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Development of an in vitro Model of Ischemia Reperfusion Injury in Kidney Transplantation

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Abstract

Kidney transplantation remains the optimal treatment option for patients with end-stage renal disease. We aim to improve organ preservation during transplantation by reducing damage from ischemia reperfusion injury (IRI) using existing clinically approved drugs. In this study, we developed and characterized an *in vitro* model that mimics the conditions of IRI in kidney transplant as a tool to screen and study the therapeutic effect of drugs in the context of IRI. We recapitulated several key outcomes in IRI in our model, including the induction of hypoxia, cell death, increased expression of damage markers (NGAL, HMBG-1) and pro-inflammatory cytokines in a sterile environment (IL-6, TNF- α). Additionally, we validated our model as a screening tool by testing select drugs for their ability to mitigate IRI. Overall, this study provides the foundation for further research that can improve organ preservation strategies, thereby increasing the quantity and quality of kidneys available for transplant.

Keywords

Ischemia Reperfusion Injury, Kidney Transplantation, Donation After Cardiac Death, Organ Preservation, Inflammation, Hypoxia, Apoptosis.

Summary for Lay Audience

Over 40,000 Canadians suffer from kidney failure. Transplantation remains the best treatment for these individuals, providing better quality of life and survival than dialysis. However, the damage to organs during the transplant surgery, known as ischemia reperfusion injury, remains a challenge to the success of kidney transplant. Ischemia refers to reduced blood flow during organ retrieval and storage, where reperfusion is the restoration of blood flow upon transplantation of the organ into the recipient. This injury causes cell death and inflammation in the transplanted organ leading to poor function. Therapeutics that prevent damage from this injury will be helpful in preserving the quality of transplanted organs and allow them to function longer. Our group hopes to repurpose an existing drug in the context of kidney transplantation, however a relevant model to test these drugs is required. In this study, we have developed and characterized a human kidney cell model that accurately encompasses the damage experienced by donor kidneys during transplantation. Additionally, we have carried out preliminary drug testing to determine the usefulness of the model to screen drugs. In future, this model will be used for a large-scale drug screen and to study drugs for their ability to prevent kidney injury during the transplant process. Ultimately, we hope to improve the quality of kidney a transplant patient receives.

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7-AAD	7-Aminoactinomycin D
ATP	Adenosine triphosphate
Bcl-2	B-cell lymphoma-2
Bak	Bcl-2 homologous antagonist killer
Bax	Bcl-2 associated X protein
DAMPs	Damage-associated molecular patterns
DCD	Donation after cardiac death
DGF	Delayed graft function
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ESRD	End-stage renal disease
HBOC	Hemoglobin-based oxygen carrier
HIF-1	Hypoxia-inducible factor 1
HMGB-1	High mobility group box-1
HMP	Hypothermic machine perfusion
IFNγ	Interferon gamma
IRI	Ischemia reperfusion injury
ITS	Insulin-transferrin-selenium
mPTP	Mitochondrial permeability transition pore
MYD88	Myeloid differentiation primary response 88
NF-κB	Nuclear factor-kappa-light-chain enhancer of activated B cells
NGAL	Neutrophil gelatinase associated lipocalin

NLR	Nod-like receptor
NMP	Normothermic Machine Perfusion
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PI	Propidium Iodide
RIPK1	Receptor-interaction serine/threonine kinase 1
RIPK3	Receptor-interaction serine/threonine kinase 3
ROS	Reactive oxygen species
SCS	Static cold storage
SNMP	Subnormothermic machine perfusion
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor alpha
TRIF	TIR domain-containing adapter-inducing interferonβ
UW	University of Wisconsin

Chapter 1

1 Introduction

1.1 Current Challenges in Kidney Transplantation

End-stage renal disease (ESRD) represents a significant global health concern as it is incurable and among the leading causes of death worldwide[1]. Globally, the incidence of ESRD has been on the rise in recent years, a trend which is also evident in Canada. As of 2019, over 40,000 Canadians were reported to be living with ESRD, representing a 33% increase since 2010[2]. Consequently, every year the rate of Canadians receiving renal replacement therapy due to kidney failure increases by ~1.6%, placing a significant burden on the healthcare system[2].

The two main types of renal replacement therapies are dialysis and kidney transplantation. Kidney transplantation is the preferred treatment option as it offers more favorable patient outcomes at reduced costs compared to dialysis[3,4]. Most importantly, as reported by the Canadian Institute for Health Information, the 5-year survival of patients who receive a kidney transplant is significantly higher at 85%, compared to 47% for patients on dialysis (depending on type of dialysis and organ received)[5]. Additionally, results from a systematic review by Tonelli et al., which examined the outcomes of 110 studies, found that kidney transplantation was associated with a reduced risk of mortality and improved quality of life compared to dialysis[6]. Several studies have also indicated that kidney transplantation is more cost effective than chronic dialysis[7,8]. For these reasons and due to the increase in patients with ESRD, the number of kidney transplants performed each year has been on the rise. For example, in Canada, there were 1,648

kidney transplants performed in 2019, a 41% increase in the annual transplantation rate from 2010, indicating an increased demand for transplantation[5].

1.1.1 Transplantable Organ Shortage and Expanding the Donor Pool

A limitation to kidney transplantation as a therapeutic approach is the shortage of acceptable donor kidneys available for transplantation compared to the number of patients with ESRD who require transplantation. As a result, the waitlist for kidney transplantation is rapidly expanding. The Canadian Institute for Health Information reported 3,261 patients waitlisted for a kidney transplant in 2019, which represents a ~37% increase from 2010[9]. Additionally, in 2019, over 1,613 patients were left waiting for a transplant. To address this growing supply and demand issue, organs from marginal donors are being used more frequently[10].

A main source of organs come from donation after cardiac death (DCD) donors. Many transplant centers worldwide have adopted the use of DCD organs as an avenue to increase the number of kidneys available for transplantation. For example, in 2019, over 30% of kidney transplants performed across Canada were from DCD donors compared to 22% in 2015, making DCD the fastest growing type of organ donation[9,11]. DCD organ donation is defined as the donation of organs from patients who have died with cardiac arrest. Unlike organs from living donation or donation after neurological death, DCD kidneys undergo a 20-to-60-minute period of warm ischemia from the time the donor's heart stops beating until the organ is procured. The additional warm ischemic injury to

the kidney while in the donor has been associated with an increased risk of poor graft outcomes post-transplantation[12].

1.1.2 Kidney Transplant Outcomes Using DCD Donors

While DCD organ donation can increase the number of organs available for transplant, these organs are also more susceptible to damage during the transplantation process and subsequently, exhibit poorer outcomes post-transplant. Most importantly, kidneys from DCD donors have a 20% increased risk of delayed graft function compared to organs from other donor types[13].

Delayed graft function (DGF) is defined as the need for at least one session of dialysis within the first week after transplantation[14]. Pathologically, DGF is a manifestation of acute kidney injury in the early post-transplantation period strongly associated with poor graft outcomes. A systematic review and meta-analysis of 33 studies comparing recipient outcomes reported a 38% increased risk of acute allograft rejection and a 41% increased risk of early graft loss in patients who experienced DGF compared to those without[15]. As such, DGF remains a prevalent concern in the transplant community and a threat to the long-term success of kidney transplantation.

1.2 Ischemia-Reperfusion Injury in Kidney Transplantation

The major mechanism that contributes to delayed graft function in kidney transplant recipients is ischemia reperfusion injury (IRI). In DCD kidney transplantation, ischemia refers to the cessation of blood flow that begins during donor death and organ procurement and continues during organ storage and implantation into the recipient,

where reperfusion refers to the re-establishment of blood flow upon implantation of the organ into the recipient. The kidney experiences two types of ischemia during transplantation: warm and cold ischemia. Warm ischemia occurs while the kidney is in the body, while cold ischemia refers to the storage period once the kidney is procured and prior to implantation. Warm ischemic periods are shorter, ranging from 20 to 60 minutes, however, damage occurs more rapidly at higher temperatures with complete loss of ATP and subsequent organ function observed after only 40 minutes[16]. Contrarily, organs can tolerate several hours of cold ischemia without significant loss of function, however, the combination with warm ischemia and damage from cold is detrimental to the organ[17]. The kidney is the third most susceptible organ to ischemic injury, only behind the brain and heart[18]. Therefore, the length and severity of IRI play a key role in determining the quality of organ being transplanted[12,19].

1.2.1 Pathobiology of Renal IRI

At the tissue level, ischemia is characterized by a decrease in oxygen and nutrient delivery to cells. Due to the lack of oxygen, there is dysfunction of the electron transport chain and cells switch from aerobic to anaerobic metabolism. In the kidney, the proximal tubular epithelial cells are most sensitive to damage from ischemic injury due to their increased metabolic demand and poor glycolytic capacity compared to distal tubular segments[20–22]. The switch to anaerobic metabolism causes a decrease in ATP production and creates an acidic environment from byproducts, such as lactic acid[23]. The shortage of ATP leads to the dysfunction of membrane bound ATPases, such as the Na^+/K^+ ATPase[24]. ATP depletion also results in mislocalization of transporters and breakdown of

tight junctions. As a result, there is an increase in intracellular Na^+ which causes water to enter the cells. Accumulation of metabolites and dephosphorylation of ATP contribute to the hyperosmolarity of ischemic cells, further promoting the influx of water and causing cells to swell, resulting in cellular edema[25].

Depleted ATP levels also result in the accumulation of intracellular Ca^{2+}, as it can no longer be pumped out of the cell and its reuptake by the endoplasmic reticulum is inhibited[26,27]. Calcium overload within the cell and mitochondria contributes to increased mitochondrial permeability and the production of reactive oxygen species (ROS)[28]. Due to the lack of oxygen present during ischemia, the formation of ROS is relatively limited[29]. However, there is also a decrease in production of antioxidant enzymes which inhibits the ability of the cell to deal with ROS upon reperfusion[30,31].

During ischemia, a number of pathways are activated to help the cell respond to hypoxic conditions. The principal defence mechanism of the cell in controlling the response to hypoxia is the hypoxia-inducible factor 1 (HIF-1) pathway[32–34]. HIF-1 is a heterodimeric transcription factor that controls the expression of hundreds of target genes[35]. The two subunits of HIF-1 are: the HIF- 1α protein, which is an oxygen regulated protein that is degraded under normal oxygen levels and HIF-1β, a constitutively expressed beta subunit. During hypoxia, the enzymes that degrade HIF-1α are inhibited, allowing it to accumulate and dimerize with the beta subunit. This in turn leads to the transcription of genes with protective functions against hypoxia, such as those involved in angiogenesis, glycolysis and tissue repair[36,37].

Upon re-establishment of renal blood flow, oxygen and nutrient levels increase within the cell. This increase leads to the normalization of cellular pH, as waste products are removed, and the cell resumes aerobic metabolism. Paradoxically, the re-establishment of homeostatic conditions leads to further damage to the cell. The normalization of pH activates cell damaging calcium-dependent proteases which accumulated during ischemia[32]. Additionally, leaked electrons in the mitochondria from ischemic conditions readily form ROS which cannot be eliminated by the impaired antioxidant system[38].

ROS damage mitochondrial proteins and lipids which, paired with swelling from the accumulation of intracellular Ca^{2+}, contributes to the formation of the mitochondrial permeability transition pore (mPTP)[39,40]. The opening of the mPTP releases cytochrome C, succinate, and mitochondrial DNA into the cytosol, that act as mediators to initiate cell death and inflammation[41], which will be discussed in subsequent sections.

1.3 Consequences of Renal IRI

There are several consequences of IRI during kidney transplantation at the tissue level that contribute to the poor clinical outcomes observed post-transplant. The main drivers of damage are activation of cell death pathways, stimulation of innate immune receptors and initiation of the inflammatory response.

1.3.1 Cell Death and Damage

During prolonged ischemia reperfusion injury, cell damage from ROS and proteolytic enzymes can trigger the activation of cell death pathways[42]. There are several forms of cell death observed during IRI in kidney transplantation. The following section

presents a brief summary of the most relevant cell death pathways in kidney transplant: necrosis, apoptosis, necroptosis and ferroptosis.

Necrosis

Necrosis is an uncontrolled form of cell death that is characterized by mitochondrial dysfunction, lack of nuclear fragmentation, cellular swelling and eventual rupture of surface membranes which leads to the release of intracellular components[43]. As such, necrosis is considered to be pro-inflammatory as released cellular proteins and DNA can act as damage-associated molecular patterns (DAMPs) to stimulate the innate immune system and further propagate the inflammatory response[44].

Apoptosis

In contrast to necrosis, apoptosis is a highly regulated and controlled cell death process characterized by cell shrinkage, pyknosis of the nucleus, controlled degradation of cellular components leading to membrane blebbing, and the formation of apoptotic bodies[45]. Apoptosis can be initiated by either the intrinsic or extrinsic pathway. During IRI, the intrinsic apoptosis pathway is initiated by the opening of mPTP, which is regulated in part by members of the B-cell lymphoma 2 (Bcl-2) family, pro-apoptotic proteins Bcl-2 associated X protein (Bax) and Bcl-2 homologous antagonist killer (Bak), which increase mitochondrial membrane permeability[46,47]. This results in the release of death molecules, including cytochrome C and ROS, into the cytosol[48]. These molecules lead to the activation of caspase 9 and downstream caspases.

The extrinsic pathway of apoptosis is also activated during renal IRI. This pathway involves the binding of ligands to death receptors on the surface of cells leading to caspase activation. The most well-characterized ligand-receptor interactions are tumour necrosis factor alpha (TNF-α)/TNF receptor 1 and Fas ligand/Fas receptor[49,50]. Binding of these ligands to their receptor triggers the activation of caspase 8 and 10[51]. Both the intrinsic and extrinsic pathways of apoptosis end with the activation of effector caspases. Effector caspases initiate the execution phase of apoptosis, which is marked by the activity of endonucleases, proteases, degradation of cellular components and the formation of apoptotic bodies[52,53].

Necroptosis

Another form of cell death observed in renal IRI is necroptosis, commonly referred to as regulated necrosis. Necroptosis exhibits characteristics of both necrosis and apoptosis. Similar to the extrinsic pathway of apoptosis, necroptosis is a programmed form of cell death initiated by the binding of a ligand, such as TNF- α, to its receptor on the surface of cells[54]. This binding recruits receptor-interacting serine/threonine kinase 1 (RIPK1) and receptor-interacting serine/threonine kinase 3 (RIPK3) proteins to form the necrosome[55,56]. However, unlike apoptosis, this process is caspase independent[57]. Downstream effects of necroptosis cause permeabilization of lysosomal membranes, resulting in the release of lysosomal enzymes which lead to cellular and mitochondrial damage, decreased ATP levels, and release of ROS[58]. The overall effects of necroptosis result in cell death that morphologically resembles necrosis, which includes cell swelling, eventual plasma membrane rupture, and release of intracellular contents which contribute to inflammation[59].

Ferroptosis

Finally, ferroptosis has recently been implicated as a major form of cell death in acute kidney injury and IRI[60]. Initiation of ferroptosis is dependent on the hyperactivity of oxidation mechanisms in conjunction with impaired anti-oxidation mechanisms[61]. While the exact mechanism of ferroptosis remains unclear, cellular events during IRI that lead to lipid peroxidation, increased ROS and iron accumulation trigger ferroptosis within the kidney[62]. Phenotypically, it is a necrotic type of cell death, similar to necrosis and necroptosis, that leads to the release of DAMPs which contribute to inflammation[63].

Markers of Cell Damage

Along with the induction of cell death pathways, injury from IRI leads to the expression and release of proteins that can be used as biomarkers of acute kidney injury. A key example explored in this thesis is the protein neutrophil gelatinase-associated lipocalin (NGAL). NGAL is expressed at very low levels in a variety of tissue types under normal conditions[64]. However, results from microarray analysis have indicated NGAL to be one of the most robustly induced proteins in the kidney following ischemic injury[65]. Additionally, *in vitro* studies have shown NGAL to be released into the culture medium upon exposure to hypoxic conditions, and clinical studies have observed that patients exhibit increased levels of NGAL in urine due to poor tubular reabsorption after acute kidney injury[64,66]. As such, NGAL has drawn attention as a potential biomarker for detecting acute injury from IRI. Other proteins that have been associated with damage from renal IRI and explored as potential biomarkers are kidney injury molecule 1, Cystatin C and interleukin-18[67].

1.3.2 Activation of the Innate Immune Response

Another major consequence of ischemia reperfusion injury during kidney transplantation is the activation of the innate immune response. As a result of cell damage and death from IRI, normal cellular components are released from cells and components of the extracellular matrix are degraded by proteolysis[18]. These components can act as endogenous ligands, known as DAMPs, to activate the innate immune response within the kidney[68]. Examples of these components that have been implicated in IRI during kidney transplantation are: the non-histone chromatin binding protein high-mobility group box 1 (HMGB-1), heat shock proteins, and extracellular matrix components bigylcan and heparan sulfide, among others[44,69–72]. The tubular epithelial cells of the kidney play an important role in the initiation of innate immune activation as they express pattern recognition receptors that can recognize DAMPs[73]. Four classes of pattern recognition receptors have been identified: Toll-like receptors (TLRs), C-type lectin receptors, retinoic acid-inducible gene-1-like receptors and nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs)[74]. For this thesis, only pattern recognition receptors that have been implicated in IRI during kidney transplantation will be described.

