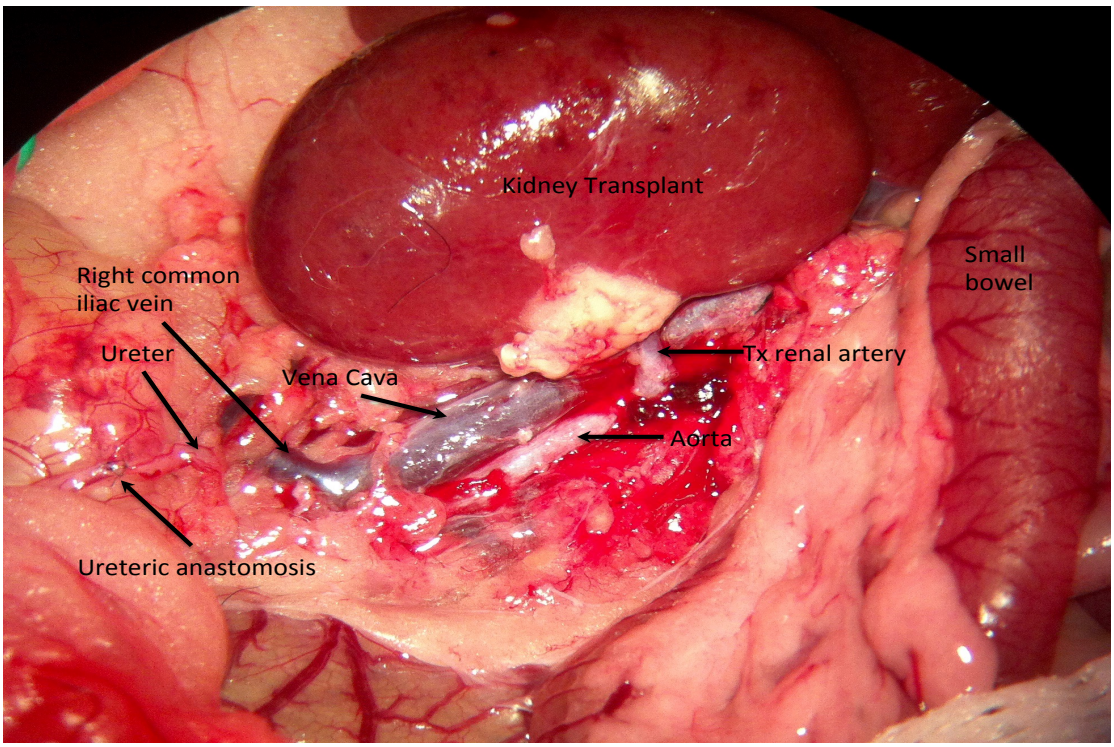


Figure 8.1: Heterotopic renal transplant, performed by the author during microsurgical training at Northwick Park Institute for Medical Research.

(2) Orthotopic transplantation: After left-sided nephrectomy, end-to-end anastomosis of the transplanted renal artery and vein onto the native renal vessels is performed. The kidney is placed within the native renal bed. Ureteric anastomosis between the transplant and native systems is made in an end-to-end fashion, sometimes over a ureteric stent (Herrero F 2004, Pietsch et al., 2004).

8.14 PREVIOUS STUDIES OF RENAL TRANSPLANTATION IN RODENTS

Some studies using MSC therapy have focused investigation upon immunomodulation and the ability of MSCs to protect against rejection, while others have examined the effects of cellular therapy against IRI +/- rejection. Reviewing the findings of these reports provides insight into the potential of cell-free therapy to mitigate injury occurring in the peri-transplant period.

8.15 MSCs AND IMMUNOLOGICAL REJECTION IN KIDNEY TRANSPLANT MODELS

De Martino et al. investigated the immunomodulatory effects of allogeneic MSCs after both syngeneic and allogeneic rat kidney transplantation (De Martino et al., 2010). MSC therapy was shown to reduce renal tubular damage by reducing inflammatory cell infiltrate in both models of transplantation (De Martino et al., 2010). This implies that acute cell-mediated rejection is mitigated by MSCs in the allogeneic transplantation group, while in syngeneic transplanted animals, enhanced immunogenicity as a result of IRI is also ameliorated by MSC treatment.

Franquesa et al. utilized the Fischer to Lewis rat transplant model of chronic allograft nephropathy to investigate the effects of BD-MSCs given intravenously 11 weeks post transplantation (Franquesa et al., 2012b). For the first 15 postoperative days, rats were treated with cyclosporine A to reduce the incidence of acute rejection. Transplanted kidneys were subjected to 150 minutes of cold ischemia, although the length of warm ischemia was not reported. After 24 weeks, MSC therapy was associated with a down-regulated inflammatory response with reduced expression of inflammatory cytokines and fewer macrophages infiltrating the renal parenchyma. Consequently, MSC treated animals were shown to have improved serum creatinine levels, superior renal histology and reduced renal fibrosis (Franquesa et al., 2012b).

Using a rat transplant model, Zhang et al. investigated the effects of combined cyclosporin A with multiple post-transplant BM-MSC intravenous injections (Zhang et al., 2007). They observed that combined cyclosporin A and BM-MSC therapy preserved short-term transplant function better than monotherapy with either treatment alone, but ultimately allograft longevity was not superior to any combination. However, as this Wistar into Lewis transplant model utilised minimal

renal ischemia and predominantly caused renal dysfunction by acute cell-mediated rejection, this finding is unsurprising. It may be that any initial renal IRI was mitigated by MSC therapy, but that ultimately immunological rejection was too powerful to be affected by either cyclosporin A or MSC administration.

8.16 MSCs AND PREVENTION OF IRI IN RODENT KIDNEY TRANSPLANT MODELS

Iwal et al investigated the effects of autologous ADRC infusion in a syngeneic rat kidney transplant model, which was published around the conclusion of this experiment. Iwal et al concluded that *ex vivo* intrarenal artery infusion of 1×10^6 ADRCs was associated with significantly improved renal function after 60 minutes of cold and 60 minutes of warm ischemia (Iwai et al., 2014). Furthermore, it was noted that intra-renal artery infusion of ADRCs, although not significantly better at protecting renal function and histology than intravenous administration, was not associated with complications previously reported with systemic administration (Iwai et al., 2014).

Hara et al. investigated the effects of BD-MSCs on renal IRI following 24 hours of cold ischemia to study the effects of prolonged cold ischemia on graft immunogenicity (Hara et al., 2011). Using a robust model of acute rat kidney rejection, they reported intravenous administration of autologous BD-MSCs to be associated with suppression of pro-inflammatory cytokines and down-regulation of adhesion molecules and chemokines. In keeping with previous studies (Zonta et al., 2010, Schrepfer et al., 2007) they noted that the vast majority of administered cells became entrapped within the pulmonary circulation and only a few reached the transplanted kidney. They concluded that BD-MSC administration was able to reduce graft immunogenicity following prolonged cold storage, and speculated that this effect was due to the inhibition of immune cell migration into the transplanted kidney. Furthermore, they concluded that BD-MSC efficacy was likely mediated via a paracrine effect (Hara et al., 2011). Unfortunately, they did not report the period of warm ischemia that the transplanted kidney was subjected to.

8.17 RATIONALE FOR RAT TRANSPLANT MODEL USED IN THIS EXPERIMENT

Previous experiments in Chapter 5 had indicated prolonged ischemic times are necessary to produce a long-standing renal injury - an essential requirement when investigating the ability of novel treatments to protect renal function in medium to long term. However, rodents have been shown to lack the artery of Adamkiewicz, a branch of the posterior intercostal artery which in man typically arises on the left at the level of the 9^{th} – 12^{th} intercostal artery (Schievink et al., 1988). This vessel is necessary to provide collateral spinal blood supply during aortic cross-clamping. Lacking this vessel, rodents undergoing prolonged suprarenal aortic cross-clamping are reported to suffer high rates of postoperative spinal paralysis due to spinal cord ischemia (Taira and Marsala, 1996, Awad et al., 2010). After discussion with experts at Northwick Park Microsurgical Institute, London, it was decided to develop a transplant model using an orthotopic transplant model, which would necessitate clamping of the renal vessels only. Syngeneic rats were used as both donors and recipients to remove immunological rejection as a confounding factor. Again, novel therapy was administered via the transplant renal artery immediately prior to reperfusion to avoid systemic distribution. Infusion of MSCs via the renal artery has previously been shown to be superior to intravenous therapy in a rat transplant model (Zonta et al., 2010).

8.2 HYPOTHESIS

(1) Prolonged periods of cold and warm ischemia will result in a predictable reduction in long-term function and histology of a renal allograft, transplanted between syngeneic rats.

(2) Administration of therapy, previously shown to mitigate warm renal IRI may improve long-term transplant function and histology.

8.3 METHODS

8.31 ANIMAL HOUSING AND HUSBANDRY

Male Fisher 344 rats, aged 12 - 14 weeks and weighing 225 – 250g were sourced from Harlan UK Ltd. Animals were housed in the JRF, University of Glasgow for at least two weeks before surgery to allow the animals to equilibrate with their new environment. Animals were housed and fed as described in Section 2.1. During equilibration before surgery, animals put on weight such that on the day of surgery, rats weighing 250 – 320g were used.