The most well-studied pattern recognition receptors involved in IRI during kidney transplantation are Toll-like receptors[75]. TLRs are a family of highly conserved transmembrane proteins that consist of an extracellular leucine rich repeat domain and an intracellular Toll/IL-1R (TIR) domain, which shares homology with the IL-1 receptor[76]. There have been 11 TLRs identified in humans, which play various roles in responding to stimuli[77]. Typically, TLRs respond to a variety of pathogen associated molecular patterns,

for example, TLR4 specifically recognizes bacterial lipopolysaccharide (LPS)[76]. In renal IRI, TLR 2, 3, 4 and 9 have been shown to be upregulated in kidney tissue and play a role in perpetuating damage from IRI[78–81]. Ligand binding to TLRs triggers an intracellular signaling cascade that culminates in the production of proinflammatory cytokines and chemokines. All TLRs signal through the myeloid differentiation primary response 88 (MyD88) adaptor protein dependent pathway, except TLR3, which employs a TIR-domain containing adaptor-inducing interferon-β (TRIF) adaptor protein (MyD88-independent pathway)[74]. Stimulation of the TLR/MyD88 pathway leads to the activation of transcription factor, nuclear factor kappa light chain enhancer of activated B cells (NF-kB), which translocates to the nucleus and promotes the production of pro-inflammatory cytokines such as TNFα, IL-6 and various chemokines[68]. The TLR/MyD88 pathway also leads to production of the precursor form of IL-1β and IL-18 (pro-IL-1β and pro-IL-18). Similarly, stimulation of the TLR/TRIF pathway can activate NF-kB, however, TRIF is also able to induce transcription factor, interferon regulatory factor 3 (IRF3), leading to the production of type 1 interferons[77].

Another pattern recognition receptor family that has recently been implicated in IRI are NOD-like receptors[82]. NLRs are a family of 20 intracellular cytoplasmic proteins that regulate the inflammatory and apoptotic responses[83]. There are two major classes of NLRs important in renal IRI, the first is non-inflammasome-activating proteins (NOD1/2), whose activation leads to the production of proinflammatory cytokines through NF-kB activity[83,84]. The second class includes inflammasome-activating proteins, most important of which is NLR pyrin domain containing 3 (NLRP3), that is highly upregulated in renal tissue after IRI[85]. These proteins contribute to the activation of

caspases and to the formation of the inflammasome complex. The inflammasome then induces cleavage of pro-IL-1β and pro-IL-18 into their active forms[18].

Ultimately, the activation of different pattern recognition receptors present in the parenchymal cells of the kidney and on infiltrating leukocytes during IRI contribute to the regulation of cell death and the inflammatory response by initiating the production of proinflammatory cytokines and chemokines.

1.3.3 Inflammation

The culmination of cell death and activation of the innate immune response is the perpetuation of inflammation within the transplanted organ. Two important aspects of the inflammatory response in renal IRI are vascular changes and the recruitment of leukocytes to the damaged tissue. Inflammatory mediators such as chemokines and cytokines, including TNF-α and interleukins, play an important role in regulating the inflammatory response to IRI[86,87].

At the vascular level, swelling of endothelial cells and degradation of the cytoskeleton from IRI disrupts endothelial cell-to-cell junctions which leads to increased vascular permeability[88]. Additionally, damaged endothelial cells produce vasoactive substances, such as platelet-derived growth factor and endothelin-1, which cause vasoconstriction, further reducing blood flow to the post-ischemic kidney[89]. Activated endothelial cells also exhibit increased expression of adhesion molecules and release of pro-inflammatory cytokines which participate in leukocyte recruitment into the allograft[90]. Specifically, the chemotaxis, adhesion, and transmigration of leukocytes, such as neutrophils, is initiated by increased expression of P-selectins and endothelial cell

adhesion molecule 1 on endothelial cells[91]. Leukocyte infiltration and activation within the transplanted kidney contributes to graft damage and rejection[90].

Overall, the effects of IRI lead to impaired function and increased immunogenicity in the newly transplanted kidney, which jeopardizes the long-term success of kidney transplant. Therefore, strategies to mitigate the complex consequences of IRI at the time of transplant are critical to improving outcomes.

1.4 Organ Preservation

The severity of tissue damage from IRI depends on the length of cold and warm ischemia and storage conditions. Consequently, ensuring optimal organ preservation at the time of transplantation is critical for mitigating the impact of IRI, especially in DCD donors. Therefore, strategies to improve organ storage conditions are at the forefront of kidney transplantation research. This section will discuss the methods of organ preservation currently employed in clinical practice, followed by the future directions in the field.

1.4.1 Current Preservation Strategies

Since the beginning of transplantation, the state of organ preservation has been regarded as a limitation to successful organ transplant[92]. Traditionally, cold storage on ice has been used to reduce metabolic activity and improve graft viability[93]. Currently, there are two main methods employed for organ storage. Both methods use an acellular preservation solution to maintain intracellular ion concentrations and prevent cellular edema during cold storage[94]. The University of Wisconsin (UW) solution is an example of an intracellular fluid-type solution with low Na^+ and high K^+ levels, that is used as the

standard of care preservation solution for abdominal organ[95]. The most common preservation method is static cold storage (SCS), which involves the flushing and storage of organs in a preservation solution on ice until the time of transplantation. This method is simple to apply in the clinical setting and has proven to be effective for short term organ storage[96].

Another storage method that has gained popularity with the increased use of extended criteria and DCD donor organs is hypothermic machine perfusion (HMP)[97]. This method involves perfusing the organ with a preservation solution at 4 ºC until the time of transplantation. HMP continuously provides metabolic substrates during storage to support the production of ATP and improve tissue energy levels[93]. The use of HMP decreases rates of delayed graft function and improve patient outcomes post-transplant compared to SCS[98], however, the organs still suffer substantial injury from cold temperatures[99]. Therefore, strategies that can limit damage from cold and IRI, as well as offer the opportunity for reconditioning of marginal organs, are the focus of current research and will be explored in the next section.

1.4.2 Therapeutic Strategies in Organ Preservation

Kidney preservation strategies that maintain the organ in a more physiological environment have gained popularity in recent research. The two main parameters being investigated for their role in organ preservation are oxygenation and increased temperature. Lack of oxygen to the kidney during the transplantation process is directly related to the severity of injury observed post-transplant. As such, methods to provide oxygen to the organ during storage are of interest. A study by Jochmans et al. compared a novel method of hypothermic oxygenated machine perfusion versus traditional HMP for

preservation of DCD kidneys during a phase III clinical trial. While there were no significant differences observed in primary graft function, incidence of delayed graft function or patient survival, episodes of acute rejection where higher in the HMP group[100].

More recently, methods that combine oxygenation with physiological temperatures have been developed. The method that has been most studied is normothermic machine perfusion (NMP), which refers to the *ex vivo* perfusion of organs at 37 ºC. Animal and pre-clinical studies have shown NMP to be effective for improving immediate kidney function, and reducing damage upon histopathological analysis[101,102]. Another method that aims to reduce damage from cold IRI, while limiting metabolic demand exacerbated by higher temperatures, is subnormothermic machine perfusion (SNMP). SNMP refers to *ex vivo* organ perfusion between 20-34ºC. Research from our laboratory and others have shown SNMP to be superior to SCS, HMP, and NMP in terms of preserving kidney function and preventing damage from IRI in pre-clinical models of DCD kidney transplantation[103,104]. While both NMP and SNMP require further investigation and validation before they can be employed in the clinical setting, increased temperatures allow for functional assessment of the organ prior to transplantation. Additionally, these methods provide the opportunity for the application of drugs and additives to prevent damage with higher efficiency than during cold storage.

As previously discussed, injury from IRI affects multiple cellular pathways, from mitochondrial dysfunction, cell death pathways and activation of the innate immune response, which damage the transplanted organ. Therefore, there are many potential therapeutic targets available for mitigating damage from IRI. Our groups and others have

seen success limiting IRI with the addition of pharmacological agents during preservation in animal models and phase II trials[105–108]. Additionally, gene therapies, such as small interfering RNAs for altering gene expression during IRI have shown some preliminary success[109,110]. However, more evidence to validate the safety and efficacy of these strategies is required before they can be implemented in clinic. Ideally, these interventions will allow for superior organ preservation, preventing IRI, and even create the possibility for organ reconditioning, thereby further expanding the pool of usable organs.

1.4.3 Drug Repositioning in Kidney Transplant

While several therapies show promise for reducing tissue damage from IRI, new drug development can take years from conception to use in patients. Therefore, an emerging strategy in the pharmaceutical industry is drug repositioning or repurposing. Drug repositioning refers to the use of a drug to treat a condition other than the one for which it was initially approved[111]. This strategy allows for more efficient transition of drugs to clinic, as dosage and safety data are already available. Therefore, clinically approved drugs which may have secondary functions that are anti-inflammatory, anti-apoptotic or can limit injury from IRI are of interest in organ preservation.

A key example of a repositioned drug is Metformin, which has recently drawn attention for its renoprotective abilities[112]. Traditionally used as an anti-hyperglycemic drug for the treatment of type II diabetes, the exact mechanism of action of Metformin is still under investigation. Studies suggest it may reduce endothelial dysfunction and activate cellular energy pathways [113–115], which may contribute to limiting damage from IRI. Indeed, several studies have uncovered the protective effects of Metformin in

cerebral and myocardial incidences of IRI[116,117]. Another drug with interesting secondary function for IRI is Glyburide. While primarily used to treat type II diabetes, Glyburide has been identified as a powerful inhibitor of the NLRP3 inflammasome[118], which may help control damaging downstream inflammation and immune activation in the context of kidney IRI. Previous work in our laboratory has examined both Metformin and Glyburide in the context of kidney IRI. Human kidney tubular epithelial cells (HK-2) were treated with 100μM of Metformin or 200μM of Glyburide prior to undergoing hypoxia and reoxygenation. Preliminary results showed an increase in ATP production in HK-2 cells treated with Glyburide and Metformin compared to control, which may further indicate their role in limiting damage from IRI. These drugs are promising examples of drug repositioning in the context of IRI during kidney transplantation. However, to identify an ideal candidate for improving kidney function and preventing damage from transplantation, a large-scale screen of clinically approved drug candidates in the context of kidney IRI is required.

1.5 Models of Ischemia-Reperfusion Injury

To screen existing drugs, a reliable model that encompasses relevant forms of inflammation and injury observed in IRI during kidney transplantation is necessary. While many *in vivo* models exist to evaluate renal IRI, the focus of this section will be *in vitro* models, which provide the most relevant platform to perform drug screening and mechanistic analysis.

First, cell type is an important consideration when selecting an *in vitro* model of renal IRI. The kidney is composed of many cell types, such as tubular epithelial cells and endothelial cells, which respond differently to IRI[119]. Most models focus on renal proximal tubular epithelial cells due to their increased susceptibility to ischemic insult and crucial functional role within the kidney[120]. Primary tubule cells can be isolated from human or animal kidneys and provide an excellent tool to study kidney injury with the most similar behaviour to cells *in vivo*[121]. However, purity of cell type, inconsistency in results and longevity of cells are limitations that compromise the suitability of primary cells for an *in vitro* model[122]. An alternative to primary cells are immortalized cell lines which produce more consistent results, are well-characterized, and can be used for a longer duration[123]. Several renal proximal tubule cell lines exist, including human (HK-2 and RPTEC/TERT), rat (NRK-2) and pig (LLC-PK1), which have been used for *in vitro* models of kidney IRI. Promising results for identifying cell death, markers of hypoxia, inflammation and injury have been achieved in IRI studies with these cell lines[124–127]. However, these cells can exhibit some gene expression patterns not observed *in vivo* and must also be monitored throughout their use for dedifferentiation and transformation[128]. Therefore, it is important to select a cell type that best suits the purpose of the studies to be performed.

Another key aspect of studying IRI *in vitro* is the method of hypoxia and reoxygenation. A broad range of methods have been used to create hypoxic environments and nutrient deprivation for the study of renal IRI. Some studies utilize hypoxia chambers to keep cells between 0% -5% O_2[105,129–131], others submerge cells under a monolayer of mineral oil to induce hypoxic conditions [79,132], both typically in combination with glucose

or nutrient deprivation. Other methods involve chemically mimicking the cellular conditions observed during IRI such as decreased pH, nutrient depletion, and ROS generation[133]. This is accomplished by culturing cells in physiological salt solutions without metabolic substrates, and in the presence of additive such as lactate acid or hydrogen peroxide[134,135].

Additionally, the length of time that cells are exposed to hypoxia and reoxygenation varies between models. The length of hypoxia ranges from 15 min to 24hr and reoxygenation lasts between 5 min to 24hr depending on the experimental model and assessments[124,125,129–131]. Similarly, depending on the aim of the study, temperatures between 4 ºC - 37 ºC have been used for storage of cells during hypoxia, whereas 37ºC is used consistently for reoxygenation[79,105,136–138]. As the temperature and length of hypoxia and reoxygenation periods affect the observed outcomes, it is important to select conditions that accurately mimic the injury being studied.

1.6 Rationale

The goal of our research is to improve the field of organ preservation by applying therapeutics that can limit the damage from IRI during kidney transplantation. Drug repositioning through large-scale screening of clinically approved drug candidates is a promising approach for identifying an effective therapeutic that can be easily translated into clinical use. Due to the lack of consistency and different uses of *in vitro* models to study renal IRI, the development of an *in vitro* model that mimics the injury observed in DCD kidney transplantation is required for effective and relevant drug testing. The development and characterization of such a model will facilitate the screening and study

of the efficacy and mechanism of action of drug candidates against IRI in kidney transplant, prior to their application in our pre-clinical models.

1.7 Hypothesis Statement

We hypothesize that our hypoxia and reoxygenation model will exhibit increased cell death, damage, and markers of inflammation characteristic of renal IRI. Additionally, we expect our model can be an effective tool to screen drug candidates for their therapeutic potential against IRI.

1.8 Specific Aims

In order to test our hypothesis, we aim to: 1) establish an *in vitro* model of IRI using human tubular epithelial cells to mimics clinical DCD kidney transplantation, 2) investigate inflammation and innate immune activity in the established IRI model and 3) to test the efficacy of the *in vitro* model as a screening tool with select drug candidates.

Chapter 2

2 Materials and Methods

2.1 HK-2 Cell Culture

Maintenance of cell line

Human tubular epithelial cells (HK-2; ATCC, VA, USA) were maintained in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12; ThermoFisher Scientific) supplemented with 10% fetal bovine serum (FBS; Gibco), 1% penicillin-streptomycin (Gibco), 1.0 mg/mL recombinant human insulin, 0.55mg/mL human transferrin and 0.5 ug/mL sodium selenite (1 X ITS; Sigma-Aldrich), 5ng/mL epidermal growth factor (EGF; ThermoFisher Scientific), referred to as complete media. Cells were kept in a humidified incubator (37 ºC; 5% CO_2; 21% O_2).

Cell culture: To passage, adherent cells were washed with warm 1X phosphate buffered saline (PBS; Wisent) prior to treatment with 1mL 0.25% trypsin-EDTA (Wisent) at 37 ºC for 1 min. Trypsin was diluted with 9 mL complete media (DMEM/F-12). Cells were collected and pelleted by centrifugation (125xg ; 5 min). Cells were resuspended in the appropriate volume of complete media (1:4) and re-plated for experiments or continued growth. All cells were used below the 20^{th} passage.

2.2 Establishment of Hypoxia and Reoxygenation Conditions

To achieve ideal storage conditions, several storage solutions with different compositions were tested (Table 2-1). HK-2 cells grown to 90% confluence were subjected to 1hr of hypoxia at 37 ºC using a hypoxia chamber (HypOxygen), 24hr of

hypoxia at 4 ºC using a GenBag and 24hr of reoxygenation at 37 ºC in a humidified incubator. Complete media, serum-free media or D-Hanks Buffer were used for warm hypoxia. Serum-free media and/or UW solution (Bridge to Life Ltd.) was used during cold storage. Finally, either complete media or complete media supplemented with UW solution from hypoxia was used for reoxygenation. After undergoing the various IRI-like conditions, cell death was measured using live cell imaging and flow cytometry. HK-2 cells grown to 90% confluence at 37 ºC in complete media were used as a control for all experiments.

In addition, for the control cell death study for the UW supplementation group during reoxygenation, 90% HK-2 cells were incubated with 25% UW solution in 75% complete media (v/v) for 24hr at 37 ºC. Cells were analyzed for viability using flow cytometry.

Table 2- 1. Storage solutions tested during phases of hypoxia and reoxygenation.

Warm Hypoxia	**Cold Storage**	**Reoxygenation**	**Abbreviation**
Serum-free media	Serum-free media	Complete media	SF
Serum-free media	UW Solution	Complete media	SFUW
Complete media	UW solution	Complete media	CompUW
Serum-free media	UW solution	Complete media + 25% UW	SFUW+
D-Hanks Buffer	UW solution	Complete media	DHUW
D-Hanks Buffer	UW solution	Complete media + 25% UW	DHUW+

2.3 *In vitro* Model of Ischemia Reperfusion Injury

The established model conditions concluded from above were used for all subsequent experiments (Figure 2-1). Fresh HK-2 cells were seeded at in a 10cm, 6-well plate, or 24-well plate depending on assay type and grown to 90% confluence. Cells were subjected to 1hr warm hypoxia at 37 ºC in a hypoxia chamber (HypOxystation®) in D-Hanks's Buffer, prepared in house (Table 2-2). Subsequently, D-Hanks Buffer was replaced with UW preservation solution (Table 2-3) and cells were subjected to cold storage at 4 ºC for 24hr using a GenBag (BD Bioscience). After cold storage, 50% of UW solution was removed and 50% was kept to supplement media for a final volume of 25% UW solution. Fresh media was added to cells, and they were grown for 24hr under normal oxygen conditions at 37 ºC. Post-reoxygenation, cells and supernatants were collected for analysis.

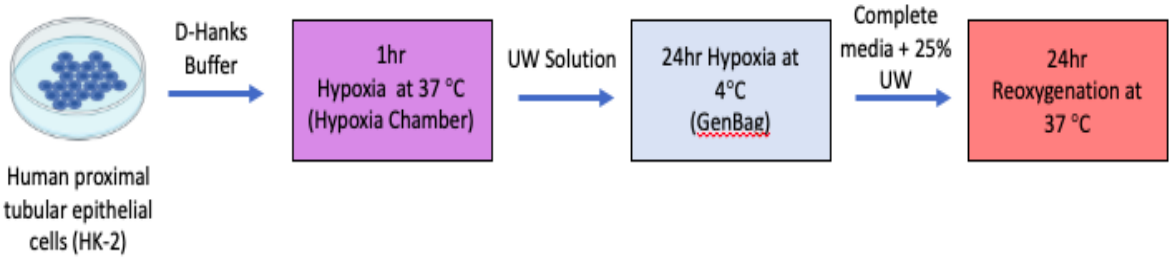


Figure 2-1. Simplified design for an *in vitro* model of ischemia reperfusion injury in kidney transplantation.