8.32 GROUP SELECTION

6 animals were randomly assigned to both control and treatment groups. Donor animals were randomly selected from the housed rat population on the day of surgery. Transplanted kidneys in control animals were treated with intra-renal artery saline vehicle infusion only, 40 minutes prior to graft reperfusion. Transplanted kidneys in treatment group animals were given an intra-renal artery infusion of PF-MVs, given exactly 40 minutes prior to transplant reperfusion.
Post surgery, animals were weighed daily and assessed for signs of distress or illness.

The first 2 successfully transplanted animals, after being subjected to 120 minutes of cold ischemia and 120 minutes of warm ischemia, were treated with saline vehicle. The next 2 animals were treated with PF-MVs. Thereafter transplanted animals were alternatively treated with saline vehicle or PF-MVs in an attempt to avoid skewed outcomes as a result of improved surgical technique.

8.331 KIDNEY TRANSPLANTATION SURGICAL PROCEDURE

Rodent anaesthesia, positioning during surgery and post-surgical care were conducted in standard fashion, as previously described in Sections 2.12 – 2.16.

8.332 Retrieval of Donor Kidney

Via a long midline incision, the left-sided retroperitoneal structures were exposed by mobilising the left colon. Damp swabs were placed to retract small and large bowel and protect the donor kidney.

To aid transplantation, it was helpful to have the maximum length of the artery, vein and ureter attached to the donor kidney. Therefore, the full length of the donor left renal artery was dissected from the renal hilum to the origin on the abdominal aorta. The suprarenal artery was divided with cautery. Gentle handling with a vascular sloop and the direct application of 1% lignocaine prevented spasm of the renal artery. The full length of the left renal vein was dissected from the renal hilum to the termination of the vein on the inferior vena cava. The major branches (suprarenal, gonadal and lumbar veins) were ligated with 9/0 nylon and divided. The left ureter was dissected free of its attachments from the renal hilum and dissected free distal to the crossing of the ureter over the bifurcation of the iliac vessels. The ureter was then transected as distally as possible, and the divided end retracted proximally, out of harm's way. Once this was achieved, the kidney itself was dissected free from the renal bed, by sharply incising its fascial attachments.

At this point, the kidney had not been subjected to any ischemia and was attached to the donor only by the left renal artery and vein. (See figure 8.2i)
A ligature was placed on the renal artery at its origin on the aorta, signifying the start of the ischemic period. (Figure 8.2ii) An arteriotomy was made, and a cannula was placed in the artery. Using a pre-placed vascular sloop to prevent leakage, the renal artery was flushed with ice-cold University of Wisconsin solution (UW), thereby flushing the static blood out of the kidney. (See Figure 8.2iii)
This typically required a flush of around 4ml of fluid. The return of this cold fluid to the donor circulation, by the left renal vein, universally resulted in donor cardiac arrest.

Once the kidney was cooled and adequately flushed, a venotomy was made in the left renal vein (8.2iv) and a loop-marking suture placed in the anterior wall. (8.3v) This helped accurate approximation without twisting during transplant anastomosis. The vein was then entirely transected, and the kidney placed in ice-cold UW for 120 minutes exactly. The body of the donor rat was discarded.

8.333 Recipient Procedure

The recipient rat was anaesthetized and positioned in standard fashion, again taking full sterile procedures. A midline laparotomy was made, and again the left-sided retroperitoneal structures were exposed by mobilising the left colon. Damp swabs were placed to retract the bowel and spleen. The native left renal artery was mobilised along its entire length as described above. The native left renal vein was mobilised up to the origin of the left gonadal vein. The native left ureter was minimally dissected from the left renal hilum. The kidney's attachments were fully excised to mobilise the kidney.

The renal artery clamped at its origin and divided distally to preserve the maximum length of the native artery. The renal vein was clamped distal to the left gonadal vein and divided at the renal hilum. The native ureter was divided as it exited the renal hilum, again to preserve the maximum possible length. The kidney was removed from the renal bed and discarded. The native vessels were flushed with saline in a retrograde fashion to eliminate any clots. (Figure 8.3vi)
After exactly 120mins of cold ischemia, the transplant kidney was placed in the recipient left renal bed (Figure 8.3vii). This signified the start of the warm ischemic period. The kidney was protected with saline soaked swabs.

Anastomosis of the vessels was begun. Firstly the native and transplant renal veins were anastomosed with 10/0 interrupted nylon sutures in a standard fashion using 16 stitches. For this, the two loop-marking sutures helped significantly in ensuring the renal vein was sutured in the correct orientation, without twisting or catching the back wall. (Figure 8.3viii) Venous anastomosis typically took 45 – 50 minutes to complete, and attention was then turned to the renal artery. This was anastomosed as described in Section 5.33. 40 minutes prior to reperfusion, the kidney was treated with 0.9ml of either saline vehicle or PF-MVs via intra-renal artery infusion, as described in Section 5.33. After exactly 120 minutes of warm ischemia, the vascular clamps were removed to re-perfuse the transplanted kidney. (Figure 8.4x)

Attention was then turned to ureteric anastomosis which was conducted as previously described with slight modifications (Herrero F 2004). Briefly, a 6/0 nylon stent was cauterised slightly to blunt each end and placed in native and transplant ureter. (See

Figure 8.4xi). Over this, the ureter was anastomosed end to end with six equally spaced, interrupted 11/0 nylon sutures. The stent was removed after the final suture was placed, but before the final suture being tied.

The renal fascia was tacked over the transplanted kidney with interrupted 10/0 sutures to hold it in the renal bed. The swabs were removed, and the intestines returned to the abdomen. The abdomen was closed with 4/0 continuous vicryl en mass, with un-dyed 4/0 vicryl to close the skin – as previously described in Section 5.33.