A summary of the experimental conditions used in subsequent experiments to validate the model and used for preliminary drug testing.

Table 2-2. 1x D-Hanks Buffer prepared for physiological storage of HK-2 cells during warm hypoxia.

Component	Concentration (mM)
NaCl	140
KCl	5
$CaCl_2$	1
$MgSO_4 – 7H_2O$	0.4
MgCl2 – 6H2O	0.5
$Na_2HPO_4 – 2H_2O$	0.3
KH_2PO4	0.4
D-Glucose (Dextrose)	6
NaHCO3	4

Table 3-3. Composition of Bridge to Life UW preservation solution[139] used for storage of cells during cold hypoxia.

Component	Concentration
Hydroxyethyl starch	50 g/L
Lactobionate	100 mM
Raffinose	30 mM
Na^+	30mM
K+	125mM
Mg^{2+}	5mM
SO_4^{2-}	5mM
PO_4^{3-}	25mM
Allopurinol	1mM
Glutathione	3mM
Adenosine	5mM
Insulin	40 U/L

2.4 Confirmation of Hypoxia

2.4.1 Measurement of HIF-1α via ELISA

To measure HIF-1α, HK-2 cells were plated in a 10cm plate and grown to 90% confluence. Control samples were collected for nuclear extraction. Hypoxia samples were subjected to 1hr warm hypoxia and 24hr cold hypoxia as previously described. Prior to reoxygenation, nuclear and cytoplasmic extracts were prepared using a nuclear extraction kit (Active Motif) as per the manufacturer's protocol. Briefly, cells were washed with 5mL ice cold PBS with phosphatase inhibitors. Cells were collected in PBS and centrifuged at 200xg for 5min. Cells were resuspended in 1x hypotonic buffer and incubated on ice for 15min. Cytoplasmic fractions were isolated by centrifuging samples at 14,000xg for 1min at 4 ºC and supernatants were collected. Nuclear extracts were prepared by resuspending cell pellets in 50uL of complete lysis buffer and incubating suspension for 30min at 4 ºC. Finally, samples were centrifuged for 10min at 14,000xg followed by collection of supernatants. Nuclear extracts were analyzed for the presence of HIF-1α protein using ELISA.

2.4.2 Non-Hypoxic Cells as a Negative Control for Hypoxia

HK-2 cells were grown to 90% confluence in a 6-well plate. At this point cells were subjected to the IRI storage conditions without hypoxia. In summary, cells were stored in D-Hanks Buffer for 1hr at 37 ºC in a humidified incubator (5% CO_2, 21%O_2), followed by storage at 4 ºC for 24hr in UW solution. Then, cells were incubated in complete media + 25% UW solution from cold stage, for 24hr at 37ºC. Viability of cells was assessed using flow cytometry.

2.5 Incubation of HK-2 Cells with TLR Ligands

2.5.1 Stimulation with Lipopolysaccharide

Fresh HK-2 cells were plated on a 24-well plate and grown to 90% confluency. Cells were treated with 1μg/mL of lipopolysaccharide (LPS) in fresh DMEM/F-12 media and incubated at 37 ºC for 12hr. Cell culture supernatants were used for analysis with ELISA.

2.5.2 Stimulation with DAMPs from Conditioned Media

Fresh HK-2 cells were plated on a 24-well plate and grown to 90% confluency. Cells were treated with 500μL conditioned media from IRI-treated cells and incubated at 37 ºC for 24hr. After the incubation period, cell culture supernatants were collected for analysis with ELISA.

2.6 Drug Testing

Fresh HK-2 cells were plated on either a 24-well plate for supernatant or 6-well plate for cell death assays and grown to 90% confluence. Metformin treatment was prepared as follows: Metformin stock solution (1.5mM) was diluted to 100μM using complete media and 50mM stock of Glyburide was diluted to 200μM in complete media. Cells were pre-treated with either 1) 100μM of Metformin or 2) 200μM of Glyburide for 6hr prior to undergoing IRI treatment as previously described. After reoxygenation, cell supernatants were obtained for multiplex ELISA analysis (Eve Technologies Corporation) and cell death was measured using flow cytometry. Drug concentrations were selected based on a previous study in our laboratory.

2.7 Cell Death Assays

2.7.1 Real-Time Cell Death via InCucyte Monitoring

After subjecting cells to warm hypoxia and cold storage in the previously described conditions, cell death was dynamically monitored during 24hr of reoxygenation using an InCucyte® (InCucyte ZOOM, Sartorius) to assess cell death in real time. To detect cell death, 1μg/mL of Propidium Iodide (PI, Thermo Fisher Scientific) was added to each well immediately prior to reoxygenation. The InCucyte captured 9 images of phase and red fluorescence at 4x magnification per well during each hour of reoxygenation. Red objects indicate PI positive staining of DNA, representing cell death. Red objects were counted by the InCucyte software per well between the 9 images and were plotted against time at each hour to produce a cell death curve.

2.7.2 Cell Death and Apoptosis Quantification with Flow Cytometry

At the experimental endpoints, select samples were analyzed for cell viability and apoptosis. Cell culture supernatants were collected into labeled FACS tubes. Each well was washed with 500μL of 1X PBS to remove any traces of FBS. Subsequently, 500μL of 0.25% trypsin-EDTA was added to each well and plates were incubated at 37ºC for 2min to facilitate detachment of the cells. Following incubation, 500μL of complete media was added to each well to inactivate the trypsin. The media containing the detached cells was collected and added into the labeled FACS tubes. The FACS tubes were centrifuged for 5min at 125xg to pellet cells. The cells were resuspended in 1X PBS and centrifuged again at 125xg for 5min. Next, cells were resuspended in 200μL of 1x Annexin-V Binding Buffer (BioLegend). After resuspension, 1μL of FITC-conjugated Annexin V (FITC-Annexin V; BD BioScience) and 3μL of 7-Aminoactinomycin (7-

AAD; eBioScience) were added to each sample to stain for apoptosis and cell death respectively. Samples were incubated with stains in the dark for 15min prior to analysis. Samples were analyzed using the CytoFLEX S (Beckman Coulter) using the following laser gains for the respective channels: 200 FSC, 100 SSC, 10 FITC, 5 PC5.5. Compensation was conducted using 1) unstained live cells, 2) heat-killed cells (90 ºC for 10min) stained with Annexin V only, 3) heat-killed cells stained with 7-AAD only and 4) heat-killed cells stained with both Annexin V and 7-AAD. FlowJo V10 (FlowJo LLC) was used to gate data for statistical analysis.

2.8 Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of damage associated molecular pattern, HMGB-1, kidney damage marker, NGAL, and inflammatory marker, IL-6, secreted in HK-2 cell culture media were measured by ELISA (MyBioSource) from control cells, post-reoxygenation (IRI) and LPS-treated cells. Cell culture supernatants were centrifuged at 3000xg for 10 mins to remove debris. The amount of HIF-1α protein present in nuclear extracts from control or HK-2 cells subjected to warm and cold hypoxia conditions (Hypoxia) was also detected using an ELISA (Abcam). HIF-1α samples were prepared as previously described. A 1:10 dilution was performed on all samples prior to assay. ELISA kits were used according to the manufacturer's protocol. Briefly, 100µL of standards and samples were incubated in the pre-coated ELISA plates for 1hr. After washing, 100µL of biotinylated detection antibody was added to the plates and incubated for 1hr. Plates were washed and 100µL of avidin-HRP working solution was added for 1hr. Following a final wash, substrate reagent was added to the plate and the colour was developed. Plates were

read at 450nm using an iMark microplate reader (BioRad). Concentrations of the target proteins in the samples were determined from the standard curve.

2.9 Multiplex ELISA

Cell culture supernatants from control, hypoxia, LPS, drug-treated and conditioned media treated cells were collected at the previously described endpoints. Samples were centrifuged at 3000xg for 10 mins at 4ºC. Immediately after centrifugation, samples were aliquoted and stored at -80 ºC. Samples were sent to Eve Technologies (Alberta, Canada) for cytokine detection using Luminex xMAP technology.

2.10 Statistical Analysis

All statistical analyses were conducting using GraphPad Prism Version 9. Student's t-test or one-way analysis of variance (ANOVA) and Tukey's post-hoc test was performed to determine statistical differences. Data is represented as the mean ± SEM of a minimum of five independent experiments (n=5), unless otherwise stated. Statistical significance was accepted at $p<0.05$.

Chapter 3

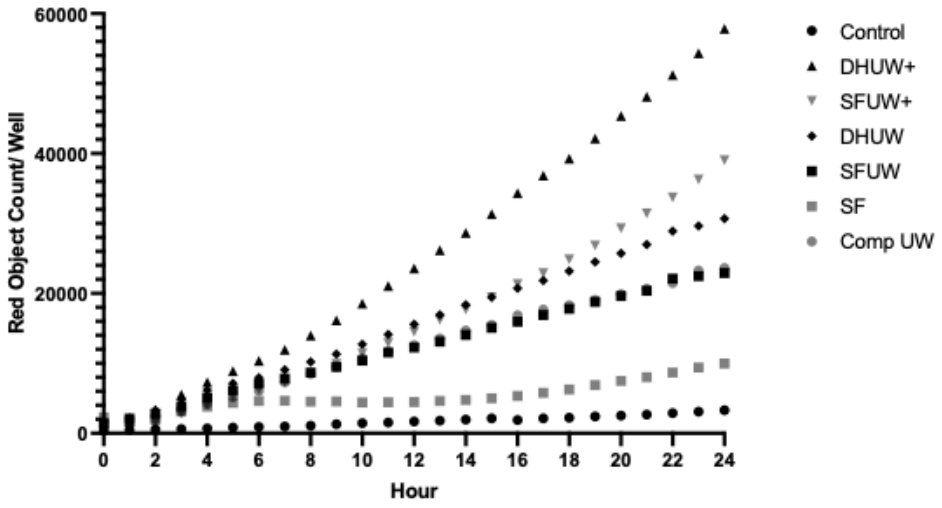
3 Results

3.1 Optimization of *in vitro* DCD IRI model conditions

To determine the optimal treatment conditions necessary to cause significant cell death, implying relevant conditions to IRI during DCD kidney transplant, HK-2 cells were subjected to hypoxia and reoxygenation in various storage solutions. Results from live-cell imaging during reoxygenation (Figure 3-1A) and flow cytometry (Figure 3-1B-D) analysis identified D-Hanks Buffer during warm hypoxia and UW solution during cold storage solution, followed by reoxygenation with UW supplementation (DHUW+) to be the most suitable conditions for storage based on cell death parameters. This condition will be referred to as simulated IRI (sIRI) in subsequent sections.

Additionally, since UW solution contains a high potassium concentration, we wanted to ensure that the cell death observed in the DHUW+ group was not due to the presence of UW solution at warm temperatures. To confirm this, we incubated fresh HK-2 cells with 25% UW solution and 75% complete media (v/v) for 24hr and measured cell death. Analysis using flow cytometry indicated that UW under warm conditions is not sufficient to elicit the cell death observed in the DHUW+ condition (Figure 3-2).

(A)


Red Object Count/ Well
60000
40000
20000
0
0
2
4
6
8
10
12
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16
18
20
22
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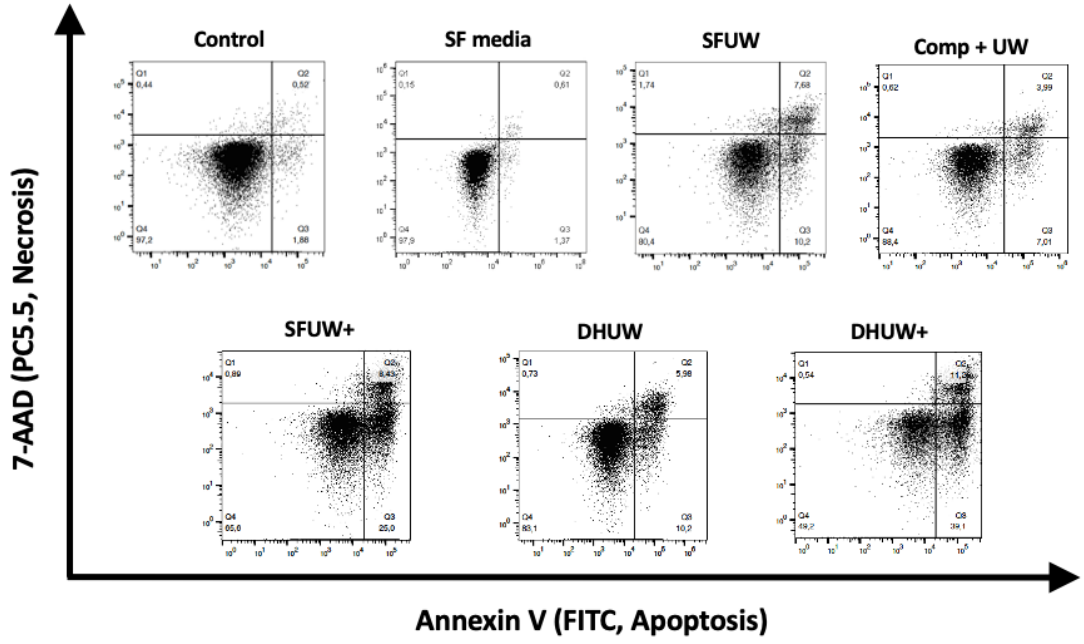
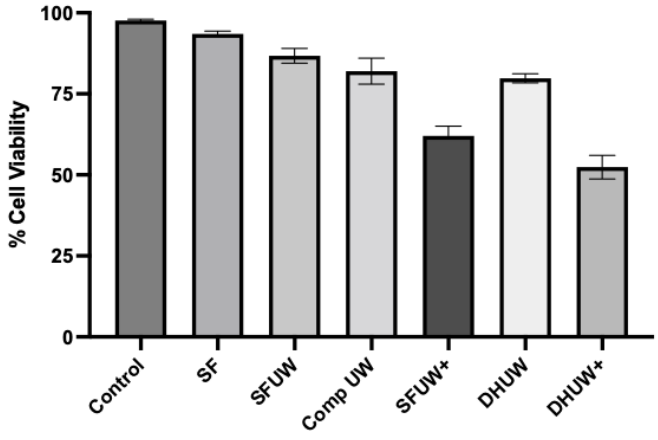
Hour
Control
DHUW+
SFUW+
DHUW
SFUW
SF
Comp UW

(B)


Control
SF media
SFUW
Comp + UW
SFUW+
DHUW
DHUW+
7-AAD (PC5.5, Necrosis)
Annexin V (FITC, Apoptosis)

(C)



(D)

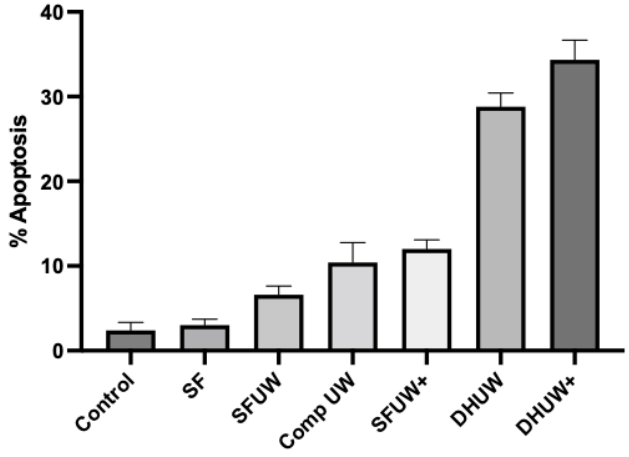
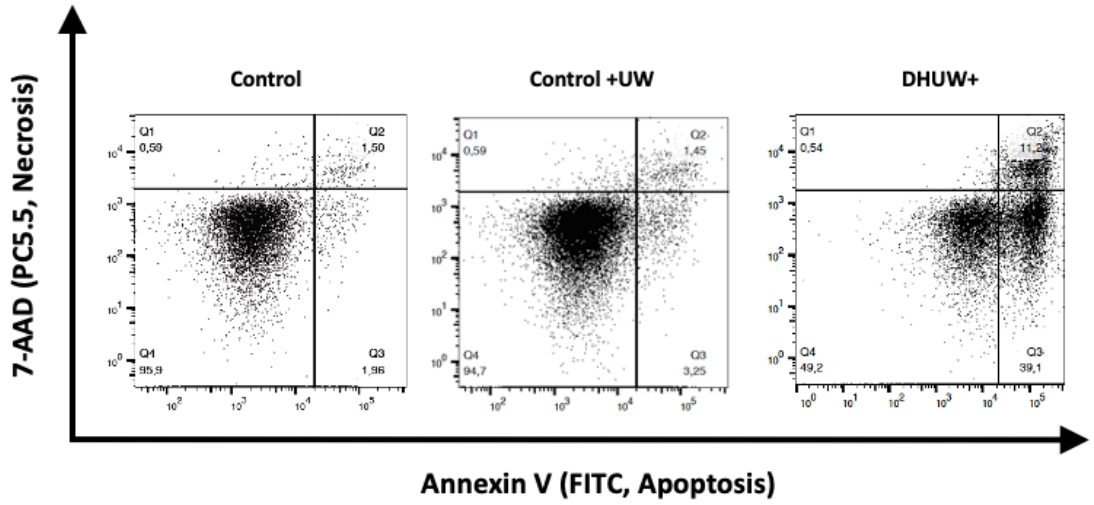


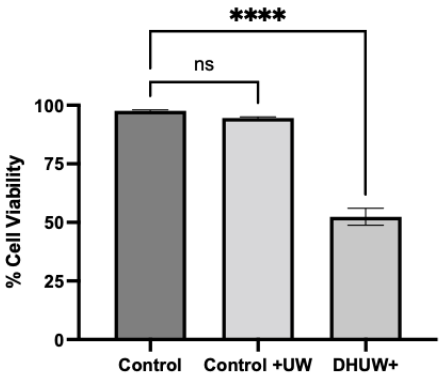
Figure 3-1. Cell death is dependent on storage solution.