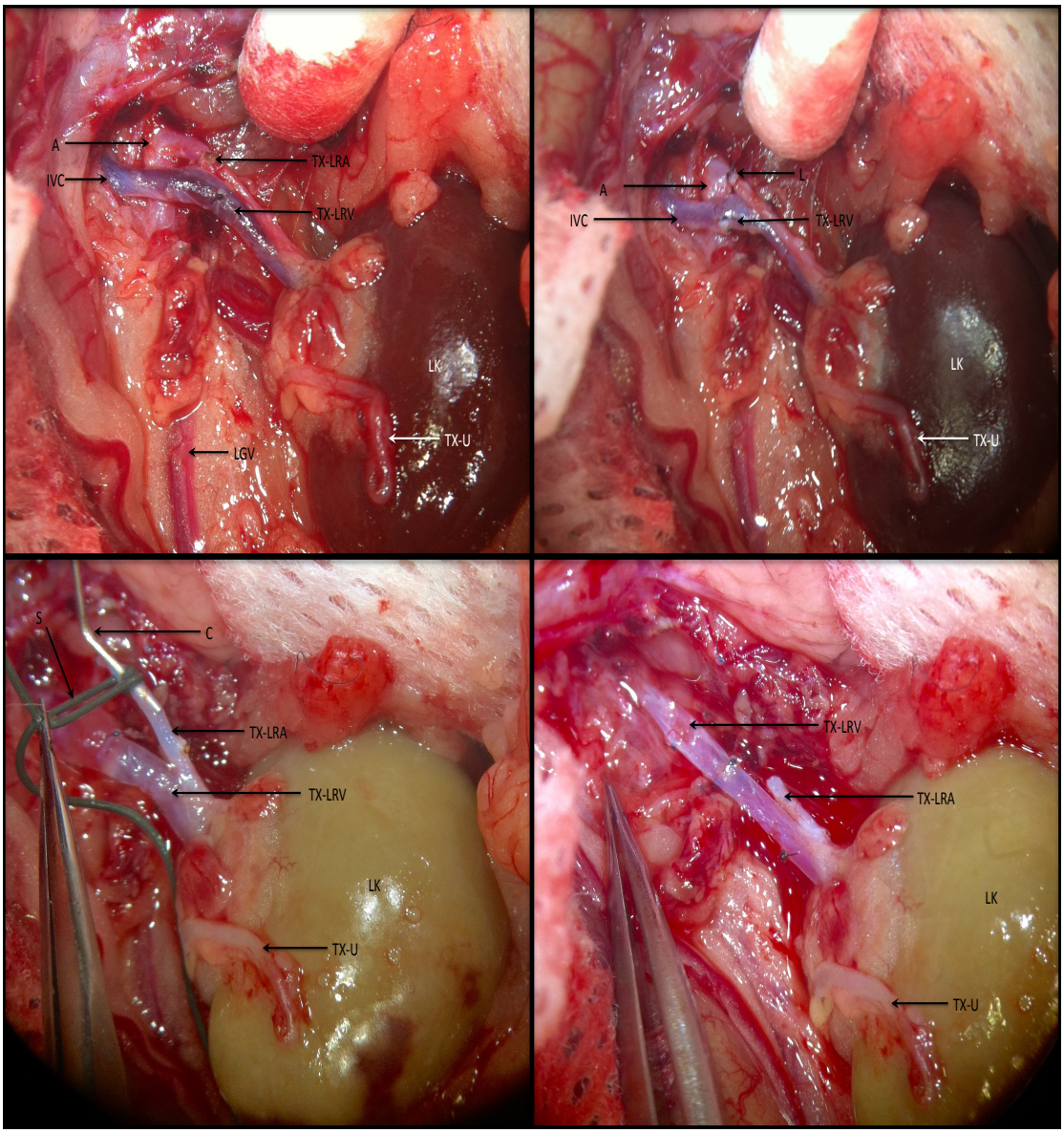


Figure 8.2: (i) The retroperitoneal structures in the donor rat are dissected to expose the transplant renal artery (TX-LRA) and renal vein (TX-LRV) and the allograft (LK). The transplant ureter (TX-U) is dissected to preserve the maximum length during the transplant process. The gonadal vessels are easily seen. (LGV) **(ii)** A ligature is placed on the renal artery is ligated at its origin on the aorta (A). The LK becomes discoloured at this point, signifying the start of the ischemic period. **(iii)** Via an arteriotomy, a cannula (C) is placed in the TX-LRA and secured with a vascular sloop (S) before the allograft is flushed with ice-cold University of Wisconsin solution. The TX-LRV bulges with UW solution and a colour change is seen in the kidney as the blood is expelled. **(iv)** The renal artery is transected at its origin. After a ligature is placed at the origin of the TX-LRV, a venotomy is made in the anterior wall of the TX-LRV.

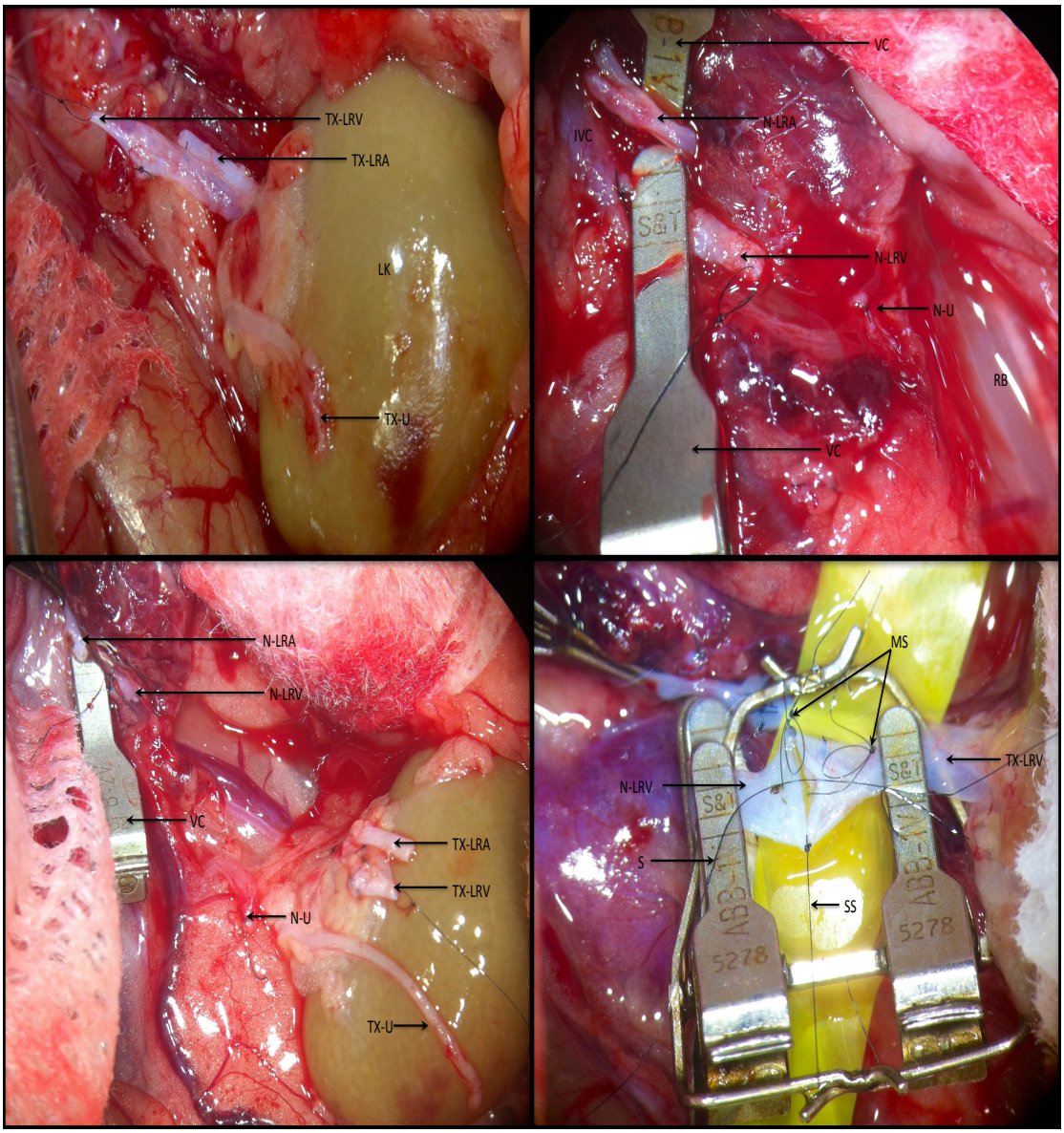


Figure 8.3: (v) A marking suture is placed in the anterior wall of the TX-LRV before the vein is completely transected, and the transplant removed and stored on ice in UW for 120 minutes. **(vi)** The retroperitoneum of the recipient rat is dissected, preserving the length of the native left renal artery (N-LRA), native left renal vein (N-LRV) and native left ureter (N-U). Vascular clamps (VC) are placed on the vessels. The native kidney is removed, leaving the renal bed (RB) free for the transplant. **(vii)** After exactly 120 minutes on ice, the transplant is placed in the recipient renal bed, and vascular anastomosis begins. **(viii)** The TX-LRV and N-LRV are placed in frame clamp, approximating the anterior vein walls using the marking sutures. Stay sutures (SS) are placed, and the vein anastomosed with interrupted 10/0 nylon. Yellow background material prevents damage to posterior structures during this process.

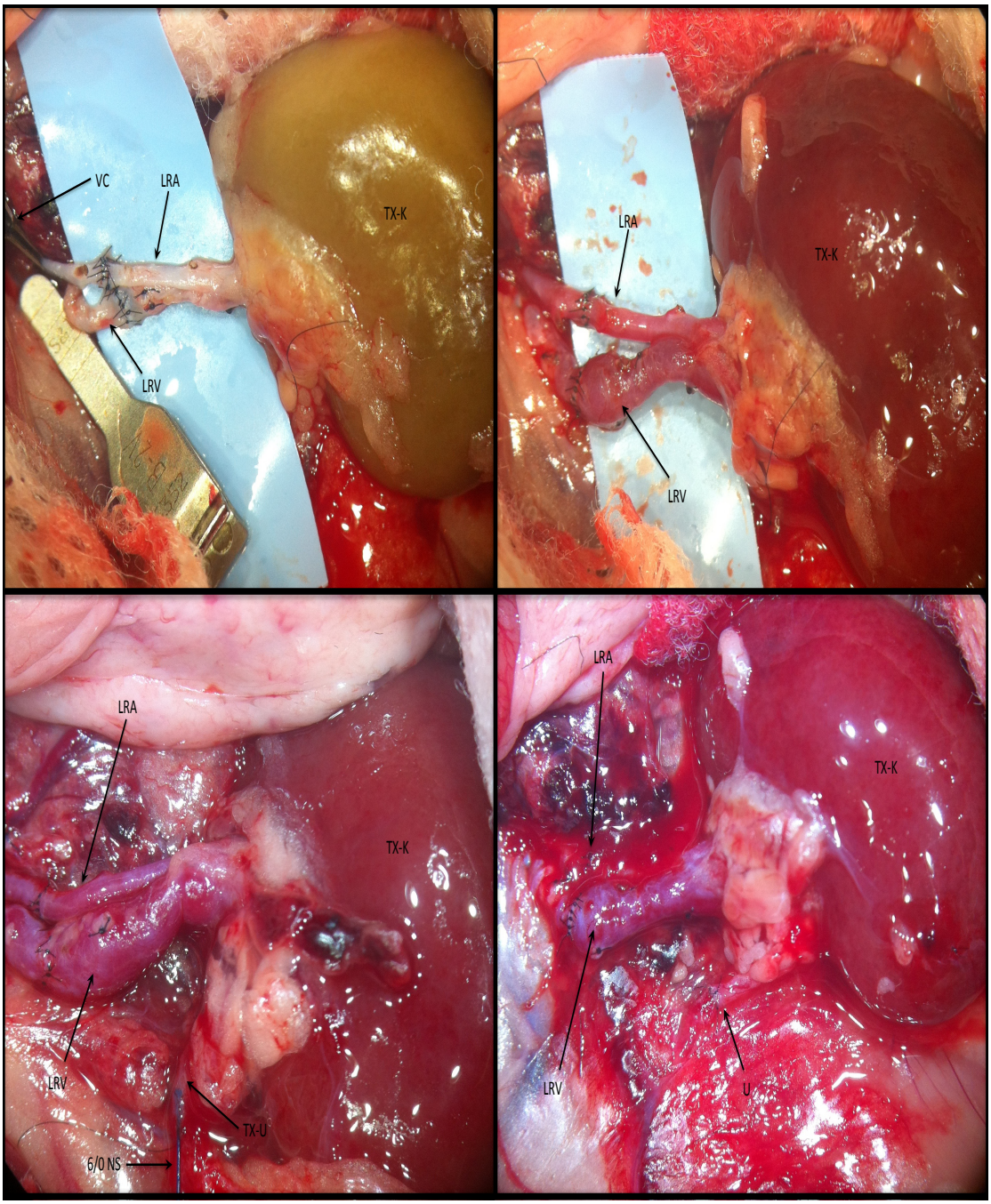


Figure 8.4: (ix) The renal artery and vein are anastomosed in standard fashion as described in Section 5.33. **(x)** The vascular clamps are removed after exactly 120 minutes of warm ischemia, and the transplant is reperfused. **(xi)** Attention is turned to the ureteric anastomosis. A 6.0 nylon stent (6/0 NS) is inserted into both transplant and native ureters to aid suture placement. It is removed before the anastomotic completion. **(xii)** The finished transplant before closure of renal fascia to secure the kidney in position.

8.34 POST-SURGICAL CARE

This was carried out in standard fashion as described in Section 2.16.

8.35 QUANTIFICATION OF RENAL FUNCTION AND RENAL INJURY

At day 14 animals underwent inulin clearance studies in order to accurately characterise the function of the transplanted kidney, according to the standard protocol described in Section 2.2. Both kidneys were weighed, before being stored in 10% formalin and RNA later solution for later analysis.

Formalin preserved kidneys were paraffin embedded and sectioned as described in Section 2.3 Histological analysis and immunohistochemistry for P16, P21 and Ki 67 were carried out as described in Sections 2.3 and 2.4

8.4 RESULTS

8.41 PILOT TRANSPLANTS

The first 2 animals to undergo transplantation were treated with saline and subjected to 120 minutes of cold ischemia, and 90 minutes of warm ischemia. (See figure 8.5) The transplant GFR values obtained from these 'pilot' studies appeared broadly comparable to GFR values from healthy animals, which suggested a possible lack of significant injury after 14 days recovery. Therefore, the warm ischemia time was increased to 120 minutes.

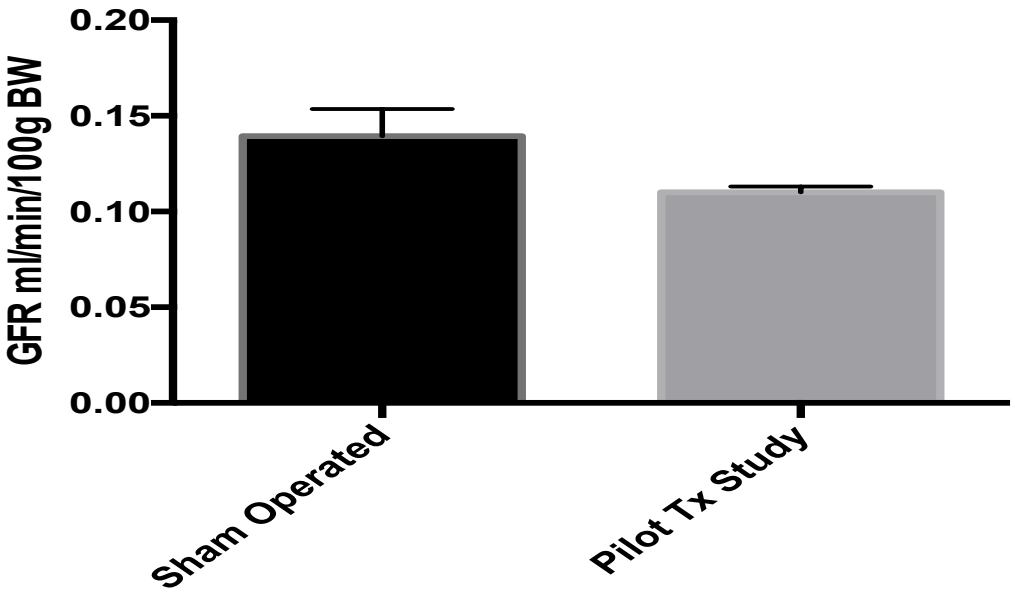


Figure 8.5: No significant difference was observed between the native renal function of sham-operated animals (n=6) and 'Pilot Study' transplanted kidneys subjected to 120 minutes of cold and 90 minutes of warm ischemia (n=2). Data after 2 weeks recovery post surgery.
Statistical analysis by unpaired Student's t-test.

8.42 POST-PROCEDURE MORTALITY RATES AND TECHNICAL COMPLICATIONS

5 of the first 6 animals to undergo transplantation with 120 minutes of cold and 120 minutes of warm ischemia did not survive 14 days post procedure.

Of these 5 animals:

• 1 animal lost >10% of its bodyweight by 7 days, and was culled. At post mortum, it was clear that there was ureteric obstruction of the transplanted kidney, due to dense inflammatory adhesions in the surgical field.

• 1 animal developed a thrombus in the renal vein at kidney reperfusion. This was not technically salvageable without subjecting the animal to >120 minutes warm ischemia and so the animal was culled.

• 1 animal bled from an unligated branch of the renal artery at kidney reperfusion. This was not amenable to surgical fixation without subjecting the animal to >120 minutes of warm ischemia. The animal was culled.

• 2 animals were found dead in their cages the following morning, for reasons that were not apparent at post mortem.

Following this, 11 further animals were successfully transplanted and survived to 14 days and were apparently healthy.

8.43 GROUP WEIGHT LOSS COMPARED TO BASELINE WEIGHT

Animals in both groups lost significant body weight until 10 days post surgery, but had recovered body weight by Day 14. (See Figure 8.6)

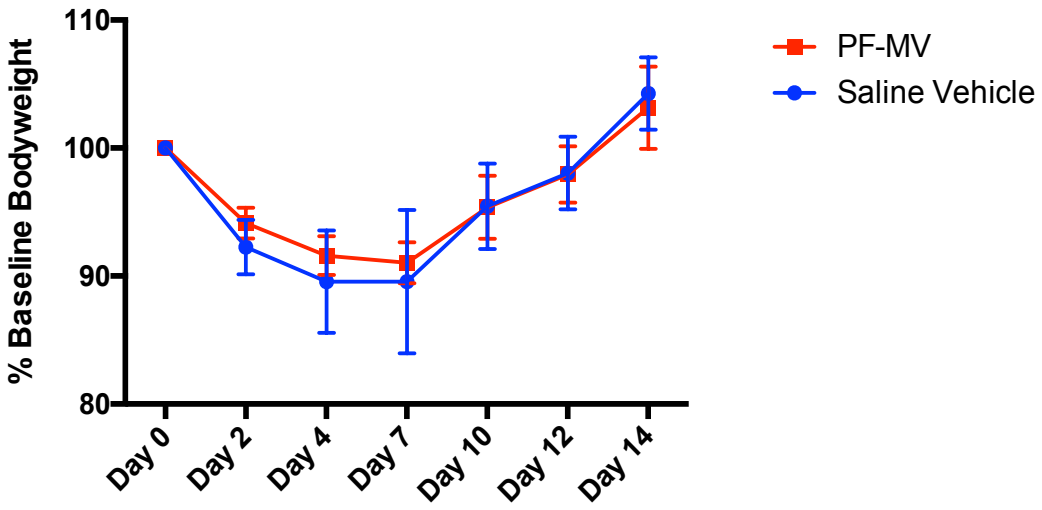


Fig 8.6: Compared to baseline, animals with both saline and MV-treated kidney transplants exhibited significant weight loss on Day 2 ($p < 0.0001$ in both groups). There remained significant weight loss for both groups on Day 4 ($p < 0.0001$ in both groups), Day 7 ($p = 0.001$ and < 0.0001 respectively) and day 10 ($p = 0.0075$and 0.001 respectively). By day 12, the body weight of animals with saline treated transplants had returned to baseline ($p = 0.1213$), whilst animals with MV-treated transplants remained below their baseline weight ($p = 0.0443$). By day 14, animals in all groups had put on a mean average of 4% body weight in comparison to baseline ($p = 0.0041$ and 0.0191 respectively).
Statistical analysis by unpaired Students t test.

8.44 COMPARISION OF PERCENTAGE WEIGHT LOSS FROM BASELINE BETWEEN GROUPS

There was no significant difference in weight loss from baseline between groups at day 2 ($p = 0.0876$), Day 4 ($p = 0.2704$), Day 7 ($p = 0.5494$), Day 10 ($p = 0.9602$), Day 12 ($p = 0.9442$) or Day 14 ($p = 0.5397$). (See Figure 8.6)
Statistical analysis by unpaired Students t test.