HK-2 cells were subjected to 1hr of warm hypoxia at 37 ºC, 24hr of cold storage at 4 ºC followed by 24hr of reoxygenation at 37 ºC in serum-free media (SF), SF, complete media or D-hanks Buffer followed by UW solution (SFUW, Comp UW, DHUW), or SFUW and DHUW with 25% UW supplementation during reoxygenation (SFUW+, DHUW+). Cell death was monitored with **(A)** live-cell imaging during the reoxygenation phase using propidium iodide to label and count dead cells (red objects). Post reoxygenation, cell death was analyzed with **(B)** flow cytometry using Annexin V and 7-AAD staining. Flow cytometry results were quantified for (**C)** cell viability (n=5) and **(D)** apoptosis (n=5). Data is represented as the mean ± SEM.

(A)



(B)



(C)

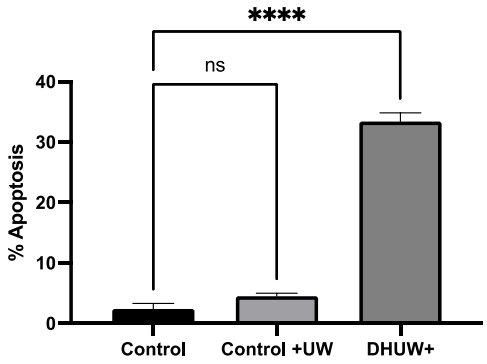


Figure 3-2. Cell death is due to sIRI conditions and not presence of UW solution.

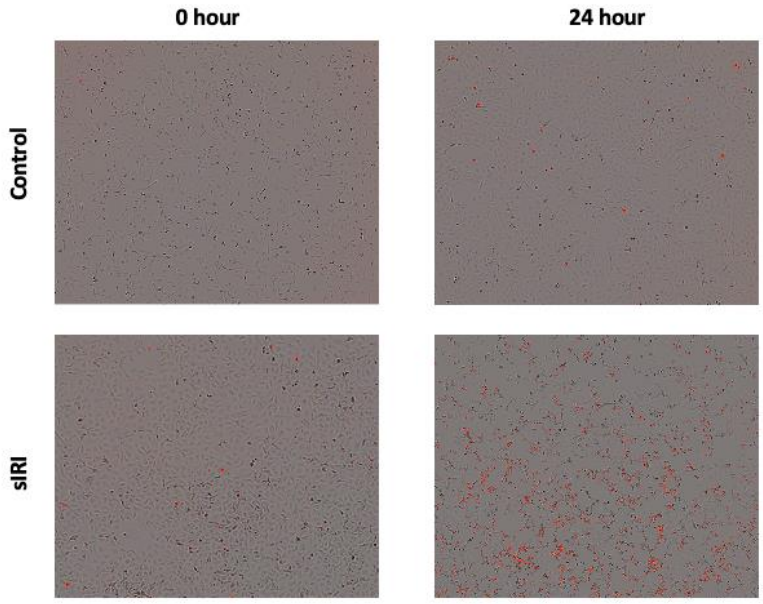
HK-2 cells were treated with 25% UW solution in complete media for 24hr at 37 ºC (control +UW) or subjected to 1hr of warm hypoxia in D-Hanks Buffer at 37 ºC, 24hr of cold storage in UW solution at 4 ºC and 24hr of reoxygenation in complete media supplemented with 25% UW solution from storage at 37 ºC (DHUW+). Post reoxygenation, cell death was analyzed using **(A)** flow cytometry by staining for Annexin V and 7-AAD. Quantification of **(B)** cell viability and **(C)** apoptosis from flow cytometry results of 3 separate experiments (n=3) showed no significant difference in death between control and control +UW conditions. Data is represented as the mean ± SEM. Means were analyzed using one-way ANOVA and Tukey's post-hoc test. **** $p<0.000001$.

3.2 sIRI conditions induce cell death in HK-2 cells

An important aspect of IRI is the activation of cell death pathways. For our model, we wanted to confirm if our applied conditions were able to induce cell death in HK-2 cells. We measured this during the reoxygenation stage by staining cells with DNA stain, propidium iodide, and monitoring with live cell imaging. Increased propidium iodide staining was detected in cells that had experienced sIRI compared to control (Figure 3-3).

Additionally, cell death was quantified after reoxygenation by detecting Annexin V and 7-AAD positive cells with flow cytometry (Figure 3-4A). Annexin V detects plasma membrane component, phosphatidylserine, that is exposed to the external environment during apoptosis. 7-AAD binds double stranded DNA from cells without an intact plasma membrane. Upon analysis, cell viability was significantly reduced in cells that had undergone sIRI compared to control (Figure 3-4B). Additionally, apoptosis, as quantified by Annexin V positive cells, was significantly increased in sIRI (Figure 3-4C).

(A)



(B)

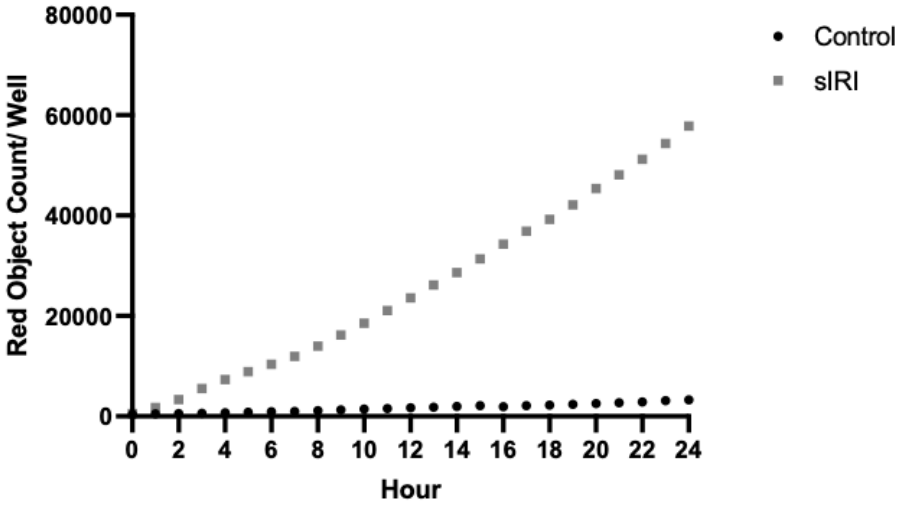
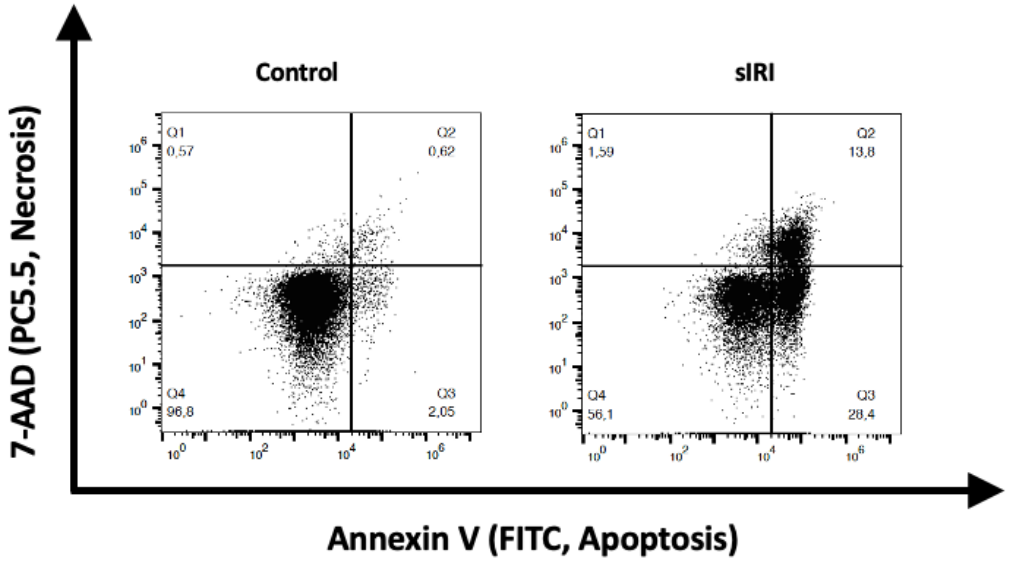


Figure 3-3. Cell death during sIRI is dependent on length of reoxygenation

HK-2 cells were subjected to control or sIRI. Prior to reoxygenation, cells were stained with propidium iodide (PI) and monitored with live cell imaging for 24hr during reoxygenation. **(A)** Images show increased red staining in IRI cells after 24hr. **(B)** A linear increase in cell death, as indicated by PI-positive cells, was observed in the IRI cells during reoxygenation compared to only a slight increase observed in control cells.

(A)



(B) **C)**

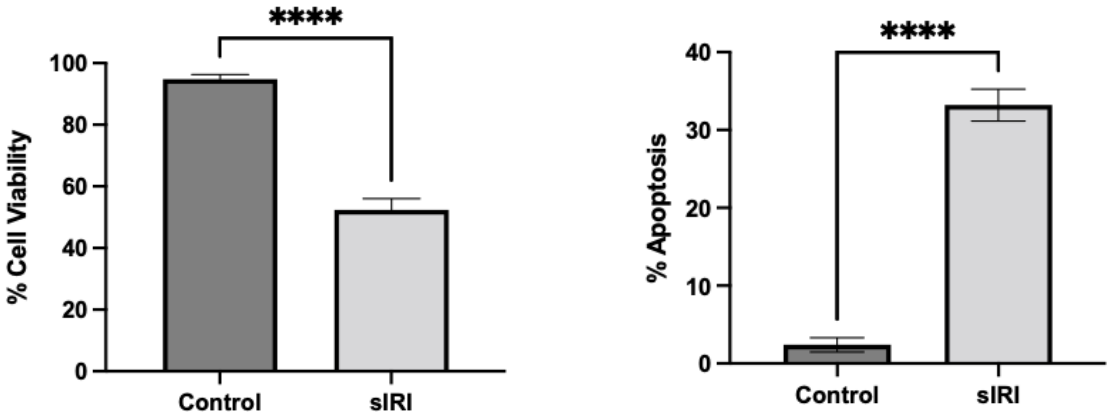


Figure 3-4. IRI model activates cell death pathways.

HK-2 cells were subjected control or sIRI conditions. Post reoxygenation, cell death was analyzed using **(A)** flow cytometry by staining for Annexin V and 7-AAD. Quantification of **(B)** cell viability and **(C)** apoptosis from flow cytometry results of 5 separate experiments (n=5) showed a significant increase in cell death in sIRI conditions compared to control. Data is represented as the mean ± SEM. Means were analyzed using Student's t-test. **** $p<0.000001$.

3.3 Hypoxic conditions are achieved in the IRI model

To confirm hypoxic conditions were achieved by our sIRI conditions, the presence of a hypoxic environment was investigated in two ways. First, the amount of a well-known marker of hypoxia, HIF-1α, a protein that is degraded under normal oxygen conditions but accumulates and translocates to the nucleus upon hypoxic conditions, was measured in cells that had undergone sIRI and control cells. Cells that experienced sIRI expressed more HIF-1α than controls (Figure 3-5), indicating they were exposed to a hypoxic environment.

Additionally, a negative control experiment was conducted to assess what percentage of cell death could be attributed to sIRI storage conditions alone. HK-2 cells were subjected to sIRI storage conditions, consisting of 1hr in D-Hanks buffer at 37 ºC followed by 24hr in UW solution at 4 ºC, in a normal oxygen environment. Results indicated no significant difference in cell death between control and no hypoxia (negative control) conditions, whereas significantly more cell death was observed in cells subjected to sIRI (Figure 3-6).

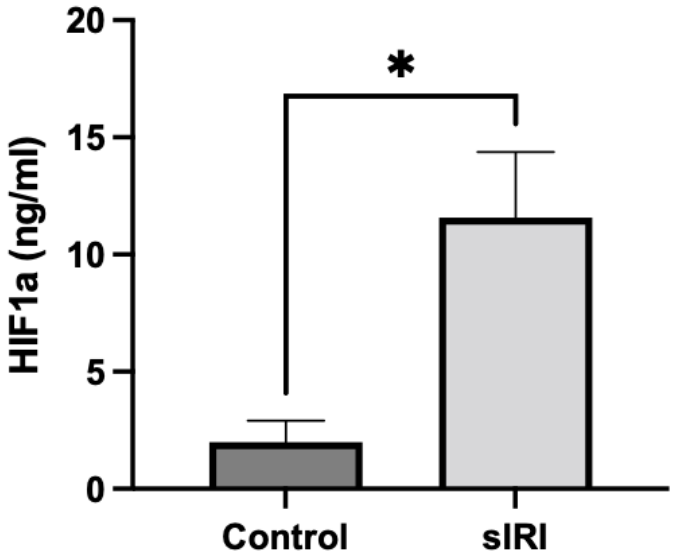
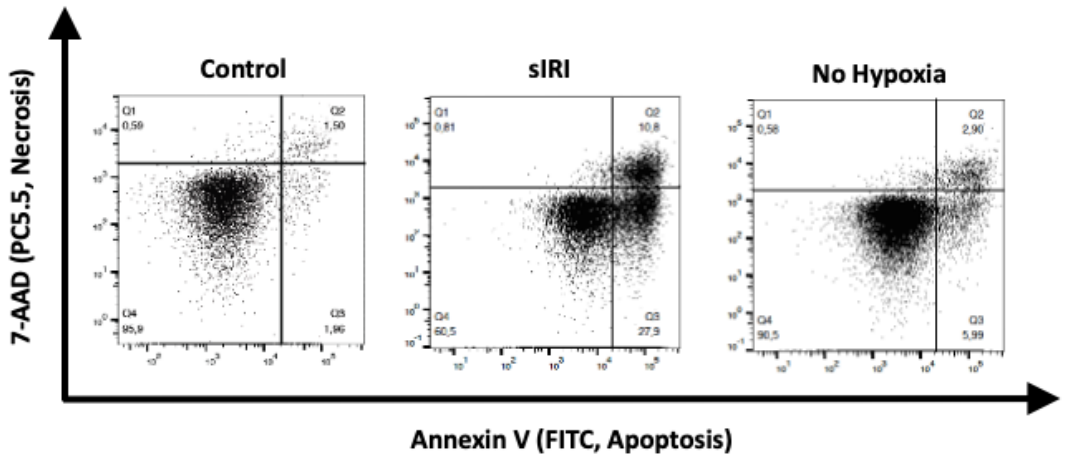


Figure 3-5. Increased levels of HIF-1α were detected in sIRI conditions.

HK-2 cells were subjected to 1hr of warm hypoxia in D-Hanks buffer at 37 ºC followed by 24hr of cold storage in UW solution at 4 ºC under hypoxic conditions. Prior to reoxygenation, levels of the protein HIF-1α in cellular extracts was assessed using ELISA. Results of 5 separate experiments (n=5) showed a significant increase in levels of HIF-1α in hypoxia-treated cells compared to untreated cells (control). Data is represented as the mean ± SEM. Means were analyzed using Student's t-test. *$p<0.05$.

(A)



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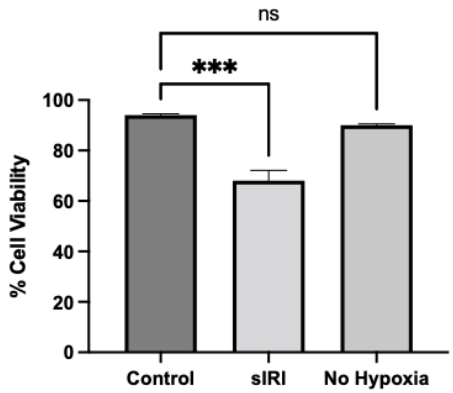

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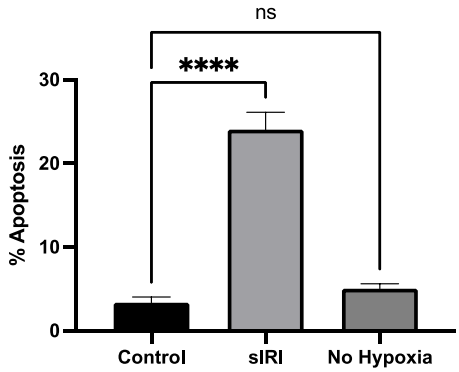

Figure 3-6. Effect of sIRI on cell death.

HK-2 cells were subjected to sIRI conditions of 1hr storage in D-Hanks buffer at 37 ºC and 24hr in UW solution at 4 ºC followed by 24hr reoxygenation in UW-supplemented complete media at 37 ºC, with (sIRI) or without hypoxia (No Hypoxia). Post reoxygenation, cell death was analyzed using **(A)** flow cytometry by staining for Annexin V and 7-AAD. Quantification of **(B)** cell viability and **(C)** apoptosis from flow cytometry results of 3 separate experiments (n=3) showed a significant increase in cell death in sIRI conditions compared to both no hypoxia and control conditions. Data is represented as the mean ± SEM. Means were analyzed using one-way ANOVA and Tukey's post-hoc test. *** $p<0.001$, **** $p<0.000001$.

3.4 sIRI leads to increased release of damage markers

Another important feature of IRI during kidney transplantation is the release of cellular material and proteins as a consequence of cell death and damage, which serve as markers of injury or can act as danger molecules to stimulate the innate immune response. To further investigate whether our model accurately mimics the conditions of IRI during DCD kidney transplantation, we measured the release of two damage markers after subjecting cells to sIRI conditions. First, we looked at damage-associated molecular pattern, HMGB-1, which is a nuclear protein that is released from cells during necrosis or necrosis-like cell death. This molecule can act as an endogenous ligand to stimulate innate immune activity. Analysis of cell culture supernatants using ELISA indicated a significantly higher level of HMGB-1 detected in sIRI-treated cells compared to controls (Figure 3-7A). Additionally, we looked at the secretion of the kidney specific injury marker, NGAL. Levels of NGAL in supernatants of sIRI-treated cells were significantly elevated compared to controls (Figure 3-7B).