8.45 GLOMERULAR FILTRATION RATE

There was no significant difference in GFR between transplants treated with saline and those treated with PF-MVs. (see Figure 8.7)

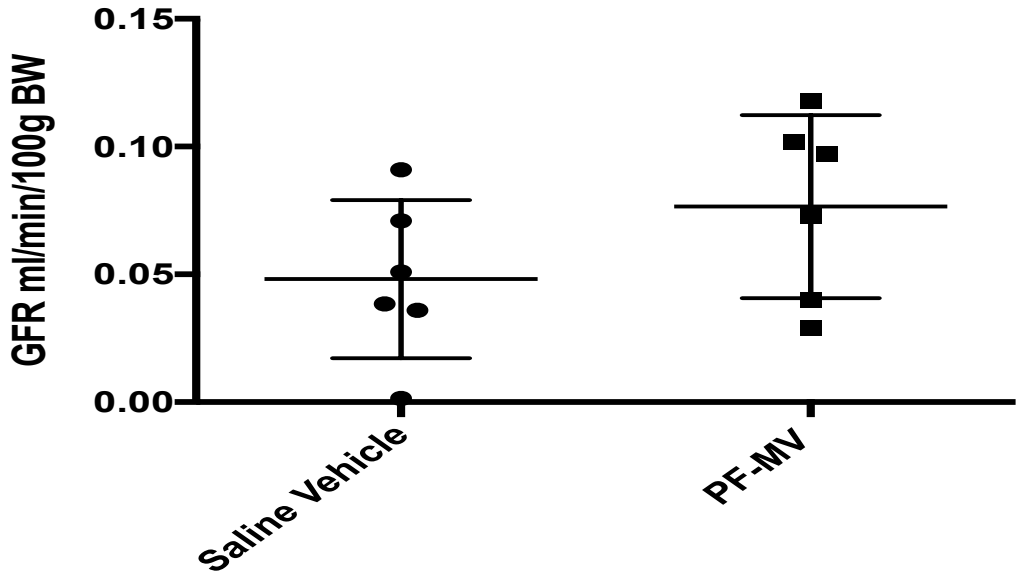


Figure 8.7: Transplant function in saline treated animals was not significantly different to those in animals with transplants treated with PF-MVs. ($p = 0.1724$)
Statistical analysis by unpaired Student's t-test.

8.46 RENAL HISTOLOGY SCORING

Epithelial Breaks

Transplanted kidneys treated with PF-MVs exhibited significantly fewer epithelial breaks than transplants treated with saline alone. (See Figure 8.8)

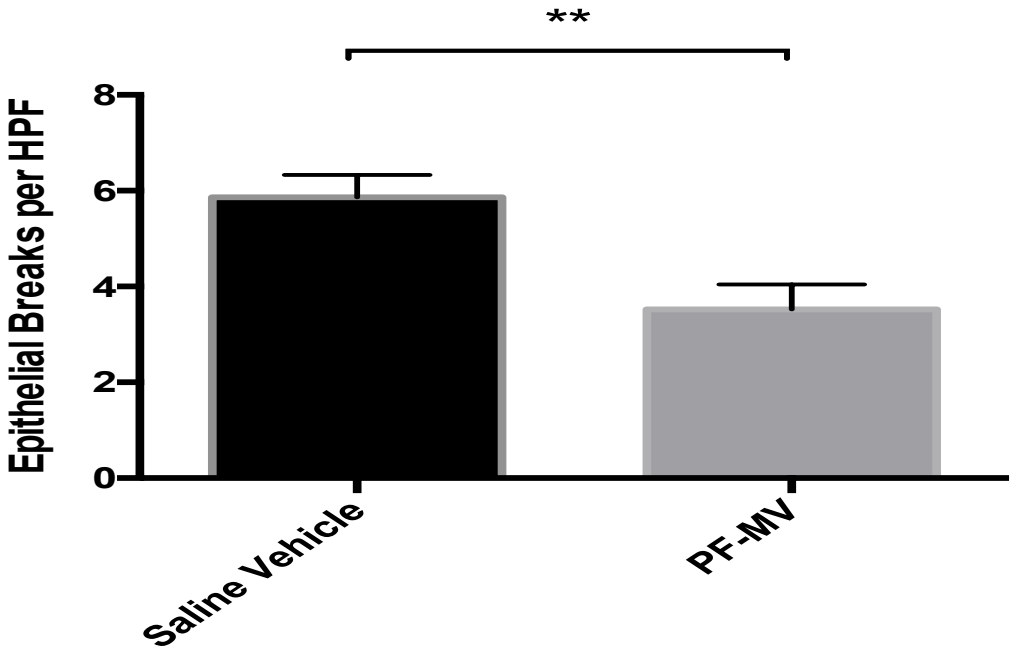


Figure 8.8: (a) Renal histology shows that transplants treated with PF-MVs exhibit significantly fewer epithelial breaks than transplants treated with saline vehicle alone (p=0.0076).
Statistical analysis by unpaired Student's t-test.

Hyaline Cast Formation

Transplanted kidneys treated with PF-MVs had significantly fewer hyaline casts when compared to transplanted kidneys treated with saline alone. (see Figure 8.9)

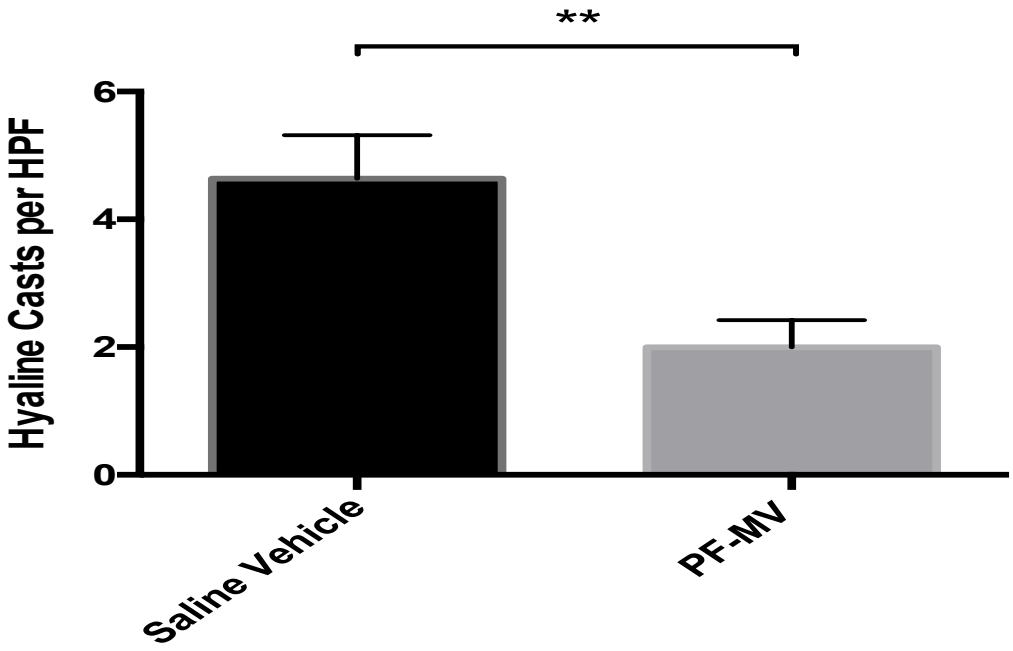


Figure 8.9: Fewer hyaline casts are observed in transplanted kidneys treated with PF-MVs than transplanted kidneys treated with saline alone (p= 0.0079).
Statistical analysis by unpaired Student's t-test.

8.5 DISCUSSION

Although recent improvements in tissue typing and immunosuppression regimes have significantly reduced the incidence of post-transplant acute rejection, long-term graft survival rates have remained unchanged (Saat et al., 2016). This probably reflects the fact that ECD and DCD renal allografts are increasingly being transplanted into older patients, and that 'marginal' organs are at increased risk of damage during the transplantation process (Ali and Sheerin, 2013).

The common pathology predominantly responsible for long-term allograft loss is chronic allograft nephropathy (CAN). CAN is a multifactorial process that results from a combination of injurious processes that begin during the peri-transplant period and ultimately lead to late transplant dysfunction and allograft loss (Marcen et al., 2009, Heemann and Lutz, 2013). Insults including cold and warm ischemia, reperfusion injury, low-grade antibody-mediated rejection together with calcineurin toxicity result in severe interstitial fibrosis and tubular atrophy (Marcen, 2009, Grinyo et al., 2010). Evidence suggests that CAN is related to chronic immunological insults, but also to uncontrollable factors at the time of transplantation including poor allograft quality and IRI that enhances allograft immunogenicity (Timsit et al., 2010).

Unsurprisingly, the complex biological processes that combine to produce acute transplant injury are difficult to simulate properly using current rodent models of renal transplantation. Most rodent models of renal transplantation focus upon a single pathological process – usually immunological rejection as a result of allogeneic transplantation, or a combination of cold and warm ischemia. In this experiment, it was decided to investigate the effects of ECV therapy against prolonged cold and warm IRI, as this is reported to be the major insult encountered by 'marginal' renal allografts. Allografts were deliberately transplanted between syngeneic rats to exclude acute rejection as a confounding factor. This allowed focused evaluation upon the effects of cold storage, warm ischemia and reperfusion injury on allograft function and histology, and the ability of ECV therapy to mitigate this. However, in future it would be relatively straightforward to introduce immunological rejection into the experiment by transplanting Fischer kidneys into the Lewis rat strain, which is a well-recognized rodent model of CAN (White et al., 1969, Shrestha and Haylor,

2014). This would allow the investigation of the effects of stem cell-based therapies against prolonged cold and warm ischemia, immunological rejection and subsequently against CAN. In addition, when performing such experiments, it would be possible to treated transplanted animals with calcineurin inhibitors, thereby investigating the effects of stem cell therapies in a more exact imitation of the clinical transplantation process.

The immunomodulatory and anti-inflammatory properties of MSCs have led investigators to study their use in animal transplant models, with the aim of reducing peri-transplant injury and hopefully lessen the need for long-term immunosuppressive medication. However, cell-free therapy confers some theoretical benefits over cellular treatments, and efficacy of cell-free treatment has been demonstrated in some different rodent renal IRI models.
While there are several reports describing the use of cell-based therapies in rodent transplant models, there appear to be no studies where an animal renal transplant model has been treated with ECV therapy in an attempt to protect allograft function against IRI. Indeed there is only one published study where ECV therapy has been given to a rodent kidney transplant model, in an attempt to reduce chronic allograft nephropathy (Koch et al., 2015).

The microsurgical challenge of anastomosing the vessels and ureters in rat kidney transplantation is known to introduce additional experimental variability when compared to results obtained with simple rat renal IRI models (Schumacher et al., 2003, Pahlavan et al., 2006, Spanjol et al., 2011). However, transplantation is deemed necessary to introduce a period of experimental cold ischemia +/- immunological incompatibility, in order to mimic the clinical process of deceased donor renal transplantation. The paucity of studies examining ECV therapy in rodent transplant models may highlight a lack of investigator enthusiasm due to the technical complexity involved in the procedure itself. However, it is also possible the lack of published data for ECV therapy in this area reflects a lack of therapeutic efficacy that is not in the public domain due to publication bias.

The model described here exposed the transplanted kidneys to 120 minutes of cold ischemia and 120 minutes of warm ischemia. This is considered a prolonged

ischemic period (Schumacher et al., 2003) and produced a severe, long-standing renal injury compared to the split renal function of age and sex matched Fischer rats. Initially, 'pilot' transplants were performed (n=2), utilising 120 minutes of cold and 90 minutes of warm ischemia, but results indicated that transplant function was not reliably diminished after 14 days recovery. The warm ischemia period was therefore extended to 120 minutes and a significant decline in transplant function observed.

Increased periods of ischemia during rodent transplantation are reported to cause greater variation in renal morphology and function (Pahlavan et al., 2006, Schumacher et al., 2003). Despite this, the variability in GFR and histology results obtained here from saline-treated rats subjected to 240 minutes of total ischemia was acceptable *(mean GFR =0.04815, SD = 0.0394, 95% Confidence Interval = 0.01568 – 0.08062)*. Interestingly, greater variability was observed in the GFR of PF-MV treated animals for reasons that are unclear *(mean GFR =0.07653, SD =0.03578, 95% Confidence Interval =0.03898 – 0.1141)*. This may have been the result of unrecognised technical complications (e.g. renal artery or ureteric stenosis) or natural variability reflecting the prolonged ischemia and the ability of PF-MVs to ameliorate this. Increasing the numbers of animals in both control and experimental groups would be a natural next investigative step, as the data indicates the experiment may be numerically underpowered to show a significant difference in transplant GFR.

Despite this, PF-MV treated animals exhibited both fewer epithelial breaks and fewer hyaline casts when compared to saline treated controls. This indicates that PF-MVs had a beneficial effect on renal morphology, but that this effect did not translate into improved GFR at 14 days post-transplant. Clearly, it is possible that GFR between groups could differ significantly at other post-operative time points, although further experiments would be required to explore this possibility.

An apparent flaw in this experiment is the lack of molecular data, which may have provided an insight into the mechanisms by which PF-MVs produce their effect upon renal morphology. As there is an absence of data regarding the biological action of ECVs in transplant models, it is difficult to speculate in what, if any, additional ways ECVs may act to counter the cold ischemia and enhanced immunogenicity that result from allogeneic transplantation.

A single study has thus far reported the use of ECVs in an animal kidney transplant model. Koch et al. harvested MSC-derived ECVs and administered them intravenously to an allogeneic rat transplant model of acute rejection on the 1st post surgical day (Koch et al., 2015). Renal IRI was only 30 minutes and no immunosuppression was given. Leucocyte infiltration in treatment and control kidneys was significantly different, although this did not appear to affect renal function or the production of donor-specific antibodies. The authors concluded that therapy with ECVs was safe but did not seem to confer any functional or histological benefit at mitigating acute cell-mediated rejection. This was unsurprising given the fact that cell-mediated rejection is an immunological process that is unlikely to be completely nullified by the administration of a single dose of therapy, the efficacy and dosing of which had not been demonstrated by the investigators previously. Furthermore, timing and route of the dose used in this study were probably sub-optimal. As a consequence, it is difficult to draw any firm conclusions regarding the potential of ECV therapy in kidney transplantation based on the findings of this study.

At the time of writing, there appear to be no other published reports investigating the use of ECV therapy in animal models of renal transplantation.
Nevertheless, some previous studies have demonstrated the ability of MSCs to mitigate damage to renal function and structure in rodent transplant models. The consensus is currently that stem cells act via paracrine mediators in the form of ECVs. Furthermore, ECV therapy appears to be equivalent in mechanism and efficacy to treatment with the parent stem cell type in a number of studies. Therefore it is possible that if applied to a rodent kidney transplant model, via a common mechanism of action, ECVs may have a similar effect to the stem cell type from which they derive.

Initially, it was planned to test both PF-MVs and ADRCs in the transplant model. However, difficulties in establishing both the technical aspects of the transplant procedure and the amount of ischemia required to produce a long-standing insult meant that this was not possible. It is unfortunate that these time constraints did not allow the testing of a therapeutic dose (7×10^5) of ADRCs in this rat transplant model.

Nevertheless, multiple previous studies have shown MSCs to mitigate warm renal IRI via anti-inflammatory and anti-oxidative mechanisms (Chen et al., 2011, Feng et al., 2010b, Sheashaa et al., 2016, Shih et al., 2013, Villanueva et al., 2013). Furthermore, studies in rodent transplant models have shown MSCs to reduce acute cell-mediated rejection, mitigate IRI and probably as a long-term consequence of these effects, reduce CAN. Unfortunately, there is a relative shortage of published work exploring the effects of ECV therapy in animal kidney transplant models, although the reasons for this are unclear. Increasing the animal numbers in the current experiment and furthermore introducing an ADRC treatment group while obtaining molecular data from stored tissue samples are obvious next steps that may allow conclusions regarding the potential of PF-MVs and ADRCs in this model of renal transplant ischemia. If proved effective, testing these novel therapies in rodent transplant models of CAN subjected to similar periods of ischemia may clarify their utility prior to testing in porcine models of renal transplantation.

CHAPTER 9

GENERAL DISCUSSION

9.1 INTRODUCTION

The experiments presented in this thesis highlight the potential of stem cells and their ECVs to reduce the peri-transplantation injury that renal allografts suffer as a result of IRI. Given the increasing use of marginal allografts, which are more prone to IRI, such therapies are urgently needed as a reduction in the initial injury may theoretically improve allograft function in the long-term.

This is important, as long-term transplant outcomes with marginal kidneys are less than satisfactory, although transplantation with such allografts has been shown to be preferable to remaining on dialysis. Nevertheless, renal allografts exhibit a long-term decline in function associated with chronic fibrosis that occurs as a result of persisting alloreactivity and calcineurin-inhibitor related toxicity. Strategies to reduce initial injury may limit allograft immunogenicity and subsequent calcineurin requirements, preserve the number of functioning nephrons and ultimately encourage renal regeneration.

Since work on this thesis began, researchers have conducted pilot studies examining the safety and feasibility of MSC use for the prevention of peri-transplantation injury. No studies reporting the use of ECVs in renal disease have been reported in human subjects at the time of writing, although the use of ECVs to treat graft versus host disease has been described (Kordelas et al., 2014). Perico et al. (Perico et al., 2011) were the first to report the use of MSCs in patients undergoing renal transplantation, concluding that infusion of autologous MSCs 7 days post-transplant did not improve outcomes after one year compared to conventional therapy. Reinders et al. (Reinders et al., 2013) reported that treatment with autologous BD-MSCs in response to acute rejection on protocol biopsy was safe, noting reductions in acute rejection and interstitial fibrosis 24 weeks post-treatment. While no conclusions were drawn regarding the efficacy of MSC therapy, the treatment appeared safe, and the findings were suggestive of systematic immunosuppression (Reinders et al., 2013). Peng et al. (Peng et al., 2013) reported that intra-renal artery infusion of MSCs at the time of surgery approximately allowed a 50% reduction in tacrolimus therapy one year after transplantation.