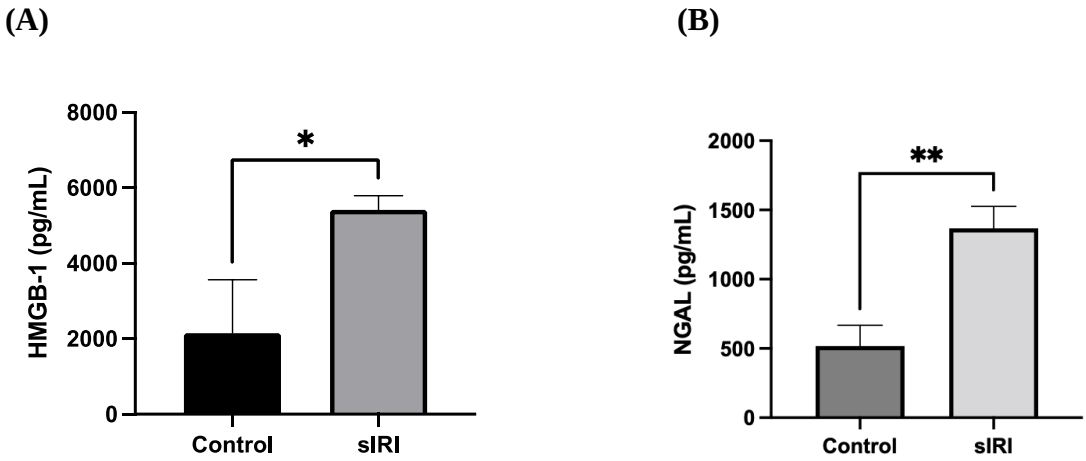

Figure 3-7. sIRI conditions lead to the release of relevant cell damage markers.

HK-2 cells were subjected to sIRI conditions of 1hr warm hypoxia in D-Hanks buffer at 37 ºC and 24hr of hypoxia in UW solution at 4 ºC followed by 24hr reoxygenation in UW-supplemented complete media at 37 ºC or no sIRI treatment (control). Levels of **(A)** high mobility group box 1 (HMGB-1) and **(B)** neutrophil gelatinase-associated lipocalin (NGAL) in cell culture supernatants were measured by ELISA, n=3**.** Data is represented as the mean ± SEM. Means were analyzed using Student's t-test. *$p<0.05$, ** $p<0.01$.

3.5 sIRI leads to production of pro-inflammatory cytokines

The inflammatory response is an important part of stimulating and perpetuating the injury from IRI during kidney transplantation. To investigate whether our model conditions were able to produce pro-inflammatory cytokines known to be involved in IRI, cell culture supernatants were analyzed for multiple cytokines using multiplex ELISA. From the cytokines tested, we observed significantly elevated levels of several pro-inflammatory cytokines in sIRI supernatants compared to control (Figure 3-8). This indicates that our model is able to produce cytokines relevant to the inflammatory response seen during renal IRI.

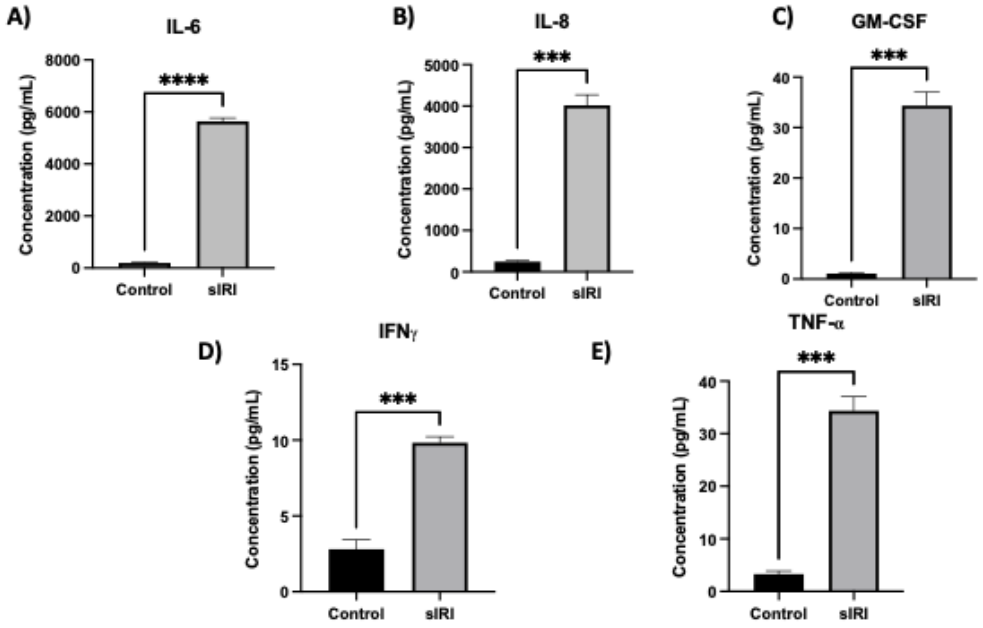

Figure 3-8. Pro-inflammatory cytokines are produced during sIRI.

HK-2 cells were subjected to sIRI conditions of 1hr warm hypoxia in D-Hanks buffer at 37 ºC and 24hr of hypoxia in UW solution at 4 ºC followed by 24hr reoxygenation in UW-supplemented complete media at 37 ºC or no IRI treatment (control). Cell culture supernatants post-reoxygenation were analyzed for pro-inflammatory cytokines **(A)** IL-6, **(B)** IL-8, **(C)** GM-CSF, **(D)** IFNγ and **(E)** TNF- α using multiplex ELISA, n=3. Data is represented as the mean ± SEM. Means were analyzed using Student's t-test. *** $p<0.001$, **** $p<0.000001$.

3.6 sIRI conditions stimulate the innate immune response

The activation of innate immune receptors on parenchymal and immune cells by DAMPs leads to the production of pro-inflammatory cytokines during renal IRI. To confirm if any signaling pathways related to innate immune activation are triggered in HK-2 cells, we used a natural TLR4 ligand, lipopolysaccharide (LPS), to stimulate TLR activity. We compared the levels of IL-6 secreted into cell culture supernatants, a downstream product of TLR4 signaling, in both LPS stimulated and sIRI treated HK-2 cells. We observed that levels of IL-6 were elevated in both the sIRI and LPS conditions compared to control cells (Figure 3-9), indicating that innate immune activity is present. Interestingly, stimulating with LPS or sIRI conditions were able to produce similar levels of IL-6.

To further assess whether DAMPs from HK-2 cells exposed to sIRI could activate naïve cells, we incubated fresh HK-2 cells with cell culture supernatants from sIRI-treated cells (conditioned media), which should contain DAMPs such as HMGB-1. Upon analysis, conditioned media was not able to elicit cell death in HK-2 cells (Figure 3-10), however, it was able to stimulate the production of pro-inflammatory cytokines to similar levels as sIRI (Figure 3-11).

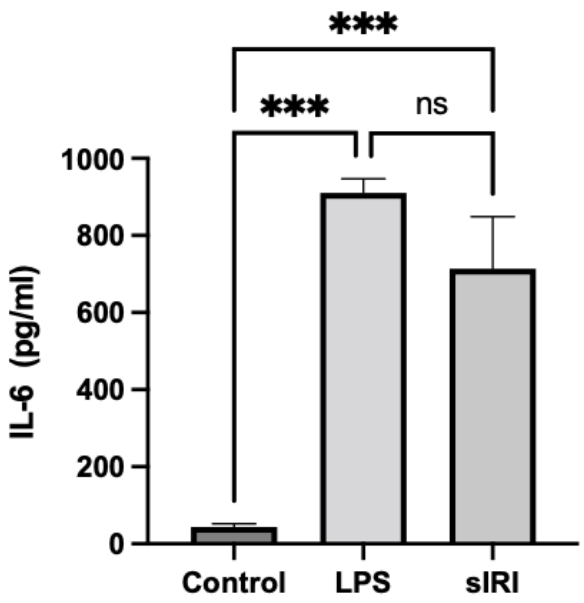

Figure 3-9. Interleukin-6 production in HK-2 cells through TLR stimulation

HK-2 cells were either incubated with 1μg/mL of LPS for 12hr (LPS) or subjected to 1hr warm hypoxia in D-Hanks buffer at 37 ºC and 24hr of hypoxia in UW solution at 4 ºC followed by 24hr reoxygenation in UW-supplemented complete media at 37 ºC (sIRI). Levels of IL-6 in cell culture supernatants (n=5) were measured using ELISA. Data is represented as the mean ± SEM. Means were analyzed using one-way ANOVA and Tukey's post-hoc test. *** $p<0.001$, ns $p>0.05$.

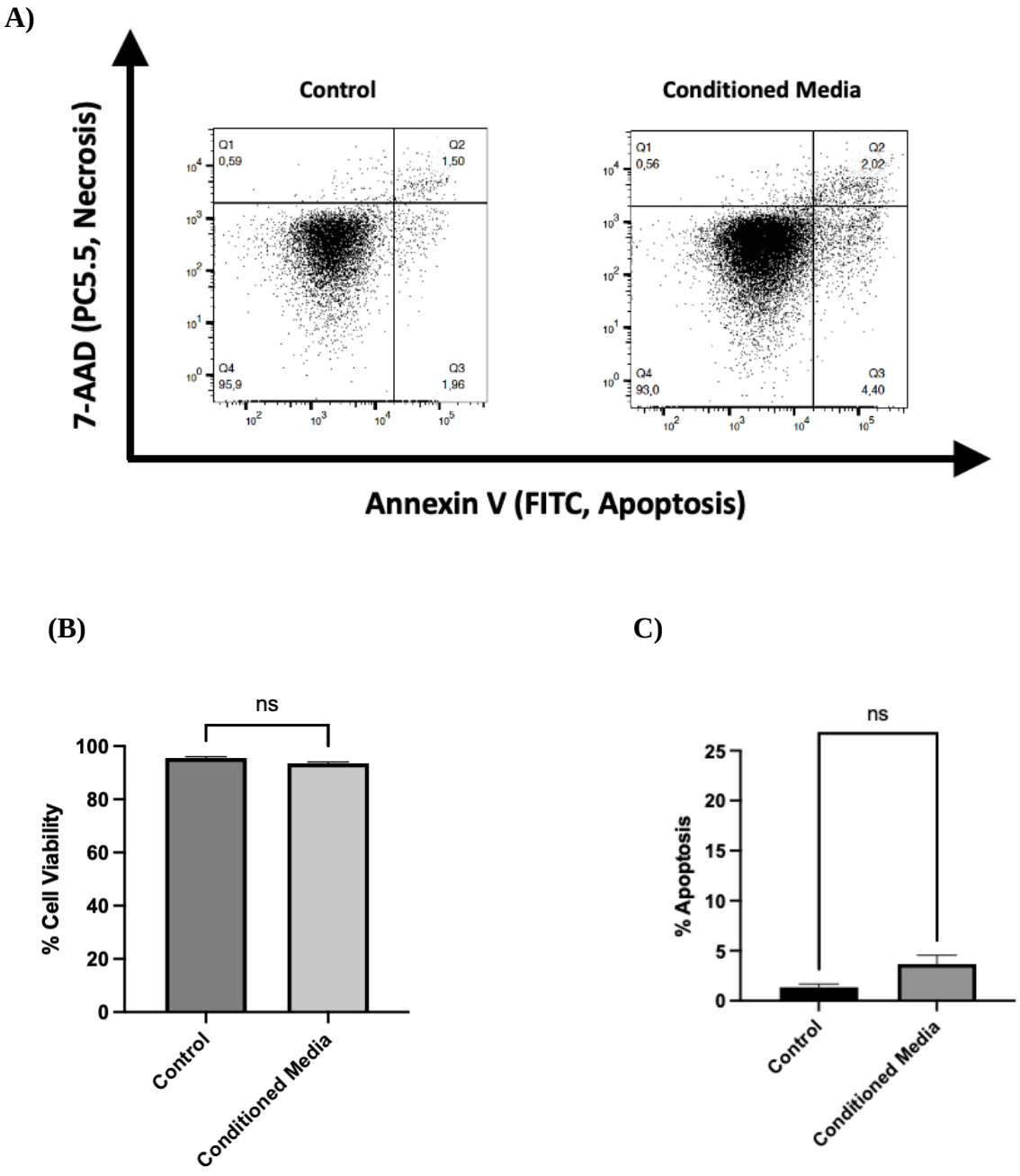

Figure 3-10. Conditioned media does not trigger cell death.

HK-2 cells were incubated with conditioned media from sIRI treatment for 24hr (Conditioned Media). Post reoxygenation, cell death was analyzed using **(A)** flow cytometry by staining for Annexin V and 7-AAD. Quantification of **(B)** cell viability and **(C)** apoptosis from flow cytometry results of 5 separate experiments (n=5) showed no significant difference in cell death between treatment with conditioned media and control conditions. Data is represented as the mean ± SEM. Means were analyzed using Student's t-test. ns $p>0.05$.

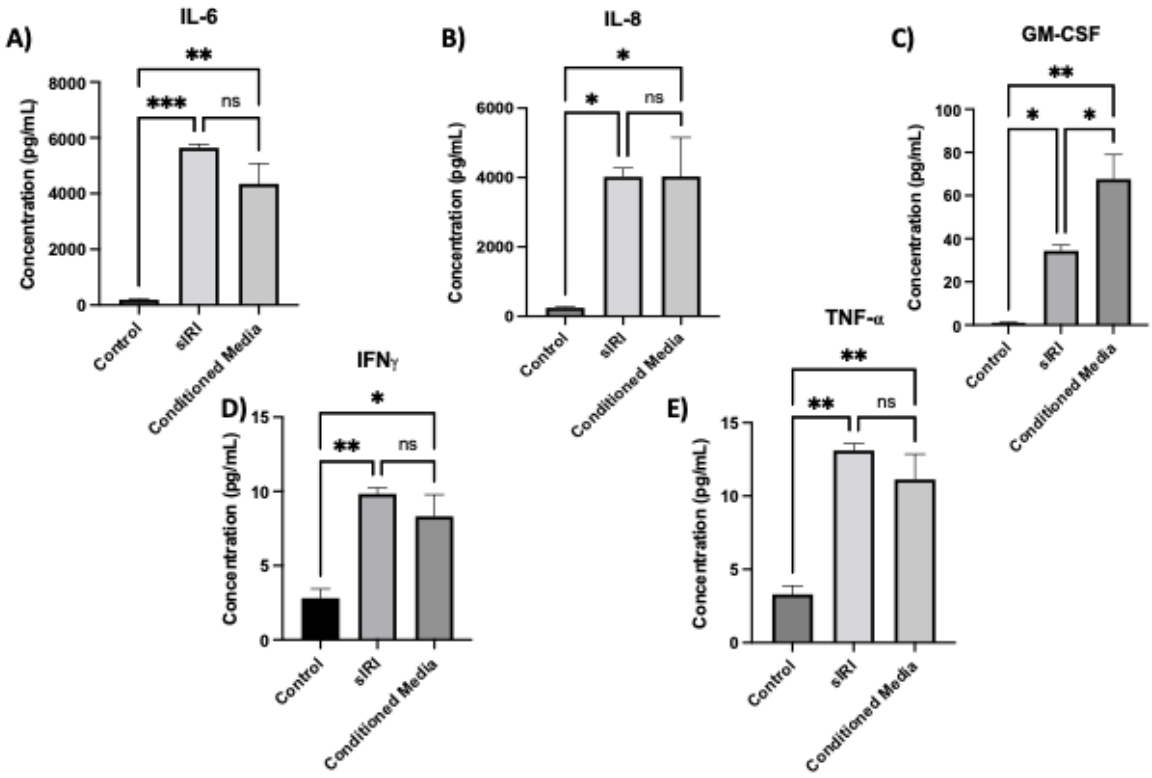

Figure 3-11.Conditioned media leads to the production of pro-inflammatory cytokines

HK-2 cells were subjected to 1hr hypoxia in D-Hanks buffer at 37 ºC and 24hr of hypoxia in UW solution at 4 ºC followed by 24hr reoxygenation in UW-supplemented complete media at 37 ºC (sIRI) or incubated with conditioned media from sIRI treatment for 24hr (Conditioned Media). Cell culture supernatants (n=3) post-reoxygenation were analyzed for pro-inflammatory cytokines **(A)** IL-6, **(B)** IL-8, **(C)** GM-CSF, **(D)** IFNγ and **(E)** TNF- α using multiplex ELISA. Data is represented as the mean ± SEM. Means were analyzed using one-way ANOVA and Tukey's post-hoc test. *$p<0.05$, ** $p<0.01$, *** $p<0.001$, ns $p>0.05$.

3.7 IRI model can be used for drug testing

As our future goal is to use the developed IRI model for large scale drug screening, we wanted to ensure it provided an effective and reliable platform for drug testing. To investigate this, we selected two candidate drugs, namely Metformin and Glyburide, which were previously explored for their role in reducing injury from IRI in our laboratory. HK-2 cells were pre-treated with either 100μM Metformin or 200μM Glyburide prior to sIRI treatment. The ability of the drug treatments to reduce cell death were analyzed with flow cytometry (Figure 3-12A). Neither Metformin nor Glyburide was able to significantly improve cell viability or apoptosis post reoxygenation (Figure 3-12B,C).

Furthermore, to test whether drug treatments were able to reduce inflammation, which is relevant to reducing damage from IRI, cell culture supernatants were analyzed for pro and anti-inflammatory cytokines with multiplex ELISA. Both Glyburide and Metformin treatment were able to significantly reduce the levels of IL-6, IL-8 and GM-CSF compared to sIRI treatment alone (Figure 3-13A-C). Additionally, Glyburide treatment was also able to significantly reduce IFNγ and TNF- α levels (Figure 3-13D, E). Interestingly, while not significant, Metformin treatment showed increased levels of anti-inflammatory cytokines (Figure 3-13F-H).

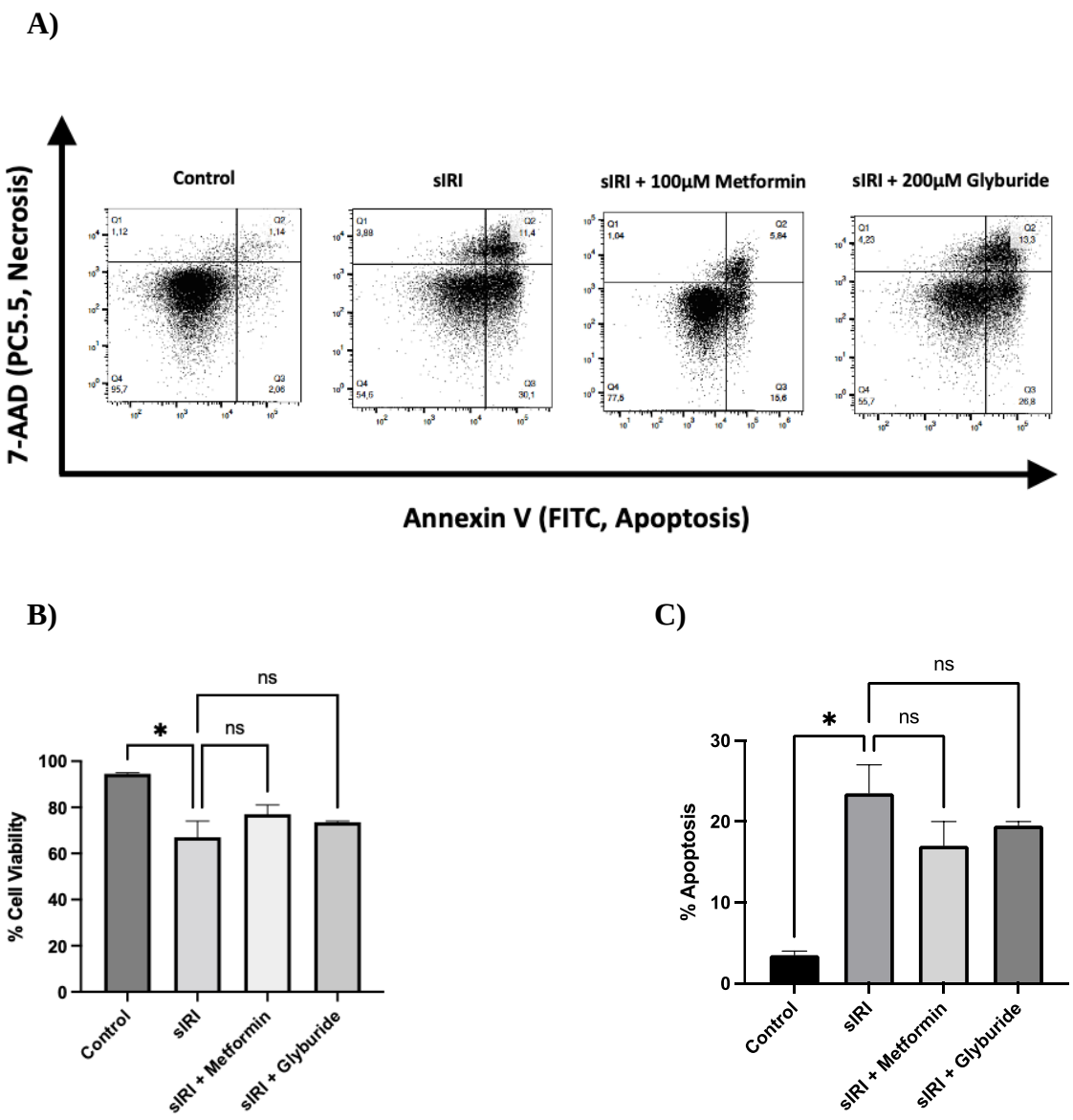

Figure 3-12. Drug treatment does not reduce cell death in IRI model.

HK-2 cells were pre-treated with either 100µM Metformin (sIRI + Metformin) or 200µM Glyburide (sIRI + Glyburide) for 6hr prior to being subjected to 1hr hypoxia in D-Hanks buffer at 37 ºC and 24hr of hypoxia in UW solution at 4 ºC followed by 24hr reoxygenation in UW-supplemented complete media at 37 ºC (sIRI). Post reoxygenation, cell death was analyzed using **(A)** flow cytometry by staining for Annexin V and 7-AAD. Quantification of **(B)** cell viability and **(C)** apoptosis from flow cytometry results (n=3) indicated no change in cell death between either Metformin or Glyburide pre-treatment compared to sIRI conditions alone. Data is represented as the mean ± SEM. Means were analyzed using one-way ANOVA and Tukey's post-hoc test. * $p<0.05$, ns $p>0.05$**.**

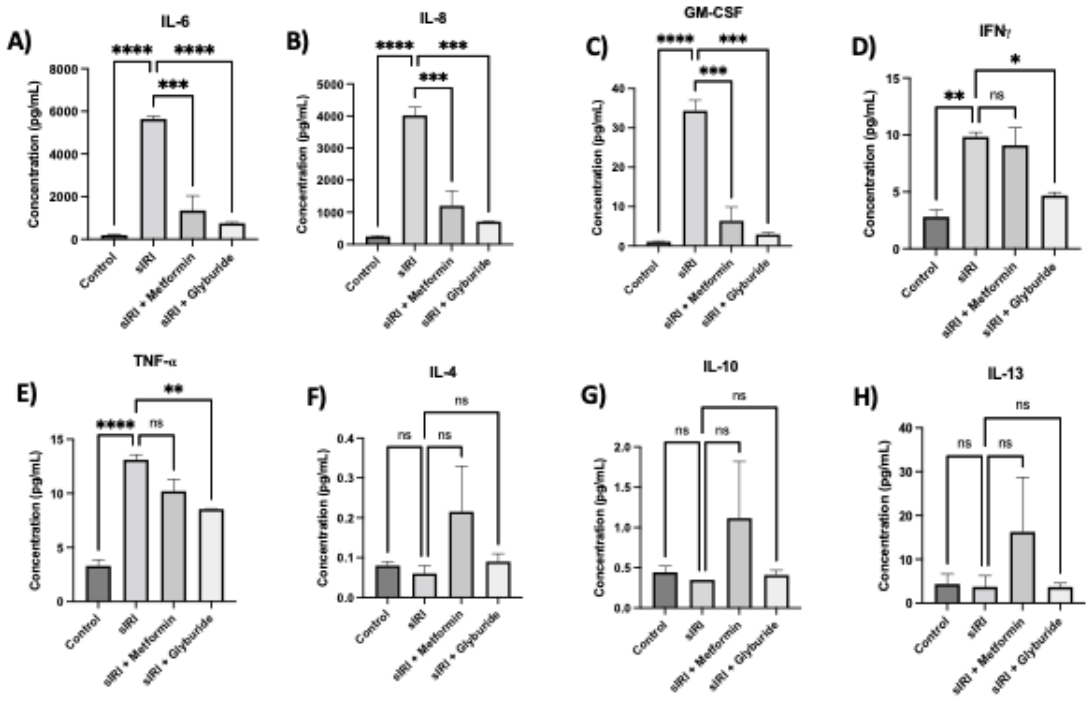

Figure 3-13. Drug treatment alters cytokine levels in IRI model.

HK-2 cells were pre-treated with either 100µM Metformin (sIRI + Metformin) or 200µM Glyburide (sIRI + Glyburide) for 6hr prior to being subjected to 1hr hypoxia in D-Hanks buffer at 37 ºC and 24hr of hypoxia in UW solution at 4 ºC followed by 24hr reoxygenation in UW-supplemented complete media at 37 ºC (sIRI). Following reoxygenation, levels of pro-inflammatory cytokines (n=3)**(A)** IL-6, **(B)** IL-8 **(C)** GM-CSF **(D)** IFNγ and anti-inflammatory cytokines **(F)** IL-4, **(G)** IL-10 and **(H)** IL-13 were measured using multiplex ELISA. Data is represented as the mean ± SEM. Means were analyzed using one-way ANOVA and Tukey's post-hoc test. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.000001, ns p>0.05.

Chapter 4

4 Discussion

IRI remains a major clinical problem in kidney transplantation as it has been linked to increased rates of DGF, prolonged hospitalization, acute graft rejection and early graft loss[140]. Improving organ preservation prior to transplantation can mitigate damage from IRI[93]. Currently, the clinical standard for organ preservation is either static cold storage or non-oxygenated hypothermic machine perfusion at 4 ºC using a preservation solution, such as UW solution[93]. While these methods are effective for reducing metabolic demand and promoting cell survival pretransplant, the grafts still experience injury from cold ischemia[141]. To improve preservation, recent research in transplantation has focused on creating more physiological conditions for organ storage. Several groups have shown success with normothermic and subnormothermic oxygenated perfusion methods[103,142,143]. However, current preservation solutions are ineffective at warmer temperatures and they cannot carry oxygen to be delivered to the organ[144,145]. Therefore, identifying an optimal perfusion solution for NMP or SNMP is of interest. Our group has successfully utilized a hemoglobin-based oxygen carrier (HBOC) during SNMP to improve kidney function, reduce acute tubular necrosis and levels of pro-inflammatory cytokines in a porcine model of DCD kidney transplantation[146]. Despite this improvement, damage from IRI still persists. As such, we aim to apply existing drugs or small molecules that can further target damage from IRI to perfusates during organ storage. Since screening and mechanistic study of drug candidates cannot be efficiently executed in animal models, an *in vitro* model that accurately mimics the damage from

renal IRI is required. In this study, we designed and characterized an *in vitro* model of IRI that mimics the injury and conditions of DCD kidney transplantation, which can be used to screen and study the therapeutic effects of drug candidates.

First, we established a model that mimicked the warm ischemia before organ procurement, cold ischemic time during prolonged storage and reperfusion post-transplant in the recipient, as observed in DCD kidney transplantation (Figure 2-1). From these conditions, we were able to induce cell death in human tubular epithelial cells (Figure 3-4), which are the main target of damage during IRI[21]. This finding was consistent with other models of renal IRI, which show between 50-65% cell death from IRI conditions depending on cell type and methods used[105,147]. Additionally, in the clinical setting, increased apoptosis of tubular epithelial cells is observed and is highly correlated with the severity of ischemia reperfusion injury[48]. Cell death is a major consequence of IRI during kidney transplantation that contributes to poor function post-transplantation and promotes further damaging factors such as innate immune activation and inflammation. Therefore, the presence of cell death in our model was important for mimicking the clinical conditions of renal IRI.

Next, we investigated the ability of our model to produce organ specific damage markers. Several biomarkers have been identified in the clinical setting for detecting acute kidney injury from IRI[148]. In this study, we specifically looked at the secretion of neutrophil gelatinase-associated lipocalin (NGAL), which has previously been shown to be upregulated and secreted by HK-2 cells in other *in vitro* models of renal IRI[149]. We

detected increased levels of NGAL in cell culture supernatants post-reoxygenation in our IRI conditions compared to control cells (Figure 3-7B). This finding correlates with clinical data, where urine levels of NGAL are significantly elevated post-transplantation and predictive of poor graft function[150]. Therefore, detection of increased NGAL levels in our model confirms a relevant form of cellular injury has occurred and provides a kidney specific injury marker that can be used to monitor cell damage.

Further, we considered the ability of our model to produce pro-inflammatory cytokines, which are key in perpetuating damage and increasing immunogenicity in the kidney post-transplant. In this study, we looked at the levels of several pro-inflammatory cytokines in cell culture supernatants post-reoxygenation. We observed significantly increased secretion of IL-6, IL-8, GM-CSF, IFNγ and TNF- α in HK-2 cells that underwent our IRI conditions compared to controls (Figure 3-8). This finding is important as these cytokines have been implicated in the literature for their role in perpetuating damage from IRI after kidney transplantation. Specifically, studies have shown a significant release of IL-6 and IL-8 post-IRI by the renal allograft[151] and that blockade of these molecules can mitigate damage from IRI[152]. Additionally, donor biopsies show increased expression of the genes for IL-6 and IFNγ post-reperfusion[153]. Similarly, donor gene expression of TNF- α and IL-6 correlate with increased risk of impaired renal function, injury and the development of chronic allograft nephropathy[154,155]. Likewise, early expression of GM-CSF by tubular epithelial cells promotes MCP-1-mediated graft fibrosis and inflammation[156]. Overall, these molecules are responsible for signaling the recruitment and activation of immune cells such as

neutrophils, macrophages, natural killer cells and T-cells into the graft, which cause further damage to the transplanted organ and increase the risk of recognition by the recipient's immune system[157,158]. Therefore, the stimulation of pro-inflammatory cytokine production by our model conditions is important to accurately replicate the environment created during DCD kidney transplantation.

Importantly, our results showed that immune activity, such as cytokine expression, was present in our model despite the absence of microbial contaminants in a sterile environment. Cytokine production is regulated by several transcription factors, including NF-kB, which is activated in response to signalling from pattern recognition receptors. Pattern recognition receptors such as TLRs typically recognize microbial products known as pathogen-associated molecular patterns (PAMPs), however, they can also be activated by endogenous ligands from cell death and damage, known as DAMPs[74]. While renal tubular epithelial cells are known to express pattern recognition receptors and participate in the immune response during IRI[75,159], we wanted to confirm TLR activation in HK-2 cells used for our model. Naïve HK-2 cells incubated with traditional TLR4 ligand, LPS, displayed significantly increased levels of IL-6 compared to control cells, but similar levels as seen in the IRI condition (Figure 3-9). This is consistent with previous studies which showed increased supernatant levels of cytokines such as IL-6 and TNF- α in response to LPS treatment[160] in HK-2 cells. Therefore, we confirmed that HK-2 cells express TLRs, and their activity is potentially triggered by our model conditions to produce cytokines.

To confirm that we produced TLR activity in a sterile environment, we needed to confirm the presence of DAMPs in our model. We examined the levels of damage-associated molecular pattern, HMGB-1, which is a nuclear protein released during necrosis-like cell death[44]. We observed significantly increased levels of HMGB-1 protein in supernatants from our IRI conditions compared to untreated cells (Figure 3-7A). This finding supports existing studies that implicate HMGB-1 as a DAMP during renal IRI that is capable of stimulating TLRs. Specifically, HMGB-1 can activate TLR4 to produce pro-inflammatory cytokines such as IL-6 and TNF- α after ischemia reperfusion injury during kidney transplantation[71,161,162] .

Next, to ensure the increase in cytokines levels was from innate immune activation, we incubated fresh HK-2 cells with the DAMPs-containing conditioned media from our sIRI conditions. We observed increased levels of pro-inflammatory cytokines IL-6, IL-8, GM-CSF, IFNγ and TNF- α compared to untreated cells, but similar levels as produced in our IRI conditions (Figure 3-11). This indicates that, mechanistically, the inflammatory response we observe in our IRI model conditions is produced through the activation of innate immune signaling pathways, such as TLRs. This represents an important criterion for our model, as immune activation status during the early post-transplant period is correlated with poor outcomes and increased risk of rejection, and is therefore an important therapeutic target in IRI[14]. Overall, our model can reproduce the immune activation and inflammatory response observed during IRI in DCD transplantation.

Finally, our goal is to use the model to screen and study the mechanism of drugs for their role in mitigating IRI. To support this, we showed that drugs can easily be applied in our model and that relevant outputs, such as cell death and cytokine levels can be monitored. Specifically, we applied pre-treatment with either Glyburide or Metformin, clinically approved drugs that have shown promising preliminary results in mitigating damage from IRI[112,163], and measured changes in cell death parameters and cytokine levels after IRI conditions. Neither Metformin nor Glyburide pre-treatment were able to improve cell viability (Figure 3-12B) or apoptosis (Figure 3-12C). Additionally, cytokine analysis indicated that pre-treatment with Metformin was able to significantly reduce levels of IL-6, IL-8, GM-CSF compared to IRI treatment alone, while pre-treatment with Glyburide significantly reduce IL-6, IL-8, GM-CSF, IFNγ and TNF- α (Figure 3-13A-E). Interestingly, while not significant, Metformin treatment also seemed to stimulate the production of anti-inflammatory cytokines IL-4, IL-10, and IL-13 (Figure 3-13F-H), however further experiments are required to confirm whether this is a true effect. These findings support the ability of our model to be used to screen and study drug candidates for reducing IRI. Additionally, further mechanistic studies can be conducted on promising drug candidates using this model to understand their ability to mediate damage from IRI.

While we have shown that our model mirrors the conditions of IRI observed in clinical DCD kidney transplantation, including key outcomes such as the induction of hypoxia, cell death and inflammation, several limitations exist. First, and most importantly, our model is a 2D tissue culture representation of renal IRI, which cannot

truly encompass the complexity of IRI at the organ level. The cell microenvironment plays a key role in how it responds to stimuli and regulates processes both in normal and pathological conditions[164]. Our model does not consider important physiological aspects such as the microfluidic environment, additional cell types present within the kidney that respond during IRI, and loss of structural integrity which may contribute to the severity of IRI[128]. Therefore, cells may behave differently *in vivo* than observed in our model. However, we were able to confirm that several parameters observed during clinical IRI were also replicated by our model, including the pro-inflammatory response. Importantly, the ligands that activate innate immune receptors leading to inflammation have been shown to be conserved across *in vitro* and *in vivo* studies[74]. Another limitation of this study is the lack of immune cells present in our model. Infiltration of leukocytes into the allograft, such as dendritic cells, neutrophils and macrophages are an important aspect of tissue damage during IRI and contribute to acute rejection post-transplantation[165]. While our model cannot recapitulate all *in vivo* elements of IRI, we selected tubular epithelial cells, which express cytokines in a sterile environment through activation of TLRs[159], and share mechanistic aspects of IRI pathogenesis *in vivo* and retain susceptibility to therapeutic intervention related to these pathways.

4.1 Future Directions and Conclusion

Therefore, despite these limitations, our model provides a unique platform to study drug candidates and their mechanism of action in the context of renal IRI during DCD kidney transplantation. Once further study of transcriptome and inflammasomal

roadmaps of this model have been conducted, it will be used for large-scale drug screening using a library of over 770 clinically approved drug candidates with pre-determined dosages and concentrations. Specifically, this library will target drugs with known anti-inflammatory, anti-TLR and anti-apoptotic effects. In addition to the parameters considered in this study, other mechanistic analyses can be conducted for drug candidates. For example, an NF-kB activity assay, and qPCR for expression of TLRs, such as TLR 2,4 and 6 which have been implicated in renal IRI, would indicate the innate immune function of drugs. Further, our model eliminates ethical concerns and financial constraints associated with using animal models for large-scale drug screening. Importantly, promising drug candidates identified using our model can be further studied for their efficacy *in vivo* using our established porcine and human discard models of DCD kidney transplantation.