Unfortunately, while these studies have been suggestive of benefit associated with MSC treatment, the small numbers of patients recruited make it difficult to draw firm conclusions (Bank et al., 2015). However, these pilot studies have been conducted predominantly using allografts that come from living donors, and the results from such allografts are known to be excellent. This is probably because live donor allografts are subjected to a minimal cold and warm ischemic times, and hence peri-transplantation injury and subsequent enhanced allograft immunogenicity are kept to a minimum. Theoretically, the impact of stem cell-based therapy in this situation is likely to be negligible, compared to the application of novel therapies in marginal allograft transplantation. Furthermore, clarification regarding the optimal route of administration, numbers of cells per dose and timing of treatment in relation to surgery is required to produce the maximum beneficial effect.

The clinical translation of stem cell-based therapies ultimately requires the safety and efficacy of treatment to be demonstrated in large animal models of transplantation prior to the implementation of widespread clinical trials. This chapter discusses how the progression to large animal work might best be achieved, highlighting improvements to the rodent experiments described here and postulating how future work might provide information that allows large animal work to be conducted in an efficient fashion.

9.2 IMPROVEMENTS TO THE RAT MODEL OF RENAL ISCHEMIA-REPERFUSION INJURY

As previously discussed, the rat model described in Chapter 5 addresses some of the disadvantages that are associated with conventional rodent models of renal ischemia. Nevertheless, there are negative aspects of the novel rat model that principally concern the characterization of GFR in terminal studies.

Continuous infusion of an exogenous tracer is regarded as being the most accurate method of determining GFR (Huang et al., 2016, Miller et al., 1952). In U-IRI-N models, such as the novel model developed here, individual ureteric cannulation is

utilised to allow the individual GFR of each kidney to be characterised. However, the size of rodents makes this technique both time-consuming and technically difficult.

In addition, hypovolemia as a result of insidious bleeding may occur during GFR studies using the method described. Any occult blood loss observed during these experiments was universally intra-abdominal, originating from either the incised abdominal musculature or from tiny retroperitoneal vessels divided during ureteric dissection. During the dissection necessary to allow the insertion of the ureteric cannulas, meticulous hemostasis was routinely performed in an attempt to prevent unnecessary blood loss. Unfortunately, any bleeding after ureteric cannulation was not immediately apparent as the intestines were returned to the abdomen post-cannulation to help maintain the steady core body temperature that is necessary to achieve consistent GFR results (Le Clef et al., 2016, Wei and Dong, 2012). The abdomen was also loosely packed with swabs to help prevent unnecessary heat loss. Routine inspection of the abdomen was not undertaken after GFR studies begun as this risked dislodging the position of the cannulas. Any bleeding was therefore not apparent until the rat's circulating volume had been reduced sufficiently to reduce urine output and thereby artificially producing lower GFR results. During this thesis, four animals were terminated prior to the conclusion of GFR studies, when it became apparent that occult intra-abdominal blood loss during the inulin clearance study had resulted in reduced urine output and inaccurate GFR results. These animals were excluded from experimental groups, which necessitated the use of an additional animal in a repeated procedure.

A second problem with the use of terminal inulin clearance studies is that only a single GFR value can be obtained from each animal (Ellery et al., 2015). Clearly, in order to gain GFR values at different times points post surgery, larger numbers of animals would be required that if multiple GFR results could be obtained from each animal at various time points without sacrificing it.

Therefore, the benefit of ureteric cannulation, namely the characterization of split renal function in a U-IRI-N model, needs to be balanced against both the inconvenience of excluding animals after technical complications during GFR studies and the requirement for large numbers of animals to provide insight into renal

function over a prolonged period of time. The fundamental principles of animal research specify that investigators actively seek to reduce the total numbers of animals utilised in their experiments, while refining techniques in order to obtain more accurate information (2011a). In keeping with this philosophy, the emergence of the 2-compartmental models of inulin clearance have led to the publication of methods whereby GFR in conscious rodents can be characterised. Practically, such models are desirable in that repeated GFR measures may be obtained from each individual animal at different time points, as the GFR procedure does not necessitate the animal to be culled. Furthermore, the use of conscious animals is beneficial as anaesthesia is reported to lower GFR and may be avoided using these techniques (Qi et al., 2004, Fusellier et al., 2007).

The fundamental concept upon which all 2-compartmental models rely is the decay of exogenous tracer from the plasma and extracellular space following the administration of a single intravenous bolus. The measurement of decay necessitates obtaining either a single timed blood sample (Katayama et al., 2010, Katayama et al., 2011) or multiple, timed blood samples (Sturgeon et al., 1998, Qi et al., 2004). The use of fluorescent or radioactive tracer facilitates the measurement of tracer decay, allowing the elimination of tracer by the renal tract to be calculated.

However, 2 compartmental models rely upon mathematical modelling to calculate both the initial, rapid redistribution of exogenous tracer from the circulation to the extracellular fluid and the subsequent slower elimination of tracer by the renal tract. Unfortunately, these less invasive methods of measuring GFR are considered inaccurate when compared to the more invasive constant infusion techniques utilised in this thesis (Sharmarlouski A, 2014). However, conventional constant infusion techniques make repeated GFR measurements from a single animal impractical.

Happily, a recent report described the continuous monitoring of GFR, by measuring the transcutaneous fluorescent decay of FITC – sinistrin in conscious animals (Schock-Kusch et al., 2011). Sinistrin is an inert renal reporter molecule that, like inulin, is considered ideal for measurement of GFR. Recent improvements to this method have been reported to give GFR values as accurate as those obtained with continuous inulin infusion (Friedemann et al., 2016). The significant advantage of

this method is that there is no requirement for a rodent to be anaesthetized, nor are repeated blood samples required. This allows multiple GFR values to be obtained from each animal, enabling the progression of renal function over a long experimental time course to be tracked.

However, such transcutaneous monitoring does not measure split renal function, which is a necessity in the U-IRI-N model where the GFR of the damaged kidney and the response to therapy is of interest.

To make improvements to the novel model described here, this difficulty may be overcome in two different ways. Firstly, it may be possible to safely perform a right-sided nephrectomy a few days after the initial IRI experiment, as the GFR data 2 weeks post procedure suggests the function of the left kidney is enough to prevent death from uremia. Surviving animals could have their renal function measured via transcutaneous as recently described (Friedemann et al., 2016). Should mortality rates after right-sided nephrectomy prove unacceptably high, it may prove necessary to reduce the initial period of left-sided ischemia although the permanent effects on renal function as a result of this would need to be clarified.

A second option to measure split GFR, without the requirement for nephrectomy, is to use a form of imaging, after the administration of a bolus of contrast medium or exogenous tracer. Recent advances in magnetic resonance imaging (MRI) have allowed both renal blood flow and GFR to be measured (Zeng et al., 2015). However, such a modality is expensive and consequently may not be practical during ‘screening' rodent research. Ratio-metric determination of GFR by two-photon microscopy has been described (Wang et al., 2010). This technique is minimally invasive and allows split renal function to be characterised. Either of these methods could be employed to measure GFR repeatedly in the same animal should right-sided nephrectomy not prove feasible. However, as both imaging modalities require the animal to be anaesthetized, transcutaneous measurement of GFR post nephrectomy would be preferable.

9.3 ORGAN RECRUITMENT OF STEM CELLS AND ECVs POST ADMINISTRATION

In this thesis, no experiments were conducted to investigate the sites to which stem cells or ECVs were recruited after infusion directly into the renal artery. However, previous studies using BD-MSCs (Cai et al., 2014) and ADRCs (Iwai et al., 2014) administered via the renal artery have shown the vast majority of cells are recruited by the kidney and furthermore persist in this location for the next 24 hours. It therefore seems likely that cells administered in the novel rodent model would be recruited into the kidney. This contrasts the findings with cells administered intravenously, which were found to localise in the lungs for at least three days (Iwai et al., 2014).

At the time of writing, there appear to be no studies that differentially track the fate of ECVs in animal models, either given systemically or administered in a local fashion. In part, this probably reflects the difficulties in labelling and tracking harvested ECVs, and furthermore the difficulty distinguishing between 'biologically active' ECVs which have become internalised in a cell, or are merely adherent to a cell surface (Feng et al., 2010a).

An in-depth discussion as to how this might be achieved is beyond the scope of this thesis. However, in broad terms, the bioluminescent labelling of ECVs has been described, using reporters such as *firefly luciferase* (Sharkey et al., 2016) or *Gaussia luciferase (Lai et al., 2015)*. This becomes fused to cell membrane domains, and consequently is incorporated into ECV membranes during production. This method may be used to track ECV fate *in vivo (Lai et al., 2015)*.

9.4 FUTURE EXPERIMENTS WITH ADRC ECVs

A body of convincing evidence has led to the recognition that the beneficial action of stem cell therapy in response to renal injury is mediated by ECVs in a paracrine fashion (Bi et al., 2007, Bruno et al., 2009b, Bruno and Bussolati, 2013, Gatti et al., 2011, Zou et al., 2014, Cantaluppi et al., 2012). One of the constant themes throughout this thesis has been that the therapeutic use of stem cells themselves should be considered to carry more risk than therapy with ECVs – not least because administered cells have the potential for malignant transformation and supra-therapeutic doses may lead to tissue capillary occlusion. The use of ECVs has not been associated with these problems, and significantly ECV application has been shown to have similar efficacy as the use of stem cells themselves (Bruno et al., 2009b).