In conclusion, our model provides the foundation for perfusate enhancement research that can improve organ preservation *ex vivo*. The identification of a drug candidate that can ameliorate the damage from IRI will help improve or replace current organ preservation in static cold conditions. Additionally, repositioning of existing clinically approved drugs will allow for the rapid translation of therapeutics to clinical use. Further, the optimization of storage conditions will allow the opportunity to protect and repair DCD organs. Therefore, we can increase the quality and quantity of organs available for transplantation which can improve the quality of life of patients suffering from end-stage renal disease.

References

1. Trillini, M., Perico, N. & Remuzzi, G. *Epidemiology of End-Stage Renal Failure: The Burden of Kidney Diseases to Global Health*. *Kidney Transplantation, Bioengineering, and Regeneration: Kidney Transplantation in the Regenerative Medicine Era* (Elsevier Inc., 2017). doi:10.1016/B978-0-12-801734-0.00001-1.
2. CIHI. *Trends in end-stage kidney disease in Canada,* 2019*. https://www.cihi.ca/en/trends-in-end-stage-kidney-disease-in-canada-2019 (2020).
3. Abbaszadeh, A., Javanbakhtian, R., Salehee, S. & Motvaseliyan, M. Comparative Assessment of Quality of Life in Hemodialysis and Kidney Transplant Patients. *SSU_Journals* **18**, 461–468 (2010).
4. Schold, J. D. *et al.* Association between Kidney Transplant Center Performance and the Survival Benefit of Transplantation Versus Dialysis. *Clin. J. Am. Soc. Nephrol.* **9**, 1773–1780 (2014).
5. CIHI. Annual Statistics on Organ Replacement in Canada: Dialysis, Transplantation and Donation, 2010 to 2019. (2020).
6. Tonelli, M. *et al.* Systematic Review: Kidney Transplantation Compared With Dialysis in Clinically Relevant Outcomes. *Am. J. Transplant.* **11**, 2093–2109 (2011).
7. Axelrod, D. A. *et al.* An economic assessment of contemporary kidney transplant practice. *Am. J. Transplant.* **18**, 1168–1176 (2018).
8. Wong, G. *et al.* Comparative Survival and Economic Benefits of Deceased Donor Kidney Transplantation and Dialysis in People with Varying Ages and Co-Morbidities. *PLoS One* **7**, e29591 (2012).
9. CIHI. Canadian Institute for Health Information. e-Statistics Report on Transplant, Waiting List and Donor Statistics, 2019. (2020).
10. Mirshekar-Syahkal, B. *et al.* Local Expansion of Donation After Circulatory Death Kidney Transplant Activity Improves Waitlisted Outcomes and Addresses Inequities of Access to Transplantation. (2016) doi:10.1111/ajt.13968.
11. CIHI. Annual Statistics on Organ Replacement in Canada. (2017).
12. Tennankore, K. K., Kim, S. J., Alwayn, I. P. J. & Kiberd, B. A. Prolonged warm ischemia time is associated with graft failure and mortality after kidney transplantation. *Kidney Int.* **89**, 648–658 (2016).
13. Rao, P. S. & Ojo, A. The Alphabet Soup of Kidney Transplantation: SCD, DCD, ECD—Fundamentals for the Practicing Nephrologist. *Clin. J. Am. Soc. Nephrol.* **4**, 1827–1831 (2009).
14. Siedlecki, A., Irish, W. & Brennan, D. C. Delayed graft function in the kidney transplant. *Am. J. Transplant.* **11**, 2279–2296 (2011).
15. Yarlagadda, S. G., Coca, S. G., Formica, R. N., Poggio, E. D. & Parikh, C. R. Association between delayed graft function and allograft and patient survival: a systematic review and meta-analysis. *Nephrol Dial Transpl.* **24**, 1039–1047 (2009).
16. Jennings, R. B., Murry, C. E., Steenbergen, C. & Reimer, K. A. Development of cell injury in sustained acute ischemia. *Circulation* **82**, 2–12 (1990).
17. Hosgood, S. A., Bagul, A., Yang, B. & Nicholson, M. L. The relative effects of warm and cold ischemic injury in an experimental model of nonheartbeating donor

kidneys. *Transplantation* **85**, 88–92 (2008).
18. Gu, L. *et al.* Initiation of the inflammatory response after renal ischemia/reperfusion injury during renal transplantation. *International Urology and Nephrology* vol. 50 2027–2035 (2018).
19. Debout, A. *et al.* Each additional hour of cold ischemia time significantly increases the risk of graft failure and mortality following renal transplantation. *Kidney Int.* **87**, 343–349 (2014).
20. Smith, S. F., Hosgood, S. A. & Nicholson, M. L. Ischemia-reperfusion injury in renal transplantation: 3 key signaling pathways in tubular epithelial cells. *Kidney Int.* **95**, 50–56 (2019).
21. Sharfuddin, A. A. & Molitoris, B. A. Pathophysiology of ischemic acute kidney injury. *Nature Reviews Nephrology* vol. 7 189–200 (2011).
22. Scholz, H. *et al.* Kidney physiology and susceptibility to acute kidney injury: implications for renoprotection. *Nat. Rev. Nephrol. 2021 175* **17**, 335–349 (2021).
23. Sugiyama, S. *et al.* The effects of sun 1165, a novel sodium channel blocker, on ischemia-induced mitochondrial dysfunction and leakage of lysosomal enzymes in canine hearts. *Biochem. Biophys. Res. Commun.* **157**, 433–439 (1988).
24. Kako, K., Kato, M., Matsuoka, T. & Mustapha, A. Depression of membrane-bound Na+-K+-ATPase activity induced by free radicals and by ischemia of kidney. *https://doi.org/10.1152/ajpcell.1988.254.2.C330* **254**, (1988).
25. Kosieradzki, M. & Rowiński, W. Ischemia/Reperfusion Injury in Kidney Transplantation: Mechanisms and Prevention. *Transplant. Proc.* **40**, 3279–3288 (2008).
26. Schumacher, C. A., Baartscheer, A., Coronel, R. & Fiolet, J. W. T. Energy-dependent Transport of Calcium to the Extracellular Space During Acute Ischemia of the Rat Heart. *J. Mol. Cell. Cardiol.* **30**, 1631–1642 (1998).
27. Roberts, B. N. & Christini, D. J. NHE Inhibition Does Not Improve Na+ or Ca2+ Overload During Reperfusion: Using Modeling to Illuminate the Mechanisms Underlying a Therapeutic Failure. *PLOS Comput. Biol.* **7**, e1002241 (2011).
28. Peng, T.-I. & Jou, M.-J. Oxidative stress caused by mitochondrial calcium overload. *Ann. N. Y. Acad. Sci.* **1201**, 183–188 (2010).
29. C, L. & RM, J. Reactive species mechanisms of cellular hypoxia-reoxygenation injury. *Am. J. Physiol. Cell Physiol.* **282**, (2002).
30. Arduini, A. *et al.* Effect of ischemia and reperfusion on antioxidant enzymes and mitochondrial inner membrane proteins in perfused rat heart. *Biochim. Biophys. Acta - Mol. Cell Res.* **970**, 113–121 (1988).
31. Pasdois, P., Parker, J. E., Griffiths, E. J. & Halestrap, A. P. The role of oxidized cytochrome c in regulating mitochondrial reactive oxygen species production and its perturbation in ischaemia. *Biochem. J.* **436**, 493–505 (2011).
32. Salvadori, M., Rosso, G. & Bertoni, E. Update on ischemia-reperfusion injury in kidney transplantation: Pathogenesis and treatment. *World J. Transplant.* **5**, 52 (2015).
33. Bonventre, J. V. & Yang, L. Cellular pathophysiology of ischemic acute kidney injury. *Journal of Clinical Investigation* vol. 121 4210–4221 (2011).
34. Zhang, L. *et al.* Hypoxia preconditioned renal tubular epithelial cell-derived extracellular vesicles alleviate renal ischaemia-reperfusion injury mediated by the

HIF-1α/rab22 pathway and potentially affected by micrornas. *Int. J. Biol. Sci.* **15**, 1161–1176 (2019).

35. Ziello, J. E., Jovin, I. S. & Huang, Y. Hypoxia-Inducible Factor (HIF)-1 Regulatory Pathway and its Potential for Therapeutic Intervention in Malignancy and Ischemia. *Yale J. Biol. Med.* **80**, 51 (2007).
36. Akhtar, M. Z., Sutherland, A. I., Huang, H., Ploeg, R. J. & Pugh, C. W. The Role of Hypoxia-Inducible Factors in Organ Donation and Transplantation: The Current Perspective and Future Opportunities. (2014) doi:10.1111/ajt.12737.
37. Luo, L., Luo, G., Fang, Q. & Sun, Z. Stable expression of hypoxia-inducible factor-1α in human renal proximal tubular epithelial cells promotes epithelial to mesenchymal transition. *Transplant. Proc.* **46**, 130–134 (2014).
38. Bayrak, O. *et al.* Nigella sativa protects against ischaemia/reperfusion injury in rat kidneys. *Nephrol. Dial. Transplant.* **23**, 2206–2212 (2008).
39. Lejay, A. *et al.* Mitochondria: Mitochondrial participation in ischemia–reperfusion injury in skeletal muscle. *Int. J. Biochem. Cell Biol.* **50**, 101–105 (2014).
40. Martin, J. L., Gruszczyk, A. V., Beach, T. E., Murphy, M. P. & Saeb-Parsy, K. Mitochondrial mechanisms and therapeutics in ischaemia reperfusion injury. *Pediatr. Nephrol. 2018 347* **34**, 1167–1174 (2018).
41. Q, Z. *et al.* Circulating mitochondrial DAMPs cause inflammatory responses to injury. *Nature* **464**, 104–107 (2010).
42. Wu, M.-Y. *et al.* Current Mechanistic Concepts in Ischemia and Reperfusion Injury. *Cell. Physiol. Biochem.* **46**, 1650–1667 (2018).
43. Hotchkiss, R. S., Strasser, A., McDunn, J. E. & Swanson, P. E. Cell Death. *http://dx.doi.org.proxy1.lib.uwo.ca/10.1056/NEJMra0901217* **361**, 1570–1583 (2009).
44. Vénéreau, E., Ceriotti, C. & Bianchi, M. E. DAMPs from Cell Death to New Life. *Front. Immunol.* **0**, 422 (2015).
45. Elmore, S. Apoptosis: A Review of Programmed Cell Death. *Toxicol. Pathol.* **35**, 495 (2007).
46. Reed, J. C., Zha, H., Aime-Sempe, C., Takayama, S. & Wang, H.-G. Structure—Function Analysis of Bcl-2 Family Proteins. *Adv. Exp. Med. Biol.* **406**, 99–112 (1996).
47. Walsh, C. M. Grand challenges in cell death and survival: apoptosis vs. necroptosis. *Front. Cell Dev. Biol.* **0**, 3 (2014).
48. Castaneda, M. P. *et al.* Activation of mitochondrial apoptotic pathways in human renal allografts after ischemia-reperfusion injury. *Transplantation* **76**, 50–54 (2003).
49. A, A. & VM, D. Death receptors: signaling and modulation. *Science* **281**, 1305–1308 (1998).
50. Kischkel, F. C. *et al.* Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *EMBO J.* **14**, 5579 (1995).
51. Cruchten, S. Van & Broeck, W. Van den. Morphological and Biochemical Aspects of Apoptosis, Oncosis and Necrosis. *Anat. Histol. Embryol.* **31**, 214–223 (2002).
52. D, M., P, Z. & J, L. Granzyme A induces caspase-independent mitochondrial damage, a required first step for apoptosis. *Immunity* **22**, 355–370 (2005).

53. IK, P., CD, L., AG, R. & KS, R. Apoptotic cell clearance: basic biology and therapeutic potential. *Nat. Rev. Immunol.* **14**, 166–180 (2014).
54. Conrad, M., Angeli, J. P. F., Vandenabeele, P. & Stockwell, B. R. Regulated necrosis: disease relevance and therapeutic opportunities. *Nat. Rev. Drug Discov. 2016 155* **15**, 348–366 (2016).
55. Zhang, D.-W. *et al.* RIP3, an Energy Metabolism Regulator That Switches TNF-Induced Cell Death from Apoptosis to Necrosis. *Science (80-.).* **325**, 332–336 (2009).
56. He, S. *et al.* Receptor Interacting Protein Kinase-3 Determines Cellular Necrotic Response to TNF-α. *Cell* **137**, 1100–1111 (2009).
57. Holler, N. *et al.* Fas triggers an alternative, caspase-8–independent cell death pathway using the kinase RIP as effector molecule. *Nat. Immunol. 2000 16* **1**, 489–495 (2000).
58. T, V. B. *et al.* Necroptosis, necrosis and secondary necrosis converge on similar cellular disintegration features. *Cell Death Differ.* **17**, 922–930 (2010).
59. Kaczmarek, A., Vandenabeele, P. & Krysko, D. V. Necroptosis: The Release of Damage-Associated Molecular Patterns and Its Physiological Relevance. *Immunity* **38**, 209–223 (2013).
60. Belavgeni, A., Meyer, C., Stumpf, J., Hugo, C. & Linkermann, A. Ferroptosis and Necroptosis in the Kidney. *Cell Chem. Biol.* **27**, 448–462 (2020).
61. Yan, H. F., Tuo, Q. Z., Yin, Q. Z. & Lei, P. The pathological role of ferroptosis in ischemia/reperfusion-related injury. *Zool. Res.* **41**, 220 (2020).
62. Linkermann, A. Nonapoptotic cell death in acute kidney injury and transplantation. *Kidney Int.* **89**, 46–57 (2016).
63. Tang, D. & Kroemer, G. Ferroptosis. *Curr. Biol.* **30**, R1292–R1297 (2020).
64. Sancho-Martínez, S. M. *et al.* Impaired Tubular Reabsorption Is the Main Mechanism Explaining Increases in Urinary NGAL Excretion Following Acute Kidney Injury in Rats. *Toxicol. Sci.* **175**, 75–86 (2020).
65. Supavekin, S. *et al.* Differential gene expression following early renal ischemia/reperfusion. *Kidney Int.* **63**, 1714–1724 (2003).
66. Mishra, J. *et al.* Identification of Neutrophil Gelatinase-Associated Lipocalin as a Novel Early Urinary Biomarker for Ischemic Renal Injury. *J. Am. Soc. Nephrol.* **14**, 2534–2543 (2003).
67. Malyszko, J., Lukaszyk, E., Glowinska, I. & Durlik, M. Biomarkers of delayed graft function as a form of acute kidney injury in kidney transplantation. *Sci. Reports 2015 51* **5**, 1–9 (2015).
68. Braza, F., Brouard, S., Chadban, S. & Goldstein, D. R. Role of TLRs and DAMPs in allograft inflammation and transplant outcomes. *Nature Reviews Nephrology* vol. 12 281–290 (2016).
69. J, L. *et al.* Neutralization of the extracellular HMGB1 released by ischaemic damaged renal cells protects against renal ischaemia-reperfusion injury. *Nephrol. Dial. Transplant* **26**, 469–478 (2011).
70. Tsung, A. *et al.* The nuclear factor HMGB1 mediates hepatic injury after murine liver ischemia-reperfusion. *J. Exp. Med.* **201**, 1135–1143 (2005).
71. Wu, H. *et al.* HMGB1 Contributes to Kidney Ischemia Reperfusion Injury. *J Am Soc Nephrol* **21**, 1878–1890 (2010).

72. Todd, J. L. & Palmer, S. M. Danger signals in regulating the immune response to solid organ transplantation. *J. Clin. Invest.* **127**, 2464–2472 (2017).
73. Kezić, A., Stajic, N. & Thaiss, F. Innate immune response in kidney ischemia/reperfusion injury: Potential target for therapy. *Journal of Immunology Research* vol. 2017 (2017).
74. Takeuchi, O. & Akira, S. Pattern Recognition Receptors and Inflammation. *Cell* **140**, 805–820 (2010).
75. Kasimsetty, S. G. & McKay, D. B. Ischemia as a factor affecting innate immune responses in kidney transplantation. *Current Opinion in Nephrology and Hypertension* vol. 25 3–10 (2016).
76. Takeda, K. & Akira, S. TLR signaling pathways. *Semin. Immunol.* **16**, 3–9 (2004).
77. Liu, M. & Zen, K. Toll-Like Receptors Regulate the Development and Progression of Renal Diseases. (2020) doi:10.1159/000511947.
78. Cantu, E. *et al.* Gene Set Enrichment Analysis Identifies Key Innate Immune Pathways in Primary Graft Dysfunction After Lung Transplantation. *Am. J. Transplant.* **13**, 1898–1904 (2013).
79. Wu, H. *et al.* TLR4 activation mediates kidney ischemia/reperfusion injury. *J. Clin. Invest.* **117**, 2847–2859 (2007).
80. Leemans, J. C. *et al.* Renal-associated TLR2 mediates ischemia/reperfusion injury in the kidney. *J. Clin. Invest.* **115**, 2894–2903 (2005).
81. Pulskens, W. P. *et al.* Toll-like receptor-4 coordinates the innate immune response of the kidney to renal ischemia/reperfusion injury. *PLoS One* **3**, e3596 (2008).
82. Zhang, J. *et al.* Elucidating the molecular pathways and immune system transcriptome during ischemia-reperfusion injury in renal transplantation. *Int. Immunopharmacol.* **81**, 106246 (2020).
83. Fritz, J. H., Ferrero, R. L., Philpott, D. J. & Girardin, S. E. Nod-like proteins in immunity, inflammation and disease. *Nat. Immunol. 2006 712* **7**, 1250–1257 (2006).
84. Bourhis, L. L., Benko, S. & Girardin, S. E. Nod1 and Nod2 in innate immunity and human inflammatory disorders. *Biochem. Soc. Trans.* **35**, 1479–1484 (2007).
85. AA, S. *et al.* An inflammasome-independent role for epithelial-expressed Nlrp3 in renal ischemia-reperfusion injury. *J. Immunol.* **185**, 6277–6285 (2010).
86. Donnahoo, K. K., Shames, B. D., Harken, A. H. & Meldrum, D. R. The role of tumor necrosis factor in renal ischemia-reperfusion injury. *Journal of Urology* vol. 162 196–203 (1999).
87. Furuichi, K. *et al.* Interleukin-1-dependent sequential chemokine expression and inflammatory cell infiltration in ischemia-reperfusion injury. *Crit. Care Med.* **34**, 2447–2455 (2006).
88. DP, B. *et al.* Impaired endothelial proliferation and mesenchymal transition contribute to vascular rarefaction following acute kidney injury. *Am. J. Physiol. Renal Physiol.* **300**, (2011).
89. DV, F. Endothelial cell responses to hypoxic stress. *Clin. Exp. Pharmacol. Physiol.* **26**, 74–84 (1999).
90. Denton, M. D. *et al.* The role of the graft endothelium in transplant rejection: Evidence that endothelial activation may serve as a clinical marker for the development of chronic rejection. *Pediatr. Transplant.* **4**, 252–260 (2000).

91. Patschan, D., Patschan, S. & Müller, G. A. Inflammation and Microvasculopathy in Renal Ischemia Reperfusion Injury. *J. Transplant.* **2012**, 1–7 (2012).
92. Cameron, A. M. & Cornejo, J. F. B. Organ preservation review: History of organ preservation. *Curr. Opin. Organ Transplant.* **20**, 146–151 (2015).
93. Jing, L., Yao, L., Zhao, M., Peng, L. & Liu, M. Organ preservation: from the past to the future. *Acta Pharmacol. Sin. 2018 395* **39**, 845–857 (2018).
94. Y, O. & T, K. Preservation solution for lung transplantation. *Gen. Thorac. Cardiovasc. Surg.* **57**, 635–639 (2009).
95. RW, J. & PJ, F. Organ reperfusion and preservation. *Front. Biosci.* **13**, 221–235 (2008).
96. GM, C., M, B.-S. & PI, T. Kidney preservation for transportation. Initial perfusion and 30 hours' ice storage. *Lancet (London, England)* **2**, 1219–1222 (1969).
97. SD, H. & JV, G. Protective effects of hypothermic ex vivo perfusion on ischemia/reperfusion injury and transplant outcomes. *Transplant. Rev. (Orlando).* **26**, 163–175 (2012).
98. Moers, C. *et al.* Machine perfusion or cold storage in deceased-donor kidney transplantation. *N. Engl. J. Med.* **360**, 7–19 (2009).
99. Tingle, S. J. *et al.* Hypothermic machine perfusion is superior to static cold storage in deceased donor kidney transplantation: A meta-analysis. *Clin. Transplant.* **34**, e13814 (2020).
100. Jochmans, I. *et al.* Oxygenated versus standard cold perfusion preservation in kidney transplantation (COMPARE): a randomised, double-blind, paired, phase 3 trial. *Lancet* **396**, 1653–1662 (2020).
101. Nicholson, M. L. & Hosgood, S. A. Renal Transplantation After Ex Vivo Normothermic Perfusion: The First Clinical Study. *Am. J. Transplant.* **13**, 1093–1252 (2013).
102. Urbanellis, P. *et al.* Normothermic Ex Vivo Kidney Perfusion Improves Early DCD Graft Function Compared with Hypothermic Machine Perfusion and Static Cold Storage. *Transplantation* 947–955 (2020) doi:10.1097/TP.0000000000003066.
103. Bhattacharjee, R. N. *et al.* Subnormothermic Oxygenated Perfusion Optimally Preserves Donor Kidneys Ex Vivo. (2019) doi:10.1016/j.ekir.2019.05.013.
104. Hoyer, D. P. *et al.* Subnormothermic machine perfusion for preservation of porcine kidneys in a donation after circulatory death model. *Transpl. Int.* **27**, 1097–1106 (2014).
105. Juriasingani, S., Akbari, M., Chan, J. Y., Whiteman, M. & Sener, A. H 2 S supplementation: A novel method for successful organ preservation at subnormothermic temperatures. (2018) doi:10.1016/j.niox.2018.10.004.
106. Bhattacharjee, R. N. *et al.* CORM-401 Reduces Ischemia Reperfusion Injury in an Ex Vivo Renal Porcine Model of the Donation After Circulatory Death. *Transplantation* **102**, 1066–1074 (2018).
107. Reilly, M. *et al.* Randomized, Double-Blind, Placebo-Controlled, Dose-Escalating Phase I, Healthy Subjects Study of Intravenous OPN-305, a Humanized Anti-TLR2 Antibody. *Clin. Pharmacol. Ther.* **94**, 593–600 (2013).
108. KJ, K., Z, P., SL, V. & PC, D. P53 mediates the apoptotic response to GTP depletion after renal ischemia-reperfusion: protective role of a p53 inhibitor. *J. Am.*

Soc. Nephrol. **14**, 128–138 (2003).
109. Yang, C. *et al.* Serum-stabilized Naked Caspase-3 siRNA Protects Autotransplant Kidneys in a Porcine Model. *Mol. Ther.* **22**, 1817–1828 (2014).
110. Fujino, T., Muhib, S., Sato, N. & Hasebe, N. Silencing of p53 RNA through transarterial delivery ameliorates renal tubular injury and downregulates GSK-3β expression after ischemia-reperfusion injury. *https://doi.org/10.1152/ajprenal.00279.2013* **305**, 1617–1627 (2013).
111. JP, J., R, B., C, R. & P, D. Drug repositioning: a brief overview. *J. Pharm. Pharmacol.* **72**, 1145–1151 (2020).
112. De Broe, M. E., Kajbaf, F. & Lalau, J.-D. Renoprotective Effects of Metformin. *Nephron* **138**, 261–274 (2018).
113. El-Mir, M.-Y. *et al.* Dimethylbiguanide Inhibits Cell Respiration via an Indirect Effect Targeted on the Respiratory Chain Complex I *. *J. Biol. Chem.* **275**, 223–228 (2000).
114. Triggle, C. R. & Ding, H. Metformin is not just an antihyperglycaemic drug but also has protective effects on the vascular endothelium. *Acta Physiol.* **219**, 138–151 (2017).
115. Rena, G., Hardie, D. G. & Pearson, E. R. The mechanisms of action of metformin. *Diabetol. 2017 609* **60**, 1577–1585 (2017).
116. El Messaoudi, S. *et al.* Effect of metformin pretreatment on myocardial injury during coronary artery bypass surgery in patients without diabetes (MetCAB): a double-blind, randomised controlled trial. *Lancet Diabetes Endocrinol.* **3**, 615–623 (2015).
117. Karimipour, M., Zarghani, S. S., Milani, M. M. & Soraya, H. Pre-Treatment with Metformin in Comparison with Post-Treatment Reduces Cerebral Ischemia Reperfusion Induced Injuries in Rats. *Bull. Emerg. Trauma* **6**, 115 (2018).
118. Lamkanfi, M. *et al.* Glyburide inhibits the Cryopyrin/Nalp3 inflammasome. *J. Cell Biol.* **187**, 61–70 (2009).
119. Chatauret, N., Badet, L., Barrou, B. & Hauet, T. Ischemia-reperfusion: From cell biology to acute kidney injury. *Prog. Urol.* **24 Suppl 1**, (2014).
120. Sabbahy, M. El & Vaidya, V. S. Ischemic kidney injury and mechanisms of tissue repair. *Wiley Interdiscip. Rev. Syst. Biol. Med.* **3**, 606–618 (2011).
121. Hauwaert, C. Van der *et al.* Isolation and Characterization of a Primary Proximal Tubular Epithelial Cell Model from Human Kidney by CD10/CD13 Double Labeling. *PLoS One* **8**, e66750 (2013).
122. Legouis, D. *et al.* Ex vivo analysis of renal proximal tubular cells. *BMC Cell Biol. 2015 161* **16**, 1–11 (2015).
123. Obinata, M. The immortalized cell lines with differentiation potentials: Their establishment and possible application. *Cancer Sci.* **98**, 275–283 (2007).
124. Rizvi, M. *et al.* Effect of noble gases on oxygen and glucose deprived injury in human tubular kidney cells. *Exp. Biol. Med.* **235**, 886–891 (2010).
125. Yang, Y. *et al.* IL-37 inhibits IL-18-induced tubular epithelial cell expression of pro-inflammatory cytokines and renal ischemia-reperfusion injury. *Kidney Int.* **87**, 396–408 (2015).
126. Luo, C. *et al.* Dexmedetomidine protects against apoptosis induced by hypoxia/reoxygenation through the inhibition of gap junctions in NRK-52E cells.

Life Sci. **122**, 72–77 (2015).
127. Chandrika, B. B. *et al.* Endoplasmic Reticulum Stress-Induced Autophagy Provides Cytoprotection from Chemical Hypoxia and Oxidant Injury and Ameliorates Renal Ischemia-Reperfusion Injury. *PLoS One* **10**, e0140025 (2015).
128. Shiva, N., Sharma, N., Kulkarni, Y. A., Mulay, S. R. & Bhanudas Gaikwad, A. Renal ischemia/reperfusion injury: An insight on in vitro and in vivo models. (2020) doi:10.1016/j.lfs.2020.117860.
129. Jiang, M., Liu, K., Luo, J. & Dong, Z. Autophagy is a renoprotective mechanism during in vitro hypoxia and in vivo ischemia-reperfusion injury. *Am. J. Pathol.* **176**, 1181–1192 (2010).
130. Wang, J., Biju, M. P., Wang, M.-H., Haase, V. H. & Dong, Z. Cytoprotective Effects of Hypoxia against Cisplatin-Induced Tubular Cell Apoptosis: Involvement of Mitochondrial Inhibition and p53 Suppression. *J Am Soc Nephrol* **17**, 1875–1885 (2006).
131. Sugiura, H. *et al.* Klotho reduces apoptosis in experimental ischaemic acute kidney injury via HSP-70. *Nephrol. Dial. Transplant.* **25**, 60–68 (2010).
132. H, S., S, G. & A, L. Nitric Oxide synthase (NOS) does not contribute to simulated ischaemic preconditioning in an isolated rat cardiomyocyte model. *Cardiovasc. drugs Ther.* **18**, 99–112 (2004).
133. Chen, T. & Vunjak-Novakovic, G. In Vitro Models of Ischemia-Reperfusion Injury. *Regenerative Engineering and Translational Medicine* vol. 4 142–153 (2018).
134. Si, J. *et al.* HIF-1α Signaling Activation by Post-Ischemia Treatment with Astragaloside IV Attenuates Myocardial Ischemia-Reperfusion Injury. *PLoS One* **9**, e107832 (2014).
135. Xu, S. *et al.* Perilipin 2 Impacts Acute Kidney Injury via Regulation of PPAR α. *J. Immunol. Res.* **2021**, (2021).
136. Suh, H. N., Lee, Y. J., Kim, M. O., Ryu, J. M. & Han, H. J. Glucosamine-Induced Sp1 O-GlcNAcylation Ameliorates Hypoxia-Induced SGLT Dysfunction in Primary Cultured Renal Proximal Tubule Cells. *J. Cell. Physiol.* **229**, 1557–1568 (2014).
137. Tang, C. *et al.* Activation of BNIP3-mediated mitophagy protects against renal ischemia–reperfusion injury. *Cell Death Dis.* **10**, 1–15 (2019).
138. An, Y. *et al.* Hypoxia-Inducible Factor-1α Dependent Pathways Mediate the Renoprotective Role of Acetazolamide Against Renal Ischemia-Reperfusion Injury. *Cell. Physiol. Biochem.* **32**, 1151–1166 (2013).
139. Chen, Y. *et al.* Preservation Solutions for Kidney Transplantation: History, Advances and Mechanisms. doi:10.1177/0963689719872699.
140. Ponticelli, C. Ischaemia-reperfusion injury: A major protagonist in kidney transplantation. *Nephrol. Dial. Transplant.* **29**, 1134–1140 (2014).
141. Zhao, H., Alam, A., Soo, A. P., George, A. J. T. & Ma, D. Ischemia-Reperfusion Injury Reduces Long Term Renal Graft Survival: Mechanism and Beyond. *EBioMedicine* vol. 28 31–42 (2018).
142. Urbanellis, P. *et al.* Normothermic Ex Vivo Kidney Perfusion Improves Early DCD Graft Function Compared With Hypothermic Machine Perfusion and Static Cold Storage. *Transplantation* **104**, 947–955 (2020).

143. Hosgood, S. A., Thompson, E., Moore, T., Wilson, C. H. & Nicholson, M. L. Normothermic machine perfusion for the assessment and transplantation of declined human kidneys from donation after circulatory death donors. *Br. J. Surg.* **105**, 388–394 (2018).
144. Iwai, S. *et al.* Impact of Normothermic Preservation with Extracellular Type Solution Containing Trehalose on Rat Kidney Grafting from a Cardiac Death Donor. *PLoS One* **7**, (2012).
145. Mallet, V. *et al.* Dose-ranging study of the performance of the natural oxygen transporter HEMO2life in organ preservation. *Artif. Organs* **38**, 691–701 (2014).
146. Bhattacharjee, R. N. *et al.* Renal protection against ischemia reperfusion injury: Hemoglobin-based oxygen carrier-201 versus blood as an oxygen carrier in ex vivo subnormothermic machine perfusion. *Transplantation* **104**, 482–489 (2020).
147. Liu, S. su, Chen, Y. yi, Wang, S. xia & Yu, Q. Protective effect of dabrafenib on renal ischemia-reperfusion injury in vivo and in vitro. *Biochem. Biophys. Res. Commun.* **522**, 395–401 (2020).
148. Nguyen, M. T. & Devarajan, P. Biomarkers for the early detection of acute kidney injury. *Pediatr. Nephrol. 2007 2312* **23**, 2151–2157 (2008).
149. Han, M., Pan, Y., Gao, M., Zhang, J. & Wang, F. JNK Signaling Pathway Suppresses LPS-Mediated Apoptosis of HK-2 Cells by Upregulating NGAL. *Int. J. Inflam.* **2020**, (2020).
150. Hall, I. E. *et al.* IL-18 and Urinary NGAL Predict Dialysis and Graft Recovery after Kidney Transplantation. *J. Am. Soc. Nephrol.* **21**, 189–197 (2010).
151. De Vries, D. K. *et al.* Early Renal Ischemia-Reperfusion Injury in Humans Is Dominated by IL-6 Release from the Allograft. *Am. J. Transplant.* **9**, 1574–1584 (2009).
152. Haq, M., Norman, J., Saba, S. R., Ramirez, G. & Rabb, H. Role of IL-1 in renal ischemic reperfusion injury. *J. Am. Soc. Nephrol.* **9**, 614–619 (1998).
153. Kaminska, D. *et al.* Cytokine gene expression in kidney allograft biopsies after donor brain death and ischemia-reperfusion injury using in situ reverse-transcription polymerase chain reaction analysis. *Transplantation* **84**, 1118–1124 (2007).
154. Nikolova, P. N. *et al.* Cytokine gene polymorphism in kidney transplantation — Impact of TGF-β1, TNF-α and IL-6 on graft outcome. *Transpl. Immunol.* **18**, 344–348 (2008).
155. Patel, N. S. A. *et al.* Endogenous Interleukin-6 Enhances the Renal Injury, Dysfunction, and Inflammation Caused by Ischemia/Reperfusion. *J. Pharmacol. Exp. Ther.* **312**, 1170–1178 (2005).
156. Xu, L., Sharkey, D. & Cantley, L. G. Tubular GM-CSF promotes late MCP-1/CCR2-mediated fibrosis and inflammation after ischemia/reperfusion injury. *J. Am. Soc. Nephrol.* **30**, 1825–1840 (2019).
157. Donnahoo, K. K. *et al.* Early kidney TNF-alpha expression mediates neutrophil infiltration and injury after renal ischemia-reperfusion. *Am. J. Physiol.* **277**, (1999).
158. Jang, H. R., Ko, G. J., Wasowska, B. A. & Rabb, H. The interaction between ischemia-reperfusion and immune responses in the kidney. *Journal of Molecular Medicine* vol. 87 859–864 (2009).
159. Kelley, V. R. & Singer, G. G. The antigen presentation function of renal tubular

epithelial cells. *Exp. Nephrol.* **1**, 102–111 (1993).
160. Gao, C., Zou, X., Chen, H., Shang, R. & Wang, B. Long Non-Coding RNA Nuclear Paraspeckle Assembly Transcript 1 (NEAT1)Relieves Sepsis-Induced Kidney Injury and Lipopolysaccharide (LPS)-Induced Inflammation in HK-2 Cells. *Med. Sci. Monit.* **26**, e921906-1 (2020).
161. Gu, L. *et al.* Initiation of the inflammatory response after renal ischemia/ reperfusion injury during renal transplantation. *Int. Urol. Nephrol.* **0**, (2018).
162. Krü Ger, B. *et al. Donor Toll-like receptor 4 contributes to ischemia and reperfusion injury following human kidney transplantation*. www.pnas.org/cgi/content/full/.
163. Lamkanfi, M. *et al.* Glyburide inhibits the Cryopyrin/Nalp3 inflammasome. *J. Cell Biol.* **187**, 61–70 (2009).
164. Cavo, M. *et al.* Microenvironment complexity and matrix stiffness regulate breast cancer cell activity in a 3D in vitro model. *Sci. Reports 2016 61* **6**, 1–13 (2016).
165. Slegtenhorst, B. R., Dor, F. J. M. F., Rodriguez, H., Voskuil, F. J. & Tullius, S. G. Ischemia/Reperfusion Injury and its Consequences on Immunity and Inflammation. *Curr. Transplant. Reports* **1**, 147–154 (2014).

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Bhattacharjee R., **Jackson A.**, et al. The Impact of Nutritional Supplementation on Donor Kidneys During Oxygenated Ex Vivo Subnomothermic Preservation *(Submitted*).