Unfortunately, time constraints prevented the isolation of ECVs from the ADRCs in this thesis. However, obtaining a preparation of ECVs from ADRCs using centrifugation and examining the effects in the novel rodent model would be an interesting next set of experiments. If the efficacy of this preparation could be established, it would be interesting to separate the ECV preparation into its three fractions using differential ultracentrifugation protocols previously described (McGuinness et al., 2016). The size of vesicles in each fraction could then be determined using either Nanosight analysis (Cantaluppi et al., 2012) and/or Nanoparticle tracking analysis (Gardiner et al., 2013) to ensure a 'clean' preparation. Testing each fraction in the novel model may then identify which of the ECV fractions contains the biologically active material that confers beneficial effects.

It is unfortunate that time constraints prevented the completion of IHC and qPCR analysis that may have provided further insight into the cellular process by which ADRCs and PF-MVs produced their biological effects in severely injured kidneys. However, a number of previous studies have indicated that MSCs produce at least some of their effects by dampening the inflammatory response to injury (Feng et al., 2010b, Chen et al., 2011, Shih et al., 2013, Furuichi et al., 2012, Wang et al., 2013b),

while PF-MVs are thought to produce their effects through separate pathways that encourage cellular regeneration (McGlynn et al., 2013, Stevenson et al., 2011).

This suggests that Pathfinder cells and MSCs produce their effects via different biologically active factors. Evidence suggests that in the case of Pathfinder cells, these are contained within microvesicles (McGuinness et al., 2016), while in MSCs the identity of the responsible ECV fraction(s) remains unclear. The characterization of ECVs from MSCs as described above would allow an investigation into the effects of a preparation containing the efficacious ECVs from both Pathfinder cells and MSCs.

Given the likely different mechanism of action of these cell types, it may be that combining the active ECV fractions would produce a synergistic effect that resulted in initial injury reduction and subsequent regeneration of specialised cell types within the damaged kidney.

The ultimate aim of these experiments is to identify any factors contained within ECVs that influence cellular processes in a beneficial way. An in-depth discussion as to how this might be achieved is outside the scope of this thesis. However, in board terms, the first step in the process would be to identify the ECV fraction(s) from specific cell types that mediate beneficial effects. One study reported characterising the mRNA and miRNA content contained within porcine ADRCs, concluding that ECVs were enriched in miRNAs involved in angiogenic and anti-inflammatory pathways (Eirin et al., 2014). However, the use of undifferentiated centrifugation resulted in the cargo of all ECV subtypes being reported, and not just the biologically active content. This highlights the importance of investigating the efficacy of particular ECV fraction. From a 'clean' preparation of efficacious vesicles, the biologically active cargo could be extracted using recognised protocols (Eldh et al., 2012). Several papers have recently reported different molecular techniques allow the cargo of different ECV subtypes to be characterised (Crescitelli et al., 2013, Chevillet et al., 2014, Ji et al., 2014). This may then enable the manufacture of a synthetic (cell-free) product for clinical application, the purity of which could be stringently tested.

9.5 *EX VIVO* NORMOTHERMIC REPERFUSION AND THE POTENTIAL IMPLICATIONS FOR CLINICAL TRANSLATION OF STEM CELL THERAPIES

Several studies have identified the adverse effects of prolonged cold ischemia on renal allografts, with the risk of transplant failure at three months reported increasing with each additional hour of cold ischemia (Debout et al., 2015). Compared to 'standard criteria' organs, ECD and DCD kidneys tolerate hypothermia poorly (Summers et al., 2013, Kayler et al., 2011), and display high rates of DGF after prolonged cold storage (Bilgin et al., 1998). DGF is itself a poor prognostic indicator for long-term allograft outcomes, probably as enhanced allograft immunogenicity increases the likelihood of subsequent episodes of rejection (Gill et al., 2016, Wu et al., 2015).

As a consequence of the change in donation trends towards marginal kidneys, clinicians have recognised the need to move away from the traditional 'cold storage' approach. Recent research has focused on the development of novel methods of organ preservation, with much interest in allograft *ex vivo* normothermic reperfusion. In the context of this thesis, this is important, as this evolving technology may provide the opportunity to better investigate stem cell-based therapies and implement them in a safer fashion.

Ex vivo normothermic reperfusion of allografts involves the continuous re-circulation of a blood-based solution through an extracorporeal organ at body temperature before transplantation. The removal of toxic metabolites and restoration of aerobic respiration is thought to 'resuscitate' organs subjected to periods of warm and cold ischemia during retrieval and transportation. These techniques have gradually been adopted in clinical transplantation for other solid organs including the heart (Ardehali et al., 2015), lung (Cypel et al., 2011) and liver (Brockmann et al., 2009, Ravikumar et al., 2016). There is growing recognition that normothermic preservation has advantages for renal allografts (Hosgood et al., 2015, Kaths et al., 2016b). The perfusion of allografts *ex vivo* has necessitated the development of isolated perfusion systems, which have been adapted from paediatric cardiopulmonary bypass circuits (Bagul et al., 2008). Additionally, leucocyte depleted blood-based perfusion solutions

have been developed, which compared to whole blood, reduce the inflammatory cascade that occurs during reperfusion, but still allow efficient transfer of oxygen (Hosgood and Nicholson, 2011, Harper et al., 2006).

A recent study using porcine kidneys has shown that continuous normothermic reperfusion after organ retrieval is associated with superior function compared to normothermic reperfusion following a period of static cold storage (Kaths et al., 2016a). However, the transportation of organs during continuous reperfusion is likely to present dangerous logistical problems, especially as problems with organ perfusion in transport could lead to long periods of warm ischemia. A more practical approach would probably involve a period of cold storage during transportation, followed by *ex vivo* normothermic reperfusion just before allograft implantation. This approach theoretically has several advantages as cold storage time is minimised and the perfused renal allograft may be macroscopically inspected and its suitability for transplantation assessed (Hosgood et al., 2015). Furthermore, the resistance to renal blood flow may be measured, and the restoration of normothermia allows aerobic respiration to commence, facilitating the removal of toxins produced under anaerobic conditions.
Recent studies have now demonstrated the benefits of this novel approach in kidney transplantation, with the initial clinical pilot study observing a DGF rate of 6% in treated ECD allografts, compared to 36% with kidneys treated with traditional static cold storage (Nicholson and Hosgood, 2013).

While further discussion regarding the benefits of normothermic kidney perfusion is outside the scope of this thesis, it is important to highlight that future efforts to improve marginal allograft outcomes are likely to involve this novel technique. Happily, the rise of *ex vivo* normothermic reperfusion creates unique opportunities for the development of concomitant strategies to incorporate novel stem cell-based treatments into clinical practice.

Firstly, *ex vivo* perfusion circuits seems to present the ideal opportunity to administer intra-renal artery therapy without the potential negative effects of systemic distribution. Novel treatment could easily be administered during allograft reperfusion, as the renal artery is readily available. Such intervention would need to

be optimised before use in the clinical setting, however, and experiments using discarded porcine kidneys could be conducted to investigate the feasibility of such an approach. This could clarify the optimal dose of stem cell therapy and avoid supratherapeutic dosing that could cause blockage of the renal microcirculation. Theoretically, it would also be possible to subject porcine kidneys to a prolonged period of warm ischemia and, using *ex vivo* perfusion circuits, to examine the response to stem cell-based therapies over 24 hours by measuring GFR via constant inulin infusion. These initial screening experiments could provide useful initial information regarding dosing protocols before trialling the efficacy of novel therapies in porcine models of kidney transplantation. Recent work by Kaths et al. has described a porcine model in which kidneys are auto transplanted after being subjected to 8 hours of static cold storage and a short period of warm ischemia during re-implantation (Kaths et al., 2016b, Kaths et al., 2016c). At day 10, animals receiving these kidneys were shown to have elevated serum creatinine and worse creatinine clearance versus controls, implying a degree of damage as a result of the storage and surgical processes. This model could be used to study the effect of stem cell therapy, administered while the kidney was undergoing *ex vivo* normothermic reperfusion. However, in order to severely damage the kidney and mimic the use of 'marginal' allografts, it may prove necessary to subject the graft to longer periods of warm ischemia than were applied in these studies.

With regard to *ex vivo* normothermic reperfusion and the parallel use of stem cell-based therapies, there is one final, important point. Endothelial membrane stiffening in response to rapid reperfusion by whole blood, as occurs in standard clinical practice during allograft reperfusion, prevents rapid clearance of the renal microcirculation. This 'no-reflow' phenomenon may prevent the adequate distribution of any novel cells, administered with therapeutic intent. However, the 'no-flow' phenomenon is ameliorated by a period of normothermic reperfusion with leucocyte depleted blood-based perfusion fluids (Kay et al., 2011). Therefore, the administration of cellular therapy to marginal kidney grafts after a period of normothermic reperfusion is thought to be superior to administration immediately after hypothermia (Iwai et al., 2014). It may well be that *ex vivo* normothermic reperfusion enhances the effects of cellular treatments by allowing better penetration of therapeutic agents into the allograft.

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APPENDIX 1